# Ectomycorrhizal fungal assessment of South African *Pinus patula* seedlings and their biological control potential to enhance seedling growth

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# Abstract

The South Africa forestry industry, covering 1.3 million hectares, is dependent on exotic pine and eucalyptus species. Nursery seedlings are not inoculated with ectomycorrhizal (ECM) fungi or other beneficial microbes. *Fusarium circinatum* is an economically important pathogen affecting seedling survival. The purpose of this investigation was to assess levels of naturally occurring ECM colonisation in South African nurseries and to determine the effects of seedling inoculation with selected ECM and bacterial isolates on plant growth and resistance to the fungal pathogen *F. circiantum*.

*Pinus patula* seedlings from 10 different South African nurseries were assessed for ECM colonisation using a grid line intersect method and molecularly identified using morphological and next-generation Illumina sequencing. Explants from ECM basidiocarps, collected from *Pinus* stands, were plated onto MMN medium to obtain isolates which were verified using molecular techniques. Mycorrhizal helper bacteria (MHB) were also isolated from these basiocarps, tested for MHB properties, siderophore production, phospahte solubilising and IAA production. ECM and associated bacterial isolates were used to inoculate seedlings and growth was assessed over a 5 month period.

Colonisation of seedlings in production nurseries was low (2-21%). Morphologically the ECM fungi *T. terrestris, Suillus sibiricus,* and the genera *Russula, Pseudotomentella* were identified. Molecularly the ECM fungi *T. terrestris, Inocybe jacobi* and the genera *Sphaerosporella* and several other ECM containing families were identified along with many saprotrophic/endophytic fungi belonging to genera such as, *Penicillium, Ramasonia* and *Talaromyces.* Inoculated seedlings showed a significant increase in growth in comparison to the un-inoculated control seedlings. ECM fungal colonisation levels of these seedlings were significantly increased and colonisation was promoted by the *Suillus* isolate, Salmon Suillus. Seedling growth in the presence of the pathogen *F. circinatum* was significantly increased and promoted by the Lactarius isolate *Lactarius quieticolor.*

Inoculation of seedlings in the nursery would ensure the production of stronger healthy plants which may be more tolerant to fusarial infection increasing survival in the plantation.

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# Chapter 1

# General Introduction

## 1.1 Mycorrhizal fungi

A large number of plants throughout the world depend on mycorrhizal fungi to survive. These mutualistic relationships are especially important in nutrient-poor ecosystems (Vellinga et al., 2009). Most mycorrhizal fungi form balanced associations where both the fungus and the plant exchange products needed for their survival and future growth. Brundrett, (2004) describes mycorrhizal associations as "*a symbiotic association essential for one or both partners, between a fungus (specialised for life in soils and plants) and a root (or other substrate-contacting organ) of a living plant, that is primarily responsible for nutrient transfer. Mycorrhizas occur in a specialised plant organ where intimate contact results from synchronised plant-fungus development*." Mycorrhiza means "root-fungus" and was originally proposed by Frank, (1885). Mycorrhizal fungi differ from other plant-fungus associations in that they form associations where they exchange materials between living cells through a specialised interface. Nearly all of these relations with plants occur within roots which are evolved to house them. Not all plants are able to host mycorrhiza; some are non-mycorrhizal and others are facultative- mycorrhizal, in that they only associate with the fungi in poor conditions (Brundrett, 2004).

There are two main types of mycorrhizal fungi: endomycorrhizal fungi, of which the most common type is the Arbuscular mycorrhizal (AM) fungi and ectomycorrhizal (ECM) fungi (Brundrett, 2004). This study will be focused on ectomycorrhizal fungi, and specifically their association with pine tree seedlings.

### 1.1.1 Arbuscular mycorrhizal fungi

Of the endomycorrhizal fungi, the most common type is the Arbuscular mycorrhizal (AM) fungi. AM fungi associate with the roots of 80% of land plants, and are found in nearly all ecosystems (Strack et al., 2003; Brundrett, 2004; Smith and Smith, 2011). AM fungi belong to the phylum Glomeromycota, believed to have evolved over 450 million years ago (m.y.a). This evolution resulted in the formation of biotrophic and usually mutualist symbioses (Smith and Read, 2008).

Arbuscular mycorrhizas are so called because of the arbuscules which they form within plant root cells; these are the main sites of nutrient exchange. They are highly branched, terminal structures formed inside of cortical root cells, which last between 4-10 days. While arbuscules are formed within the plant cell they remain outside of the cell cytoplasm being surrounded by the plant cell's plasma membrane. An apoplastic space is thus formed between the plasma membrane and fungal cell wall (Strack et al., 2003; Brundrett, 2004; Smith and Smith, 2011). AM fungi also often form intracellular storage organs such as lipid-rich vesicles (Brundrett, 2004).

Morphologically there are two main types of AM fungi: coiling (Paris) and linear (Arum). These are determined by the type of growth that occurs within the colonised root. Linear AM fungi form associations where hyphal growth occurs principally longitudinally within the air channels between the cortex cells. Coiling AM fungi spread predominantly by intracellular hyphae in coils (Smith and Read, 2008). AM fungi are of great importance to the agricultural and horticultural industries and are the main association formed by the majority of South African indigenous vegetation (Hawley and Dames, 2004).

### 1.1.2 Ectomycorrhizal fungi

This study will be focused on ectomycorrhizal fungi, and specifically their association with pine tree seedlings. ECM fungi, unlike AM fungi, are more restricted and only associate with certain plant families (Brundrett, 2004). The majority of these families are the ecologically important woody perennials in temperate, boreal and tropical forests (Smith and Read, 2008; Nehls et al., 2010; Pickles et al., 2012; Tedersoo et al., 2012). ECM fungi are ubiquitous within these forest ecosystems (Cairney, 2012). Approximately 3-5% of higher plants or 6000 plant species

are able to form ECM associations, with potentially over 20 000 ECM fungal species (Pickles et al., 2012; Tedersoo et al., 2012; Garcia et al., 2014). This number rose from the previously estimated 5 500 (Monlina et al., 1992) fungal species with the advent of molecular identification techniques (Pickles et al., 2012).

The fungi associate with the finest roots of these trees. The main biological function of ECM fungal symbiosis is the exchange of fungus-derived mineral nutrients for plant-derived photosynthetically fixed carbohydrates (Taylor and Alexander, 2005; Nehls et al., 2010; Cairney, 2012; Tedersoo et al., 2012; Garcia et al., 2014). This association represents approximately 39% of the microbial biomass and between 10-35% of the respiration within boreal forests (Tedersoo et al., 2012). ECM fungi are characterised by 3 main structural components; the sheath or mantle, the Hartig net (Figure 1.1) and the extraradical or external mycelium (Smith and Read, 2008).

The mantle consists of a fungal hyphal network which encloses the root. Depending on the way that the hyphae develop the mantle can be either pseudoparenchymatous or plectenchymatous. Characteristically pseudoparenchymatous mantles are densely packed with highly differentiated hyphae while plectenchymatous mantles consist of loosely interwoven hyphae with their linear structures still evident. Many ECM fungi produce mantles which are hydrophobic. This is achieved by ~100% root tip colonisation which effectively isolates the root from the soil environment. Thus it is assumed that the fungus controls the interface and fluxes in and out of the root (Taylor and Alexander, 2005). The mantle has also been found to act as a storage compartment for nutrients within the fungus (Garcia et al., 2014)

The Hartig net is the point of contact for nutrient exchange between the fungus and the root and consists of hyphae growing in between the epidermal and cortical cells of the root. It is the point for nutrient exchange due to the large surface area it provides which allows for efficient metabolic transfers (Taylor and Alexander, 2005). ECM fungi are usually characterised as being one of two main morphological types - epidermal or cortical. Epidermal ECM fungi are the typical associations made with angiosperms, where the Hartig net is confined to the epidermal layer of cells (Figure 1.2). Cortical ECM fungi usually form as a result of associations with gymnosperms such as members of the Pinaceae family. In this form the Hartig net penetrates and occupies multiple layers of cells in the cortex of the root. As illustrated in figure 1.2, these morphological types are defined by the plant with which the fungus associates and if it has a wide enough host range the same fungus can form both types with different hosts (Brundrett,

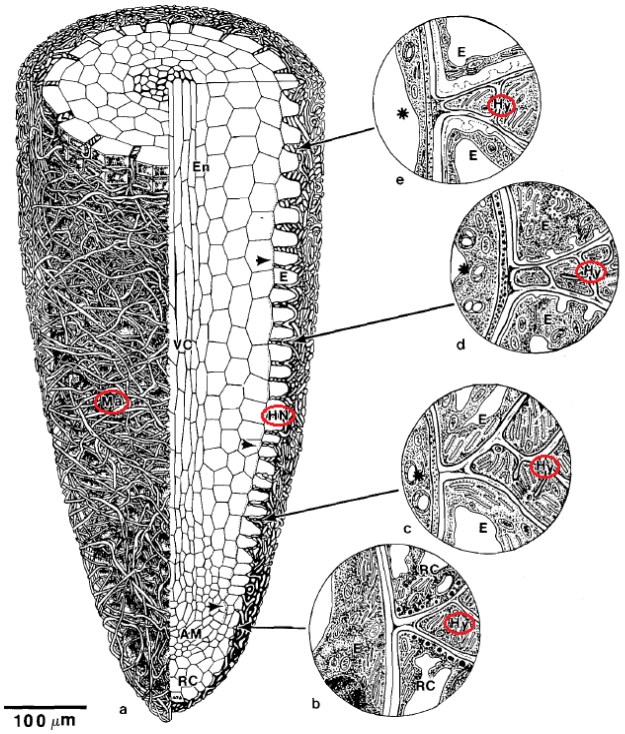



Figure 1.1: Schematic Drawing of the ECM association between the fungus *Alpova diplophloeus* and *Alnus crispa*. (a) 3D drawing of a mycorrhizal root showing the mantle (MA), Hartig net (HN), and epidermal cells (E), which progressively become bigger the further back from the apex they are. (b) Root cap (RC) region illustrating the hyphae (Hy) penetrating between the cells of the root cap. (c) Hartig net region illustrating the hyphae with rough endoplasmic reticulum and labyrinthine wall branching penetrating between the epidermal cells. (d) Mature Hartig net region illustrating the fungal hyphae penetrating as far as the modified wall of the exodermis (*). Epidermal cells show wall modifications (e) Older Hartig net region with the fungal hyphae showing reduced numbers of cysternae of endoplasmic reticulum and mitochondria. Epidermal cells show many modifications including wall material depositions, vacuolation and a decreased number of mitochondria while the hypodermal cells (*) is also more vacuolated. The fungal components are highlighted with red circles (adapted from Smith and Read, 2008).

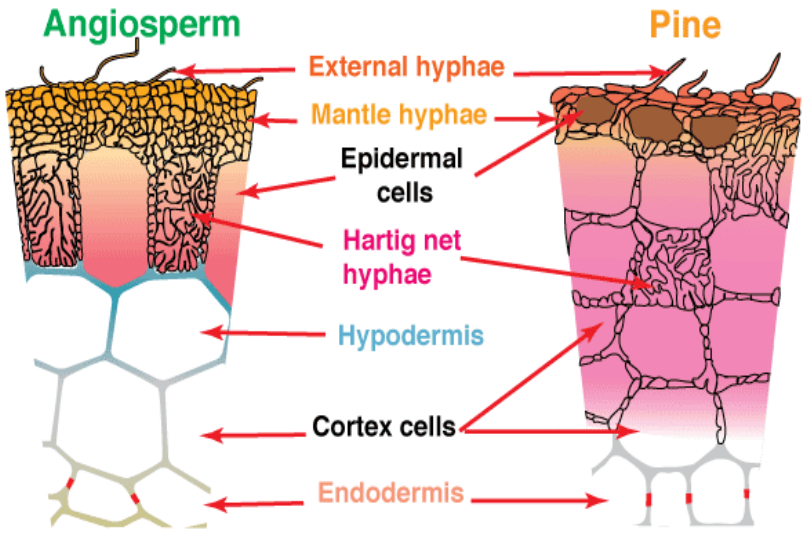



Figure 1.2: Schematic of the Hartig net growth differences between angiosperm host plants, where growth rarely goes beyond the first layer of epidermal cells, and gymnosperms such as pines, where the Hartig net penetrates multiple layers deep in the inner cortex. (Taken from Brundrett, 2008)

2004).

The extraradical or extramatrical mycelium (EMM) is the outwardly growing hyphae which provide the vital connections with the soil (Finlay, 2004; Smith and Read, 2008). The EMM is considered to be the primary site of nutrient and water uptake, and as with the mantle provides a significant sink for carbon (Garcia et al., 2014). Depending on the ECM fungal taxa, the pattern of EMM production differs from a few millimeters to occupying vast volumes of soil (Taylor and Alexander, 2005). These systems of EMM are structurally and functionally complex and comprise of both hyphal and rhizomorphic components (Agerer, 2001).

The way in which these hyphae and rhizomorphs are organised divides up ECM fungi into 5 main exploration types. The first of there is the contact exploration type, characterised by smooth mantles, with few emanating hyphae. The colonised root tips are in close contact with the substrate surrounding them and hyphae are commonly found in contact with nearby leaf litter. Secondly, there are the short distance exploration types. These, are enveloped by large amounts of emanating hyphae, this and the fact that the hyphae do not form rhizomorphs are defining characteristics. The majority of ascomycetous ECM fungi are regarded as short explo-

ration types (Agerer, 2001).

Thirdly, there are are the medium distance exploration type. It breaks up into 3 subtypes; fringe, mat and smooth. The fringe subtype form fans consisting of outreaching hyphae and hairy rhizomorphs which continuously branch out and interconnect. This subtype has extensive contact with the soil. The mat subtype can occupy a large area. As individuals they have a limited range, with mostly undifferentiated rhizomorphs. Smooth subtype ECM fungi produce internally undifferentiated rhizomorphs which on rare occasions have a central core of thick hyphae, their mantles are smooth with very few EMM (Agerer, 2001).

Fourthly, there are the long distance exploration types. These also have smooth mantles and produce highly differentiated rhizomorphs, which allows them to explore a much larger range of soil. The fifth and final exploration type is known as pick-a-back exploration type. The ECM fungi do very little exploration of their own but exploit the long distance exploration types instead, by growing within their rhizomorphs or mantles (Figure 1.3). The majority of the fungi in this type belong in the family Gomphidiaceae (Agerer, 2001).

Along with acquiring nutrients EMM are considered to be important providers of carbon into the soil and are thus an important part of below-ground food webs. It has been estimated that up to 29% of the net carbon acquired from the host is allocated to the EMM, although this depends on the taxa involved and other abiotic factors (Bidartondo et al., 2001). The allocation of carbon is far from uniform and the majority of the carbon assigned to the EMM goes to the growing mycelial front. A large percentage, up to 64%, of the carbon assigned to the EMM is respired, and a lesser percentage is lost in the form of exudates (Cairney, 2012).

#### 1.1.2.1 Nutrient uptake

When ECM fungi colonise plant roots they increase the accessibility and facilitate the movement of nutrients such as nitrogen, phosphorus and potassium, and water. They also increase the host's tolerance to salinity, drought, some pathogens, and toxic heavy metals (Smith and Read, 2008; Ma et al., 2010; Rosenstock et al., 2014). ECM fungi play a pivotal role in the soil's microbial and biochemical processes. The carbon they acquire from hosts is allocated via the mycorrhizal mycelium as dissolved organic carbon into the forest ecosystem. Along with colonised roots ECM fungi can account for up to 40-50% of the dissolved organic carbon in forest soils (Finlay, 2004; Rosling, 2009).

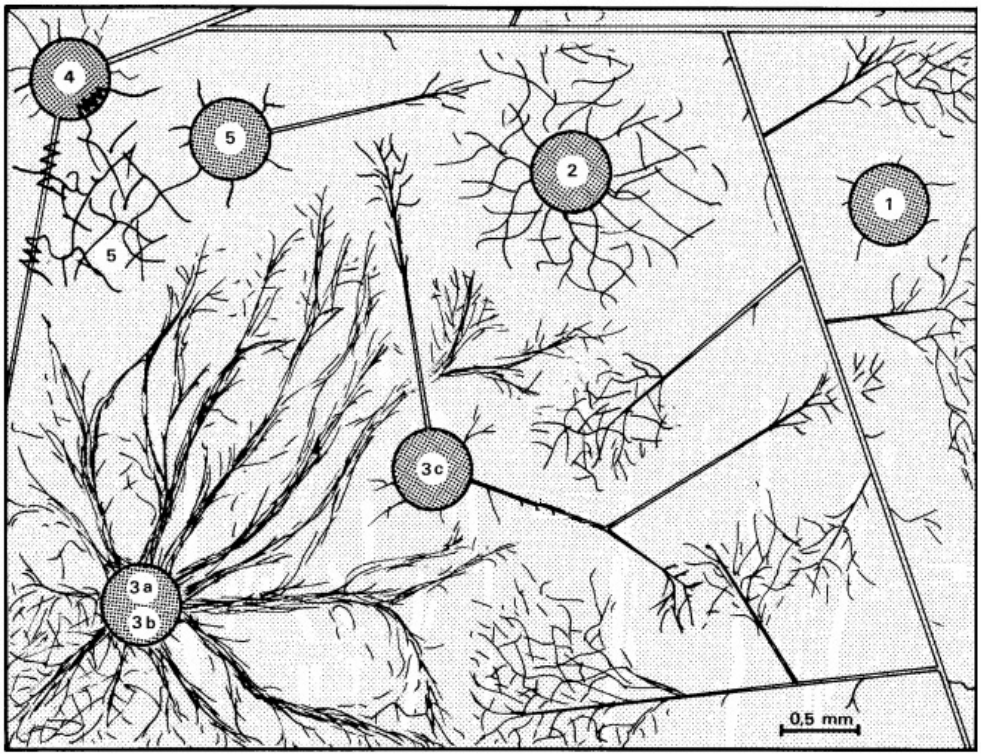



Figure 1.3: Schematic drawing representation of EMM exploration types, via cross sections of ECM fungi and EMM rhizomorphs. 1. Contact exploration; 2. short distance exploration; 3. medium distance exploration (a) fringe, (b) mat, (c) smooth; 4. long distance exploration; 5. pick-a-back exploration, shown as hyphae in contact with and intruding into rhizomorphs and ECM of long distance exploration types. All figures are to scale. (Adapted from Agerer, 2001)

ECM fungi access these nutrients from both mineral and organic sources depending on the environment, availability and species. In mineral soils, such as those found in pine forests, mycorrhizal fungi dominate the total fungal community (Rosling, 2009). In forestry plantations, especially *Pinus* plantations, there are large accumulations of acidic leaf litter. It is in this leaf litter that ECM fungi are in contact with and acquire a large portion of their nutrients (Dames et al., 1999). In natural forest soils the major nutrients required by plants are often found as macromolecules which need to be broken down into forms usable by the organisms within the soil and plants. If organic, enzymes are often employed while acids are usually used for the breakdown of inorganic nutrients such as phosphate (Taylor and Alexander, 2005; Pritsch and Garbaye, 2011). In forest ecosystems nitrogen is considered the most limiting nutrient, closely followed by phosphate, and potassium. (Dames, 2002; Rosling, 2009; Cairney, 2011). Its availability severely impacts a wide range of biogeochemical processes in forest ecosystems (Inselsbacher and Näsholm, 2012).

#### 1.1.2.1.1 Nitrogen

ECM fungi preferentially take up nitrogen (Pritsch and Garbaye, 2011). EMM explore the soil for nitrogen sources and form networks of hyphae with a high capacity for the acquisition and retention of nitrogen (Wallander et al., 2014). As with all nutrients, nitrogen is found in the soil in both organic and inorganic forms. Organic forms of nitrogen present in soil are predominantly made up of proteins or large polysaccharides, such as chitin (Pritsch and Garbaye, 2011), which is found in fungal cell walls and arthropod exoskeletons. Up to 20% of the soil nitrogen is contained in either dead or living fungal hyphae (Lindahl and Taylor, 2004). Amino acids are also major organic nitrogen sources. They account for approximately 80% of the nitrogen supply in soils while ammonia and nitrate each contribute 10%. In Boreal forests, where ECM fungi associate with the majority of the higher plants, organic nitrogen is the main form of nitrogen available to plants. The soil supply, and not root or hyphal uptake, limit plant acquisition (Inselbacher and Näsholm, 2012). ECM fungi produce enzymes such as phenol oxidases and peroxidise which are used to degrade the complex protein-phenol complexes with tannins in which organic nitrogen are commonly found (Pritsch and Garbaye, 2011).

Inorganic nitrogen is less common in forest soils. Depending on the type of soil it is usually found as nitrate or ammonia, in calcareous and acidic soils respectively (Rosling, 2009). Large amounts of inorganic nitrogen are detrimental to the ECM community and especially to EMM

growth (Wilkson et al., 2012; Wallander et al., 2014). This was best demonstrated by Wallander et al. (2014) who tested the effect of the addition of nitrogen fertilizer. They found that nitrogen leaching peaked quickly after the addition of fertilizer and declined slowly but continuously over the 16 month exposure period. The addition of this fertilizer resulted in EMM growth decline especially if the fertilizer was nitrogen combined with phosphate.

#### 1.1.2.1.2 Phosphate

Phosphate is the second most limiting nutrient in soils (Cairney, 2011). Phosphate limited sites often have the lowest tree growth but the highest ECM fungal growth. This usually results from ECM fungal communities responding to the limitation of phosphate by increasing the colonisation and acquisition of phosphate containing minerals (Rosenstock et al., 2014). Phosphate is an important nutrient as it is critical for energy metabolism, the synthesis of nucleic acids, membranes and for photosynthesis (Plassard et al., 2011).

Host trees have their own adaptations to deal with low phosphate levels yet 80% associate with ECM fungi (Plassard et al., 2011). ECM fungi have the ability to enhance their host trees phosphate absorption by growing, and acquiring phosphate past the root depletion zone. While ECM fungi do enhance the roots phosphate concentration, it is often retained within the fungal hyphae. The amount of absorbed phosphate transferred over to the host compared to the amounts retained with the fungal tissues depends on the availability of phosphate and the relative growth rate of the host and the fungus. ECM fungi can retain as much as 90% of the nutrient, mostly in the ECM fungal root tips in the form of polyphosphates (Cairney, 2011: Plassard et al., 2011).

As with nitrogen, phosphate is available in two different forms: organic (Po) and inorganic (Pi). The solubilisation of Pi and the hydrolysis of Po by ECM fungi mostly occur at the growing mycelial front. The levels of absorption differ with different EMM sections as the undifferentiated and younger mycelia have a higher absorption rate in comparison to the rhizomorphic EMM. Ion adsorption is mostly restricted to the younger EMM. EMM growth is influenced by the availability and starvation of phosphate which increases and decreases its growth respectively (Cairney, 2011).

Also as with nitrogen, Po is more common within the soil. It is commonly found in the form of phosphate monoesters such as mononucleotides and sugar phosphates, phosphate diesters, such as nucleic acids, phospholipids and inositol phosphates. In comparison Pi is mostly present as

mineral and dissolved phosphates such as inorganic phosphates. The concentration of Pi in the soil is generally low, approximately 30-65% with a concentration of <10 μM (Cairney, 2011; Plassard et al., 2011).

Organic phosphorus is hydrolysed by a series of different extracellular wall-bound enzymes produced by ECM fungi. The most commonly produced enzyme is phosphomonoesterases, or acid phosphate. It associates with ECM fungal root tips and EMM. Phosphomonoesterases are inversely related to Pi, which in high concentrations prevent their synthesis. While it is inhibited by Pi it is also not promoted by the presence of Po. Rather, acid phosphate production is increased when there is a high level of plant litter. Multiple other factors also influence enzyme production such as temperature, season, toxic metal pollution, rainfall, the availability of nitrogen, liming and soil type (Cairney, 2011; Pritsch and Garbaye, 2012). Phosphodiesterases are another enzyme produced though these do not endure in the soil for very long. Not very much is known about this enzyme's efficacy in enhancing phosphorus acquisition (Cairney, 2011). Phytases are produced to hydrolyse the more difficult Po sources. Its activity is reduced by the presence of high levels of Pi. These enzymes work by releasing phosphate groups from phosphorus sources and are widely distributed in ECM fungi (Cairney, 2011; Pritsch and Garbaye, 2012).

Inorganic phosphorus is solubilised by low molecular weight organic acids (LMWOA) (Cairney, 2011). Of these acids, oxalic acid is the one most commonly produced by ECM fungi. Other LMWOA produced are citric, succinate and malonic acids. These acids induce mineral dissolution more efficiently than the ions of inorganic acids (Rosling, 2009). ECM fungi significantly contribute to the mineral weathering processes in forest soils because ECM fungi are able to acquire phosphorus from poor soluble sources such as natural rock phosphates. Apatite is an example of this. The presence of apatite within the soil stimulates the growth of EMM when phosphorus is limiting. The anionic forms of the LMWOA are considered effective in combination with proton release. The amounts of individual organic acids produced by ECM fungi vary with fungal genotype. As with the enzymes that hydrolyse Po, LMWOA are excreted close to and to some extent behind the root tips (Rosling, 2009; Cariney, 2011; Plassard et al., 2011). Both the host plant roots and ECM fungi possess high affinity plasma membrane Pi transporters, HcPT1 and HcPT2, which regulate the uptake of Pi. Their production, induced by low Pi levels in the soil, is to help absorb the small amounts available (Cairney, 2011).

Phosphorus transfer to the host is highest during the early colonisation stages. As with absorp-

tion in the EMM hyphae, phosphorus transfer is not uniform along the entire colonised root tip. Transfer is at its highest in the median zone, which is the area where the Hartig net is thought to be the most active. The transfer process can occur passively via a controlled concentration gradient if the levels of phosphorus within the ECM fungi are high enough. Transfer requires sufficient efflux of phosphorus into the interface apoplast between the fungus and the host and absorption occurs across the plasma membranes of the root epidermal and cortical cells. The ECM fungi often retain significant amounts of the phosphorus. Phosphorus translocation from the absorption sites at EMM tips to the colonised root tips is not well understood, but believed to be transferred via vacuoles (Cairney, 2011).

#### 1.1.2.1.3 Potassium

In higher plants potassium ions ($K^+$) are the most abundant cation present in the cytoplasm. It makes up 2-10% of a plants dry biomass. Potassium ions are the most important macro molecule for all organisms. Potassium is vital for metabolic processes and the ions play an important role in physiological functions such as the neutralization of negative charges, osmoregulation, plasma membrane polarization and growth. They also help the plant adapt to environmental stresses such as increasing drought or salinity tolerance (Garcia et al., 2014; Garcia and Zimmerman, 2014). Potassium ions are not only important for the proper functioning of organisms but are also required for homeostasis and the correct transfer of other nutrients to the host plants, especially Pi (Garcia et al., 2014; Garcia and Zimmerman, 2014).

In Norway spruce ECM fungi only provided 5-6% of the total $K^+$ under limiting conditions, which are frequent in forest environments (Garcia et al., 2014). Potassium ions are not limited within the soil but their availability is very low because of their strong mineral adsorption. Depending on the type of soil, potassium concentrations range from approximately 0.1 to 1 mM. In the fungus potassium ions are chiefly stored in vacuoles, as found in *Suillus luteus*. Many *Rhizopogon* species showed that vacuoles are important for the sequestration of $K^+$, thus vital if the forest is subjected to prolonged periods of $K^+$ deprivation (Garcia and Zimmerman, 2014).

### 1.1.2.2 Carbon

It is well established that mycorrhizal species exchange a large portion of the nutrients they acquire for carbon, photosynthetically produced by their host species. They rely on their host

plant for nearly all their carbon requirements (Simard et al., 2002). There are difficulties in determining the exact amount of carbon transferred from plants to ECM fungi. An accurate assessment would require information about the fungus' Hartig net, mantle thickness and the quantity/extent of the EMM would need to be determined under near natural conditions. It would also have to take into account the variation of ECM and host plant association from species to species (Smith and Read, 2008). That being said, it is well known that a large fraction of the carbon photosynthetically fixed by the plant is allocated below-ground to the ECM fungi which can vary from 10 to 50%, depending on the fungus and the amount of nutrients the fungus is providing (Simard et al., 2002; Wallander et al., 2011; Garcia and Zimmerman, 2014).

But first, how does the carbon move from the plant to the ECM fungi? While many ECM fungi are culturable, very few are able to utilise complex polymers such as those found in leaf litter and humus in forests. They are dependent on simple sugars such as the monosaccharides glucose, mannose and fructose. Preferentially, ECM fungi utilise glucose which is produced along with fructose when the sucrose is exported from the root cells to the interfacial apoplast space. Once there it is hydrolysed by plant wall-bound invertase enzymes. As stated, glucose is preferentially absorbed by the fungus until the build up of fructose becomes too high and begins to inhibit the invertase from hydrolysing the sucrose. The fungus then converts the glucose into large quantities of glycogen, and smaller amounts of trehalose, while fructose is converted into mannitol (Simard et al., 2002; Smith and Read, 2008). This process is explained in greater detail in figure 1.4.

The greatest amount of carbon is allocated to newly formed colonisation points during the end of the growing season - late August in the northern hemisphere, under low nutrient availabilities (Simard et al., 2002; Hobbie, 2006; Smith and Read 2008; Höberg et al., 2010). ECM fungi have the greatest ability to attract carbon from the plant soon after colonisation which reduces increasingly as they age up to roughly 90 days (Smith and Read, 2008). The allocation of carbon is also affected by the season (Smith and Read, 2008). Höberg et al., (2010) conducted a high temporal-resolution tracing of $^{13}C$ from the photosynthate in the canopy down to soil organisms in a young boreal *Pinus sylvestris* forest. They found up to a 500% increase in below-ground carbon allocation occurred during the end of the growing season in comparison to the start in early spring. They also found that this labeled carbon was primarily found in the biomarkers in fungal fatty acids. Their conclusion was that the production of fungal sporocarps was totally dependent on this late season carbon allocation. This is supported by the main flush of root

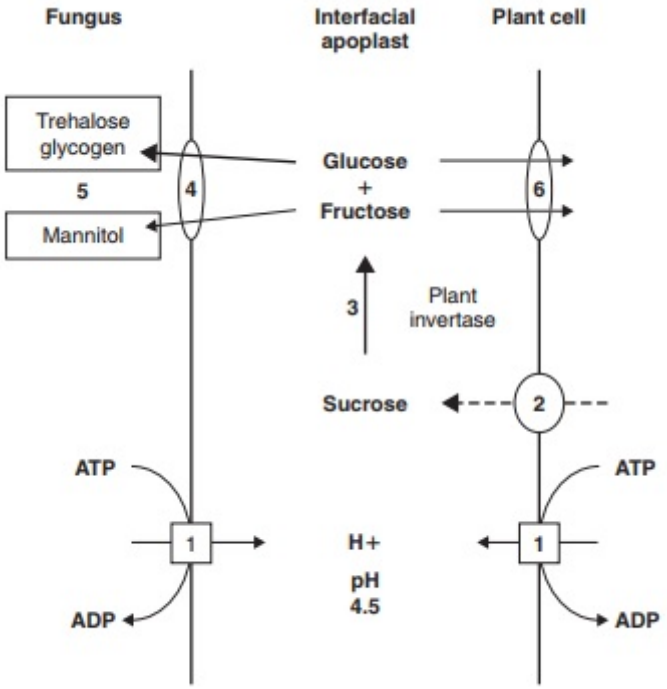



Figure 1.4: Diagram of ECM-plant interface describing important processes involved in the sugar transfer between host and ECM fungus. 1. Both plant and fungus converted ATP to transfer $H^+$protons into the interfacial apoplast, causing a lowering of pH and a proton motive force needed for active transport. 2. Sucrose delivered to the roots is exported from the plant cell and is 3. hydrolysed by the plant invertase, a cell wall-bound enzyme, into fructose and glucose. 4 Glucose, as the preferential carbon source, is absorbed first via a hexose transporter. Fructose is also absorbed but only when levels of glucose are low and the levels of fructose threaten to inactivate the invertase. 5. Glucose is converted by the fungus into mostly glycogen and smaller amounts of trehalose, while the fructose is converted into mannitol. 6 is at yet unconfirmed but there has been evidence showing that plants have ways of reabsorbing some of the lost sugar, via upregulation of a monosaccharide transporter. (Taken from Smith and Read, 2008)

growth, high EMM activity and sporocarp production occurring in late summer. This increase in allocation late in the season is believed to be due to the end of "growing" as stem elongation and bud setting are complete (Smith and Read, 2008).

In addition to age and seasonality, the nutrient status of the soil also affects the allocation of carbon to ECM fungi, with allocation being at its highest at low nutrient availabilities. Thus making allocation sensitive to influences such as increased nitrogen (N) fertilization and environmental N deposition, ozone and $CO_2$ (Hobbie, 2006). In their study Höberg et al., (2010) also found that while there were no short term effects of soil N fertilization, there were effects detected 1 year later as the carbon allocation was reduced by 60%. This is likely due to an increase in nitrate in leaves which was negatively correlated to the amount of carbon which was allocated to the roots and subsequently ECM fungi. Thus, as the nitrogen levels increase, less carbon is allocated to the roots for storage and ECM fungal growth, and is used for plant growth instead (Hermans et al., 2006). The higher exogenous N levels negate the need for ECM association (Wallander et al 2011).

ECM fungi are important sinks for the photosynthetically produced carbon. In all plant systems, but especially in boreal and temperate forests, they play a vital role in the carbon balance of the plant and the biosystem. The $CO_2$ accumulation capacity of a plant is determined by their carbon input, photosynthesis, and the respiration, or carbon output, of plant-dependent symbionts, such as ECM fungi (Heinonsalo et al., 2010). Since a large proportion of the below ground allocated carbon is used to produce EMM, carbon is the key factor in determining the amount of EMM produced by ECM fungi (Wallander et al., 2011; Ekblad et al., 2013). EMM can receive up to 20-29% of the carbon produced by its host; of this recently fixed and allocated carbon between 43-64% is respired (Simard et al., 2002: Heinonsalo et al., 2010). Thus, EMM is an important driver of soil biological processes (Heinonsalo et al., 2010). EMM play a vital role in the soil food web as they are the dominant mechanism of carbon entry and distribution throughout the soil (Smith and Read, 2008; Höberg et al., 2010; Wallander et al., 2011).

#### 1.1.2.3 Diversity

The diversity of macro-organisms is commonly affected by latitude and longitude. But this is not true of ECM fungi. The diversity of ECM fungi, and especially fungal richness, is governed by temperature rather than distance from the equator. ECM diversity is dependent on tempera-

ture and precipitation and peaks in temperate and boreal forest biomes (Tedersoo et al., 2012). The phylogenetic ECM fungal community composition is most strongly influenced by the host plant's species and age (Ma et al., 2010; Tedersoo et al., 2012). Tedersoo et al. (2012) found that the species of the host plant resulted in a 33.8% variation in the ECM fungal community.

Knowledge about fungal diversity within South Africa as a whole is limited. The only truly comprehensive survey of South African fungi was undertaken by Doige, (1950), listing 4748 fungi - only a fraction of the expected total diversity. South African vegetation is largely associated with endomycorrhizal fungi, such as AM, Ericoid and Orchid mycorrhiza, but focus will be placed on ECM fungi. The majority of ECM fungi within South Africa can be found associated with *Pinus* and/or *Eucalyptus,* which contribute to the exotic forest plantations. Both tree genera along with their ECM associations were introduced, allowing these trees to establish successfully (Vellinga et al., 2009; Wood, 2017). The surveys which have identified ECM fungi, such as those performed by Lundquist (1986) and van der Westhuizen and Eicker (1987), were based only on above-ground diversity, recording the presence of sporocarps. As a result, a large portion of the below-ground diversity was missed as the diversity and frequency of fungi above and below-ground differ substantially. Few researchers in South Africa have performed molecular and morphological studies, which provide a more comprehensive picture of the ECM fungal community. Hawley et al. (2008) performed such a study, of *Pinus patula* in the Sabie region, Mpumalanga; this identified 11 ECM fungal species with 7 dominant field types, including genera such as *Amanita* and *Thelephora.* Wood (2017) contains a comprehensive list of non-native ECM fungi identified in South Africa, listing 25 genera. No country-wide comprehensive study of ECM fungi has been performed in South African to date.

Of indigenous South African ECM fungi, there is little evidence outside the desert truffles, investigated and described in Trappe et al. (2008) and Adeleke and Dames (2014). That's in comparison to the approximately 400 indigenous species identified in tropical Africa (Verbeenken and Buck, 2002). Thus, while there are few indigenous ECM fungi, they are mostly found in forestry plantations associated with their host plants, even when surrounded by indigenous South African forests. Some evidence of ECM fungal naturalisation was found in Australia by Dunk et al. (2012) and Jairus et al. (2011). Yet, when a similar study was performed by Hawley and Dames (2004) on locally important indigenous forest trees in the Eastern Cape, South Africa, no ECM fungal associations were found. As such, plantations associated with ECM fungi pose little threat to the natural ecosystems outside of *Pinus* or *Eucalyptus* plantations

(Wood, 2017).

## 1.2 Mycorrhizal helper bacteria

Mycorrhizal symbiosis has been considered as a bipartite relationship between the plants and mycorrhizal fungi. More accurately it is a tripartite relationship between the host plant, the mycorrhizal fungus and the soil microorganisms found within the rhizosphere, which are known to also interact with both the plant and fungus (Finlay, 2004; Frey-Klett and Garbaye, 2005). The rhizosphere is defined as the zone of influence produced by plants on the associated microorganism and soil components. It is characterised by altered microbial diversity and microorganism activity. It is physically, chemically and biologically distinct from the remaining surrounding soil as it is directly influenced by the plant via the exudates it produces (Rigamonte et al., 2010). Through secreted exudate signals and nutrient interaction, rhizospheric bacteria play a major role in the development of mycorrhizal symbiosis (Deveau et al., 2012).

Bacteria that are directly involved in the formation of mycorrhizal symbiosis were first discovered in the studies by Bowen and Theodorou (1979). They showed that the presence of certain bacteria either promoted or inhibited the colonization of *Pinus radiata* by *Rhizopogon luteolus*. The bacteria associated with positive mycorrhizal establishment function are known as Mycorrhizal Helper Bacteria (MHB) (Tarkka and Frey-Klett, 2008). MHB fall into one of two categories; those that stimulate the formation of mycorrhizal associations, and those that interact positively with the mycorrhiza which already have established associations. The bacteria in these categories involve different taxonomic groups but perform similar functions in the tripartite relationship (Rigamonte et al., 2010).

MHB are usually found in the mycorrhizosphere. The mycorrhizosphere is the special environment where the microbial populations are shaped by the pressure exerted by exudates produced by the mycorrhizal roots and hyphae. It contains mycorrhizas, EMM and associated organisms which differ from both the rhizosphere and remaining soil (Tarkka and Frey-Klett, 2008: Rigamonte et al. 2010). MHB are a taxonomically diverse group which have been found everywhere in a range of different habitats closely associated with mycorrhizal fungi. They have been isolated from ECM fruiting bodies, colonised roots, galls, termite mounds and even heavy metal-contaminated soil (Tarkka and Frey-Klett, 2008; Mestre et al., 2016).

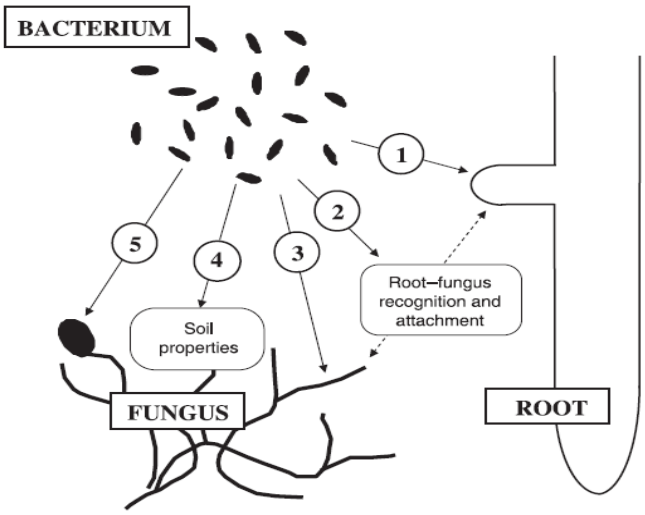



Figure 1.5: Simplified representation of a plant's rhizosphere indicating 5 possible ways in which MHB can promote mycorrhizal establishment: (1) effect on the root receptivity to mycorrhizal fungi, (2) effect on the root-fungus recognition and attachment, (3) effect on the fungus survival and growth, (4) effect on the physio-chemical properties of the soil and (5) effect on the germination of fungal propagules (according to Garbaye, 1994)

MHB belong to many different bacterial groups and genera such as Gram negative proteobacteria, Gram positive *Firmucutes* and actinobacteria (Rigamonte et al., 2010; Aspray et al., 2013; Kurth et al., 2013). While there is a wide range of MHB some species are far more prominent than others. Garbaye and Bowen (1989) found approximately 106 bacterial colony forming units per gram (fresh weight) of mycorrhiza. The majority of these were *Pseudomonas fluorescens* strains. While not all the bacteria isolated were MHB, 80% were found to have a positive effect on the establishment of mycorrhiza while the remaining 20% were either neutral or had a negative effect.

MHB do not only help mycorrhizal fungi colonise host plant roots (Figure 1.5) but also induce the germination of mycorrhizal spores and increase pre-symbiotic growth. They detoxify negative substances, inhibit the growth of competitors and antagonists, alleviate soil mediated stress, and help improve the nutrition of the fungus. Some increase the aggressiveness of the fungus against other fungi by inducing the production of phenolic compounds such as hypaphorine. MHB also stimulate the growth of lateral root in plants which provide new colonization sites for the mycorrhizal fungi (Rigamonte et al., 2010; Aspray et al., 2013; Kurth et al., 2013). MHB improve the nutrition of mycorrhizal fungi in a number of ways, such as the solubilising

of minerals. The minerals are solubilised by the secretion of protons or complexing agents such as low molecular weight anions or siderophores. They inhibit the growth of competitors via direct competition and as a result decrease the levels of anti-fungal metabolites within the soil. Most of these functions are preformed for the exchange of nutrients in the form of exudates (Rigamonte et al., 2010).

The stimulation of pre-symbiotic growth has been exemplified multiple times under laboratory conditions. Mycorrhizal fungal growth was found to be especially receptive to the bacteria when large amounts of citric acid were produced by the MHB as a substrate for the fungus (Aspray et al., 2013). Deveau et al. (2010) investigated what it is the MHB and ECM fungi produce that stimulates their mutual growth using *P. fluorescens* BBc6R8 and *Laccaria bicolor* S238N. *L. bicolor* was found to produce trehalose which chemoattracted and promoted the growth of the MHB. *P. fluorescens* was found to produce thiamine in concentrations high enough to promote ECM fungal growth. It is the production of these kinds of energy-rich metabolites by ECM fungi which induce the formation of mycorrhizospheres and the selection of the bacteria within them.

While all of these are modes of action for MHB have been reported it must be noted that these activities are not ubiquitous for all MHB isolates. Some bacterial isolates are only MHB under certain conditions and can inhibit fungal growth under different conditions. For example actinobacteria are frequent colonisers of mycorrhizospheres. They are known for their opposition to other microbial species and are a rich source of antifungal compounds. Therefore, depending on the conditions, they either inhibit or promote mycorrhizal symbiosis. *Streptomyces* sp AcH505 was co-cultivated with the ECM fungi *Amanita muscaria* and *Suillus bovinus* and was able to increase both of their rates of mycorrhization (Kurth et al., 2013). When acting as a MHB, this bacterial strain promotes the extension of fungal mycelium due to its production of the metabolite auxofuran but also simultaneously reduces the fungal mass because of thinning. When *Streptomyces* sp AcH505 was co-cultivated with *Hebeloma cylindrosporum* fungal growth was reduced. This bacteria is also an example of a cross group MHB, as it also stimulated the fine root production within the plant, reduced the plant's defence response to allow mycorrhizal colonisation to occur and increased the plant's resistance to grey mould (Kurth et al., 2013).

The greater majority of MHB are considered to be fungal specific rather than plant specific (Rigamonte et al., 2010; Kurth et al., 2013). With ECM fungi MHB have only been described

to associate with basidiomycetes. For example *Paenibacillus* sp. EJP73 has been shown to be a MHB in the *Lactarius rufus – Pinus sylvestris* ECM fungal association in both laboratory and greenhouse experiments (Aspray et al., 2013).

## 1.3 Pines in South Africa

Within South Africa (SA) natural forests are rare as they occur over only 8% of the country's surface (van Wilgen and Richardson, 2012). These few indigenous forests have poor resources thus the forestry industry is dependent on plantations of pine and eucalyptus species (Wingfield and Knox-Davies, 1980).

The forestry industry, whilst associated with negative environmental impacts such as increased evapotranspiration and decreased streamflow, translates into significant economic benefits for SA. This sector is a major job creator; the forestry industry currently employs around 201 025 people, ~77 000 directly and 30 000 indirectly. The pulp, paper, sawmilling, and timber board industry employ many more. As a whole the forestry industry provides a means of earning a livelihood for 2.3 million people within the country's rural communities. Plantations, timber processing and supporting industries play a vital role in SA's energy security. The total forestry sector's turn over in 2012/13 was approximately R21.6 billion. SA plantations produced 16.2 million tonnes of commercial roundwood worth R6.7 billion in 2009 (DAFF annual report 2012/13, DAFF annual report 2014/5).

The forestry industry does not just provide economic benefits. It also significantly increases the country's above-ground biomass; produces many aesthetic and recreational benefits; and conserves many of the *Pinus* species. Pines are indigenous to the northern hemisphere where they have become endangered. The Central American and Mexico Coniferous Resources Co-operative (CAMCORE) was established with the goals of identifying threatened tree species, collecting seeds from these vulnerable populations and distributing them for *ex situ* conservation and growth studies. Within SA, CAMCORE is a major player for conservation as all major growers are members and species such as *P. radiata* which are highly threatened in their native habitat flourish within SA (van Wilgen and Richardson, 2012).

*Pinus* species have been planted within South Africa for the past 300 years. They were first introduced by settlers as a source of timber but also as a way to tame or improve the South African

landscape and are now dominant features. Organised forestry plantations with a focus on *Pinus* became the norm in the early 20th century. Forestry plantations that focused on conifers peaked in 1997 which saw 798 000 ha planted, but have been declining slowly since, with 600 000 ha of pine planted in 2009 (van Wilgen and Richardson, 2012).

The majority of *Pinus* plantations have been planted within the fynbos and grassland areas with mean annual rainfalls exceeding 800 mm, in areas which are usually unsuited for other forms of agriculture. Pines have thrived in these areas as they are well adapted to fire-prone environments (van Wilgen and Richardson, 2012). They constitute approximately 50% of the South African forestry industry (Roux et al., 2007) and are dominant in the northern and southern regions of SA. The most common planted species are *Pinus patula, P. elliottii, P. radiata, P. taeda* and less commonly *P. pinaster* (DAFF Timber report, 2010/11).

*Pinus patula* Schlecth. was first introduced to SA in 1907. It is the most commonly planted *Pinus* species in SA and comprises 50.5% of the total softwood planted area with 312 447 ha. Specifically, this species occurs mostly in north and south Mpumalanga, southern KZN and the Eastern Cape. *P. elliottii* Champ. is the second most common *Pinus* spp, covering 28% (173 358 ha). It is planted throughout SA except in the Western Cape. *P. radiata* D. Don is planted virtually exclusively in the Western Cape and currently covers 60 605 ha and *P. taeda* L. covers 19 724 ha (Roux et al., 2007; Coutinho et al., 2007; DAFF Timber report, 2014/15; van Wilgen and Richardson, 2012).

### 1.3.1 *Pinus* and ECM fungi

Initial planting of *Pinus* spp within SA was very rarely successful. This barrier was only overcome by the introduction and eventual spread of ECM fungi into the southern hemisphere, suggesting that ECM fungi are necessary co-evolved mutualists. Currently, pine plantations throughout SA and the southern hemisphere are dominated by non-native ECM genera such as *Rhizopogon, Suillus, Thelephora, Pisolithus. S*pecies such as *S. luteus, R. vulgaris* and *Thelephora terrestris* are commonly found associated with *Pinus* species, specifically with *P. radiata* (Vellinga et al., 2009; Dickie et al., 2010). The presence of these fungi allows pine trees to flourish in a varying range of climates, and under poor soil conditions (LePage et al., 1997).

Due to their now global distribution ECM fungi commonly co-invade with seedlings into novel habitats. Yet, as stated earlier there is little evidence of ECM fungi jumping from their natural

*Pinus* hosts to local indigenous trees outside of their natural habitats in North America and Europe (Vellinga et al., 2009).

#### 1.3.1.1 Fossil record

Many ECM fungi exclusively associate with the Pinaceae family as they originated around the first appearance of Pinaceae and appear in the fossil record in the Eocene epoch 50 m.y.a (Pickles et al., 2012). LePage et al. (1997) described the first ECM fungal fossil found in the middle Eocene Princeton chert in British Columbia. Fossilized ECM were found associated with the roots of *Pinus,* complete with a Hartig net which extended into the endodermis of the roots, a pseudoparenchymatous mantle and simple septate extraradical hyphae. The roots associated with the fungus lacked root hairs and had dichotomized to form large, coralloid clusters. This provides evidence that ECM fungi were well established as plant mutualists 50 m.y.a in association with the first appearance of Pinaceae.

Phylogenetic analysis suggests that their main genera and families originated some time during the early cretaceous period approximately 130 m.y.a and that they evolved multiple times from mostly saprotrophic fungi (Tedersoo et al., 2010; Pickles et al., 2012). This evolutionary time period put ECM fungi into the same time frame as Pinaceae, which were experiencing major diversifications during this period. Pinaceae evolved in the mid to late Jurassic to Cretaceous periods (Finlay, 2004; Berbee and Taylor, 2010; Pickles et al., 2012).

### 1.3.2 Pitch canker (*Fusarium circinatum)*

*Fusarium circinatum* (teleomorph = *Gibberella circinata*) is the causal fungus of pitch canker in forestry plantations throughout the world. This fungus is considered to be one of the most important pathogens of *Pinus* species and *Pseudotsuga menziesii* (Wingfield et al., 2008; Mitchell et al., 2011). Fifty-seven different species of *Pinus* are susceptible. This pathogen represents a significant threat and is an important limitation to countries which grow the non-native pines intensively in plantations. It is frequently associated with reduced yields and productivity and high levels of tree mortality thus causing significant economic losses (Crous, 2005; Wingfield et al., 2008; Steenkamp et al., 2014). *F. circinatum* was first recorded in 1946 by Hepting and Roth, in south eastern U.S.A, from there it has now become a major pathogen, especially in California.

It was first detected in South Africa in 1990 in a single forestry nursery in Mpumalanga as the cause of root disease on *P. patula* seedlings and cuttings (Wingfield et al., 2008; Mitchell et al., 2014; Steenkamp et al., 2014), believed to have originated from infected Mexican seed (Mitchell et al., 2011). Since then it has spread to most pine growing forestry nurseries in the country. Within 10 years of its discovery in SA, it has become one of the greatest constraints to the pine industry, especially to *P. patula* (Mitchell et al., 2011). Uniquely Pitch canker started off as a severe nursery pathogen in SA and was only isolated from established trees 15 years after its initial discovery, from 5-9 year old *P. radiata* trees in the Western Cape and then on 12-15 year old *P. radiata* in the George area and *P. greggii* in the Eastern cape and Kwa-Zulu Natal (Coutinho et al., 2007; Steenkamp et al., 2014). The pathogen continues to spread.

It poses a threat to plant establishment as mortalities usually commence 3 months after planting, with the greatest mortality occurring after winter and good initial growth (Mitchell et al., 2011; 2012). This is best exemplified by Crous, (2005) who performed an experiment on 16 plots in Mpumalanga over 2 years to determine the extent of in-field mortality due to *F. circinatum*. Survival in all of the plots was extremely low with the majority of the mortality occurring between 30 and 140 days after planting. It was estimated that *F. circinatum* was responsible for between 18.5 to 31.5% decline in survival of the monitored plots with 42% of dying trees testing positive for the presence of the pathogen. The impact of Pitch canker on tree survival within South Africa has been estimated to cost the forestry industry in excess of R12 million a year due to its affect on both *P. radiata* and *P. patula* (Mitchell et al., 2011).

#### 1.3.2.1 Symptoms

Pitch canker infects trees of all ages, any vegetative or reproductive part and at any time of the year. The first symptoms on established trees are usually the wilting and discolouration of needles which eventually turn red and fall off, resulting in branch dieback to the point of infection. The dieback is caused by the girding cankers which obstruct the water flow to that part of the tree. The tissue associated with these cankers is typically resin soaked and often oozing resin from the infection site (Figure 1.6d). While infection can occur at any point on the tree, new current-year-growth is more susceptible to infection, often due to wounds. Susceptible wounds can be caused by anything from pruning and insect activity to baboons striping the bark off trees (Wingfield et al., 2008; Mitchell et al., 2011; 2012; Steenkamp et al., 2014).

*Fusarium circinatum* infection of the reproductive structures results in female flower and mature cone death. Infection of younger cones results in smaller than normal, deformed cones which often abort before reaching maturity (Coutinho et al., 2007; Wingfield et al., 2008). Seeds can be infected either externally or internally and in some cases produce asymptomatic seedlings (Mitchell et al., 2011).

In South Africa Pitch canker is a major nursery pathogen causing significant economic losses and large-scale seedling death. In seedlings, symptoms typically present themselves by the initial wilting of the tips, then discolouration (usually purpling) of the area beneath the growing tip and as the disease progresses the seedlings turn brown and die (Figure 1.6 a-c) (Mitchell et al., 2011). During the later stages of infection damping off and root and collar rot occurs (Mitchell et al., 2011; Steenkamp et al., 2014).

#### 1.3.2.2 Biotic and abiotic factors influencing disease severity and transmission

Symptom expression and the disease severity are generally associated with susceptibility of host species, along with other biotic conditions (Coutinho et al., 2007; Wingfield et al., 2008).

Insects are considered to be important agents of fungal transmission. In South Africa there is little information available about the role insects play in transmitting Pitch canker to susceptible trees (Wingfield et al., 2008; Mitchell et al., 2011). Crous, (2005) found that tree death attributed to insects was associated with white grubs and the bark beetle, *Hylastes angustatus*. Coutinho et al., (2007) found that many of the trees infected with Pitch canker were also infested with the weevil, *Pissodes nemorensis*. Isolations from the infected tissues, weevil galleries, and adult insects were analysed and consistently produced *F. circinatum*. Thus identified insects could potentially contribute to favourable biotic conditions for the transmission of Pitch canker in South Africa.

A number of abiotic factors can increase disease severity. Climate plays a role in the infective ability of *F. circinatum*. Low temperatures decrease the fungal growth, while low humidity levels can prevent infections from occurring even if the temperature is optimal. The opposite is true for high humidity, which increases infection efficiency. Nutrient levels can affect susceptibility, specifically high nutrient levels, derived from either the soil or leaf litter, which increases disease severity. Site specific factors that increase plant susceptibility include plant stressors, such as drought or water logging, and shallow soils. Plantation management also plays a role with

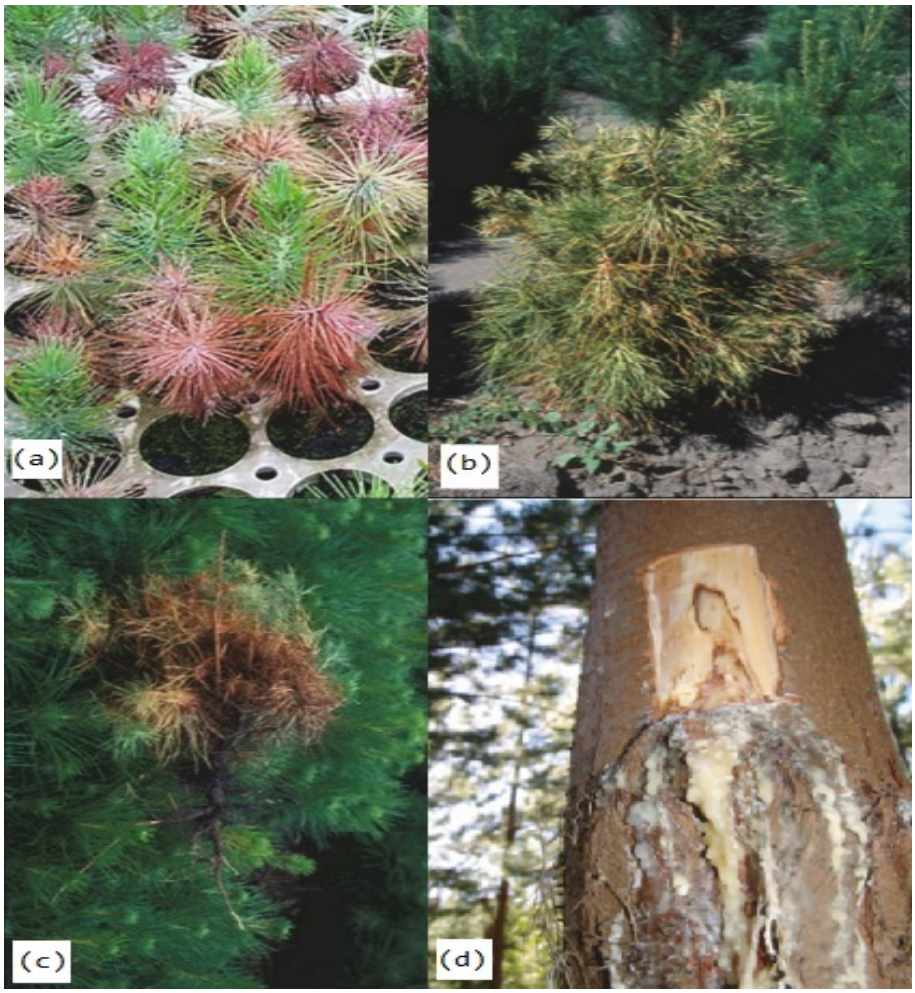



Figure 1.6: Symptoms of *F. circinatum* on young *Pinus* trees. (a) Dead *P. patula* seedlings from natural infection. (b) Young *P. radiata* tree exhibiting dieback of branch tips symptoms. (c) Branch on fully grown *Pinus* tree showing branch dieback. (d) Resinous canker, on the main stem of a full grown *Pinus* associated with pruning wound (a,b,c adapted from Wingfield et al., 2008) (d, adapted from Coutinho et al., 2007)

high plantation densities leading to increased susceptibility to disease. Air pollution, specifically high ambient ozone concentrations, augment the development of cankers in susceptible trees (Wingfield et al., 2008). This is believed to be the cause of the high incidences of Pitch canker which occur along highways in California (Wingfield et al., 2008). In disease resistant trees, air pollution and *F. circinatum* result in stunted tree growth and a decreased root mass (Carey and Kelly, 1994).

#### 1.3.2.3 Current control options

Currently there are no absolute means of controlling Pitch canker in plantations and nurseries. The approach currently being used is an integrated management system of quarantine measures, nursery and silviculture maintenance and the selection of clones which are less susceptible to the pathogen (Wingfield et al., 2008). Once *F. circinatum* is established in a nursery it is nearly impossible to eliminate.

Control in nurseries usually consists of soaking the seeds to sterilise them in diluted ethanol, sodium hypochlorite, hydrogen peroxide and hot water (90s at 55 °C). Trays are also sterilised with steam although the most effective method with planting *P. patula* is to only use new trays. Some fungicides are also used and seeds are imbibed with biological control agents such as *Pseudomonas chlororaphis* before cold stratification (Mitchell et al., 2011). Mitchell et al., (2004) tested the effects of the fungicide Benlate and the biological control agent *Trichoderma harzianum* on field survival when applied at different times, in the nursery and at planting. No improvements in field survival were recorded when added in the nursery. Some improvement in the plant survival rate was seen initially but was no longer significant 360 days after planting implying any benefits received from these products are short lived. Similar results were seen by Crous, (2005) where the single application of fungicide or fungicide plus insecticide at the time of planting only increased the survival rate by 13 and 29%, respectively.

Control options in plantations are limited to reducing wounds and the avoidance of drought prone areas. A large amount of tree wounding in plantations occurs due to insect or baboon activity, thus control steps aimed at reducing these need to be taken. Care should also be taken in pruning the trees and when cones are removed, they should be clipped and not sheared off (Wingfield et al., 2008).

One solution is to improve the genetic tolerance of susceptible species such as *P. patula* with

tolerant species clones or hybrids (Mitchell et al., 2012). Large numbers of *Pinus* species are routinely screened for tolerance to *F. circinatum* by infecting open pollinated seedlings from orchard clones and assessing lesion development in controlled greenhouses. Mitchell et al., (2014) compared the tolerance of *P. patula* seedlings to established trees by inoculating 9 year old trees from 96 species in the field and comparing their tolerance levels to seedlings of the same species. They found that breeding for tolerance to Pitch canker is possible by identifying the more tolerant clones based on the performance of their open-pollinated progeny. Roux et al., (2007) focused on finding more tolerant hybrids of different species. They found that the most tolerant hybrids were *P. elliottii* x *P. caribaea* and *P. patula* x *P. oocarpa* and the most susceptible species were *P. patula, P. greggii* and their hybrids.

The breeding of tolerant clones and hybrids is a lengthy process and is not an immediate solution. One possible approach is to investigate integrated pest management systems which incorporate ECM fungi and associating micro-organisms, such as plant growth promoting rhizobacteria in combination with more resistant clones, to improve the resistance of *Pinus* to Pitch canker. ECM fungi have been shown to provide a wide range of benefits to their host trees, which indirectly infer bioprotection.

## 1.4 Aims

Currently the South African nurseries which produce *Pinus* seedlings do not inoculate with ECM fungi at any stage of the production. The presence of ECM fungi, if any, is due to natural colonisation from nearby plantations and residual species within the system. As ECM fungi alter in terms of the benefits they are capable of impacting, it is important to know the levels of colonisation and identity of the ECM fungi present in nurseries.

In chapter 2 the identity and level of ECM colonisation present in 10 South African nurseries were assessed and quantified, by achieving the following aims: (1) establishing the colonisation levels for each nursery, (2) determining if nursery practices have an effect on colonisation levels via a survey, (3) morphological identification of ECM fungi present, and (4) molecular identification of the ECM fungi and other endophytes and/or saprotrophic fungi associated with *Pinus patula* roots using Illumina sequencing.

In chapter 3, local ECM fungal isolates and MHB are assessed for their ability to promote the

growth of *P. patula* seedling and biological control potential against the pathogen *F. circinatum,* by achieved the following aims: (1) locate, culture and identify local ECM fungi, (2) isolate and identify MHB from said ECM fungi, and (3) determine whether ECM fungal inoculum and potential MHB alone or in combination can increase *P. patula* seedling growth and inhibit the pathogen, *F. circinatum*.

In chapter 4 the implications for the use of ECM fungi identified in this study in forestry nurseries is discussed. This includes their potential effects on seedling microbiomes and commercial production of ECM inoculum, along with future work and final conclusions.

# Chapter 2

# Screening of ectomycorrhizal and other associated fungi in South African forestry nurseries

## 2.1 Introduction

*Pinus patula* is the most commonly planted *Pinus* species in South Africa and represents 51% of the total softwood planted area of the South African forestry industry (Roux et al., 2007; DAFF Timber report, 2010/11). Thus, it is important to produce the highest quality *P. patula* seedlings possible for the continued existence of this economically important industry.

Root growth is crucial for the establishment of planted seedlings. Seedlings undergo high levels of stress just after out-planting if their root systems are not able to provide the necessary amounts of water and nutrients to ensure survival. To overcome this transplant shock, seedlings require high root system size and distribution along with root-soil contact and hydraulic conductivity (Grossnickle, 2005; Quoreshi et al., 2008). One way to ensure the seedlings have healthy and large root systems is for seedlings to form symbiotic associations with ectomycorrhizal (ECM) fungi. These fungi have been shown to extend a plant's root system and thus increase the plant's access to nutrients and water, produce plant growth hormones, protect against plant pathogens (Kropp and Langlois, 1990; Quoreshi et al., 2008; Kipfer et al., 2012; Onwuchekwa et al., 2014) and help seedlings overcome transplant shock (Ricon et al.,

2007; Sanchez-Zabala et al., 2013). These attributes contribute towards producing a higher quality nursery stock (Sanchez-Zabala et al., 2013).

Many studies have shown ECM inoculation to have significantly improved nursery seedling growth and survival after outplanting. For example Sanchez-Zabala et al., (2013) found significantly improved height in the field of *P. pinaster* seedlings inoculated with *Lactarius deliciosus, L. quieticolor, Pisolithus arhizus* and *S. luteus* in comparison to the controls. Similar results were found by Ricon et al., (2007) who also reported significantly improved height along with nutrient accumulation in *P. halepensis* Mill. seedlings after inoculating with the ECM fungal species, *Amanita ovoidea,* three isolates of *Suillus collinitus* and *Rhizopogon roseolus.* One and 2 years after outplanting the survival and height of the same seedlings were found to have been significantly increased by two of the isolates of *S. collinitus* in comparison to seedlings inoculated with the other ECM fungi and uninoculated seedlings. Thus, while ECM fungi are highly beneficial to seedlings in and out of the nursery, it is important to select appropriate ECM fungi for a specific host in order to induce optimum seedling growth and health (Ricon et al., 2007; Quoreshi et al., 2008; Kipfer et al., 2012; Sanchez-Zabala et al., 2013).

Numerous studies have shown improved growth of *Pinus* seedlings highlighting the importance of inoculation of ECM fungi as a standard nursery practice. Seedlings do become colonised with ECM fungi in the field, but in low levels and over longer periods of time. ECM fungal colonisation, and the higher nutrient reserves these seedlings have as a result, continue to benefit the seedlings in the years following outplanting (Ricon et al., 2007; Sanchez-Zabala et al., 2013). In South Africa, ECM inoculation in nurseries is rarely practiced. Thus, the main aims of this chapter are a) to determine if *P. patula* seedlings from 10 different South African nurseries (known to not use ECM inoculum) have any natural ECM fungal colonisation and b) if so, what ECM fungi are colonising them via morphological and molecular techniques.

## 2.2 Methods

### 2.2.1 Assessment of ectomycorrhizal colonisation

*P. patula* seedlings mature enough for outplanting into plantations were provided by the Institute for Commercial Forestry Research (ICFR). Eighteen seedlings from the 10 major forestry

nurseries in South Africa were provided. To provide confidentiality each nursery was given an identifying letter, A - J, and seedlings were identified using the nursery letter and their own identifying number. The roots of each seedling were washed free of growth media and separated from the stem. The percentage colonisation for each seedling was determined using a modified line intersect method (Tennant, 1975). The roots were suspended in water over a 2 x 2 cm grid and photographed. The photos were then analysed and every time a root intersected, touched or crossed a gridline a 1 was recorded. A 2 was recorded if a curved portion of root lay over a gridline. If any section of root which came into contact with a gridline was colonised by ECM fungi this was recorded along with its mark representing a gridline intersection. The seedling root lengths were then determined using the equation below (Equation 1). The percentage colonisation was the percentage of intersection marks which represented roots with ECM fungal colonisation from the seedling's overall number of intercepts (Tennant, 1975). These results were then statistically analysed.

Equation 1

Root length (R) = Number of intercepts (N) x 1.5714 (Length conversion factor)

### 2.2.2 Nursery survey

A survey was sent out to the 10 different nurseries asking about their different growing practices; such as substrate use, seedling tray sanitation techniques, anti-microbial treatments, the use of any seed treatments, and ECM inoculum use. These were then compared to the colonisation results to determine if any of these practices could possibly be the cause for the differences between nurseries that were observed. The full survey is attached in Appendix C.

### 2.2.3 Morphological identification

Colonised root tips were collected and photographed using a dissecting microscope (Olympus BX50). The roots were then separated into different morphotypes based on mantle morphology and anatomical characteristics. The key morphological features examined were the shape of branches, the mantle colour, surface texture, presence of rhizomorphs and emanating hyphae (Table 2.1). Further examination of the mantle was performed by peeling mantle sections from colonised whole roots which were mounted in lactoglycerol and examined using light

microscopy (LEICA CME). These sections were examined for surface and inner mantle hyphal arrangements, pigmentation, specialised hyphae and rhizomorph hyphae (Figure 2.1), as described by Agerer, (1987-2012). Characterisation for each morphotype was based on a minimum of 5 different root tips (Hawley *et al.,* 2008).

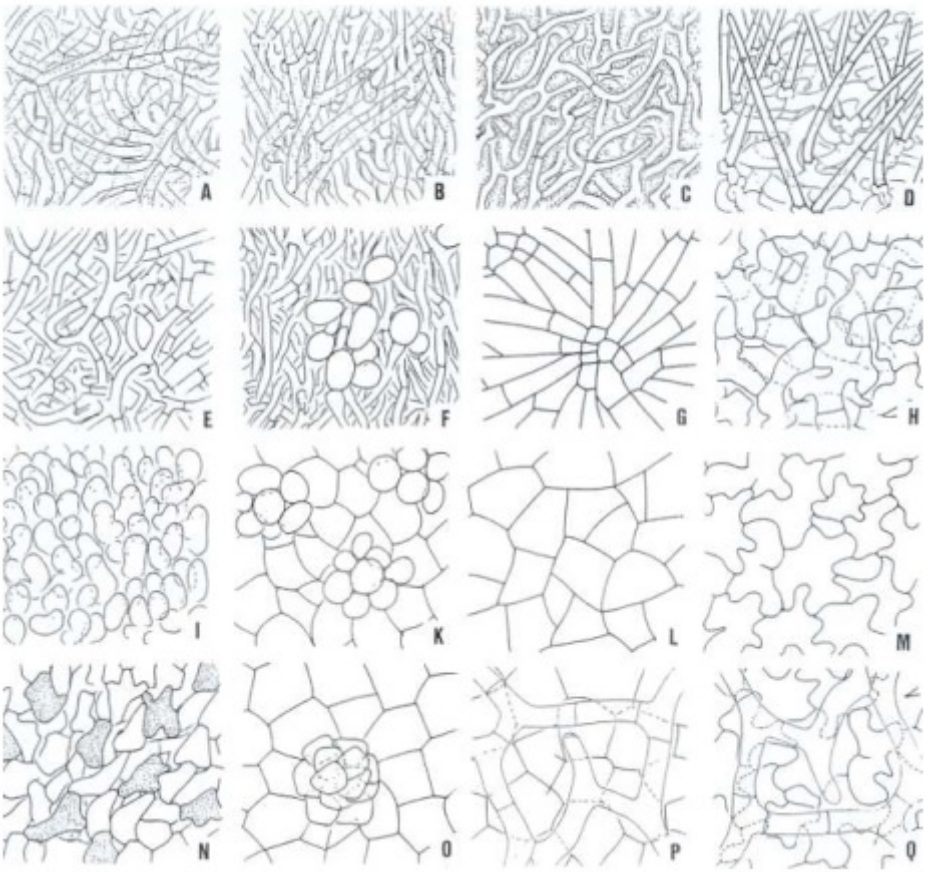



Figure 2.1: Mantle types as seen from mantle scrapings. (a) plectenchymatous, (b) irregular plectenchymatous, (c) plectenchymatous in gelatinous matrix, (d) net-like plectenchymatous with prominent cystidia, (e) repeatedly branched and net-like hyphae, plectenchymatous, (f) round cells on a plectenchymatous layer, (g) arrangement is star-like, tightly plectenchymatous, (h) transitional plectenchymatous to pseudoparenchymatous, irregular, (i) plectenchymatous, hymeniform, (k) angular pseudoparenchymatous, with mounds of round cells, (l) angular pseudoparenchymatous, (m) epidermoid pseudoparenchymatous, (n) pseudoparenchymatous, shape variable and staining with sulfo-vanillin, (o) angular pseudoparenchymatous, with mounds of flattened cells, p) angular pseudoparenchymatous, with hyphal net, (q) epidermoid pseudoparenchymatous, with hyphal net (Taken from Agerer, 1987-2012)

Table 2.1: Characteristics recorded for each of the ECM fungal morphotypes (Adapted from Agerer, 1987-2012)

| **ECM fungal root tip morphology** | **ECM fungal mantle morphology** |
|---|---|
| **Branching type (ramification)**<br>• Simple<br>• Pinnate<br>• Irregular<br>• Dichotomous<br>• Coralloid<br>• Tubercle-like | **Inner surface** (hyphal arrangement) (Figure 2.1)<br>• Specialised cells (dimensions)<br>• Cystidia, lactifers, tannin cells<br>• Hyphal dimensions (Width and length of cells) |
| • Length and diameter of branches | **Middle layers** (hyphal arrangement) (Figure 2.1)<br>• Hyphal dimensions<br>• Specialised cells (dimensions and frequency)<br>• Wall thickness |
| **Axis**: last branch axis (last order branch) | **Outer surface** (hyphal arrangement) (Figure 2.1)<br>• Hyphal dimensions |
| **Shape of ramified ends**<br>• Straight<br>• Bent<br>• Tortuous<br>• Beaded | **Rhizomorph type and specialisation**<br>• Emanating hyphae (wall thickness and shape)<br>• Specialised hyphae: Cystidia, lactifers, tannin cells |
| **Mantle surface texture**<br>• Smooth<br>• Reticulate<br>• Grainy<br>• Spiny<br>• Cottony or woolly | **Other:**<br>• Surface oil droplets<br>• Crystals<br>• Soil particles |
| • Mantle colour | |
| **Rhizomorphs:**<br>• Colour, attachment and shape<br>• Attachment: restricted, angled or fanned<br>• Shape: filamentous, smooth or hairy | |
| • Emanating hyphal features | |

### 2.2.4 Molecular identification

ECM fungal identification was first attempted on single colonised root tips, but when sequenced using Sanger sequencing, the ECM fungal ITS genes were not the dominant sequence and mixed sequences were often produced. To overcome this problem cloning was attempted. Cloning was not able to provide enough information about the ECM fungal community found on the *P. patula* seedlings. To form a more comprehensive analysis of the fungal community as a whole present on colonised roots next-generation Illumina sequencing was performed.

#### 2.2.4.1 Molecular analysis via cloning

##### 2.2.4.1.1 DNA extraction

All visibly colonised root tips from 5 seedlings representing the nurseries A, I, C, D, and G, seedlings: A 452, I 11, C 11, D 663, G 452, were collected and kept in RNAlater (Sigma R0901-100ML-PW) until DNA extraction.

The DNA extraction was performed using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research D6005) according to the manufacturer's protocol. Prior to the lysis step, the ECM fungal colonised root tips were homogonised in 200 µl of sterile water using a micro-pestle to assist in the extraction of genetic material. The fungal root tip mixtures were then transferred to ZR BashingBead$^{TM}$ Lysis tubes along with 650 µl of lysis solution. The tubes were secured to a bead beater (Labnet) and processed for 5 minutes, to ensure cell membrane disruption, and then centrifuged (Hanzhou Allsheng Instruments, Supermini Centrifuge) at 10 000 rpm for 1 minute. From the supernatant 400 µl was transferred to a Zymo-Spin$^{TM}$IV Spin Filter and collection tube and centrifuged at 7 000 rpm for 1 minute, to filter out any cell debris which was not pelleted out. To the filtrate produced, 1 200 µl of DNA Binding Buffer was added. The DNA was then collected by adding 800 µl to a Zymo-Spin$^{TM}$ IIC Column within a collection tube and centrifuged at 10 000 rpm for 1 minute, causing the DNA to bind to the membrane within. The flow through was discarded and the step was repeated using the remaining 800 µl. Next, the DNA collected was washed by adding 200 µl of DNA Pre-Wash Buffer to the spin column and then 500 µl of the DNA Wash Buffer centrifuging at 10 000 rpm in between each addition of buffer. To ensure all the ethanol from the wash buffer was removed, the spin column was then transferred to a new column and re-centrifuged for an additional minute. To elute and collect the DNA, the spin columns were placed in 1.5 ml centrifuge tubes and placed in a dry bath set to

50°C for 2 minutes to encourage evaporation of any remaining ethanol. After this 25 µl of sterile water, also warmed at 50°C, was added directly to the membrane and after 2 minutes incubation at room temperature, was centrifuged at 10 000 rpm for 30 seconds. This was repeated with another 25 µl, to ensure all DNA was eluted from the spin column membrane, producing a final volume of 50 µl. The successful extraction of DNA was confirmed by visualisation on a 1% agarose (Promega V4121) gel stained with ethidium bromide (Merck 1.11608.0030) and photographed using a UV Transilluminator (Bio Rad ChemiDoc$^{TM}$, Universal Hood II).

#### 2.2.4.1.2 PCR

The Internal Transcribed Spacer (ITS) region of rDNA gene was amplified via PCR using the primers ITS1F and ITS4 (Table 2.2) (White et al., 1990). The 25 µl PCR reaction consisted of: 12.3 µl of sterile distilled water, 5 µl of 5X KAPA HiFi GC Buffer, which contained 2 mM $MgCl_2$ (1X), 5 µl of the template DNA, 0.75 µl of 10 mM dNTP mix, 0.75 µl of each primer and 0.5 µl of the KAPA HiFi HotStart DNA Polymerase at a concentration of 1 U/µL (Lasec KR0369). The PCR amplification process followed the cycle parameters described in table 2.3 below. The size of the amplified genes and success of the PCR were determined via visualisation on a 1% ethidium bromide (Merck 1.11608.0030) agarose (Promega V4121) gel sized against a 100 bp ladder (Promega G210A).

All agarose gel visualizations performed in this study were performed with a 1% agarose ethidium bromide stained gel and bands were sized against a 100 bp ladder.

Table 2.2: Nucleotide sequence of primers used to identify ectomycorrhizal fungi via cloning.

| **Primer** | **Sequence** |
|---|---|
| ITS1F | 5'- CTTGGTCATTTAGAGGAAGTAA -3' |
| ITS4 | 5'- TCCTCCGCTTATTGATATGC -3' |

Table 2.3: Cycling parameters for PCR of ITS rDNA region.

| **Conditions** | **Temperature (°C)** | **Time (seconds)** | **Cycles** |
|---|---|---|---|
| Initial Denaturation | 95 | 300 | 1 |
| Denaturation | 98 | 30 | 25 |
| Annealing | 47 | 45 | |
| Extension | 72 | 60 | |
| Final Extension | 72 | 420 | 1 |

#### 2.2.4.1.3 Cloning

The PCR products were then ligated into the plasmid cloning vector pGem®-T Easy vector (3015 bp) (Promega A1360). The ligation reaction contained 5µl of 2x Rapid Ligation Buffer, 1 µl of the pGem®-T Easy vector, 2 µl of the PCR product, 1 µl T4 DNA ligase at a concentration of 3 Weiss units/µl and 1 µl of sterile water to make up a final volume of 10 µl. This reaction was then incubated overnight at 4°C to allow for a maximum number of transformants.

Competent cells for the transformation were made using *Escherichia coli* strain DH5 alpha. A single colony of *E . coli* was inoculated into 5 ml of luria broth (LB) and incubated on a shaker at 37°C, shaking, for 2-3 hours, till the optical density at 600 nm was ~0.6. The cells were then placed on ice for 10 minutes, then centrifuged at 6000 rpm for 3 minutes at 4°C. After this, the supernatant was discarded and the remaining pellet of cells was then resuspended in 10 ml of cold, sterile, 0.1M $CaCl_2$, which creates pores in the cell's membrane thus making them competent. The cells were then left on ice for an additional 20 minutes and recentrifuged at 6000 rpm for 3 minutes to form a pellet and then resuspended in 5 ml of cold sterile 0.1M $CaCl_2$15% glycerol solution. The addition of the glycerol acts as a cryoprotectant for the now competent cells. The cells were then dispensed into pre-chilled 1.5 ml microcentrifuge tubes and kept at -80°C till needed (Brooks, personal communication).

The ligation reactions were then transformed into competent cells, by first incubating the ligation with 60 µl of competent cells for 30 minutes. The mixture was then placed in a water bath at 42°C for 45 seconds to heat shock the cells into allowing the foreign DNA into the cells. The cells were then put back on ice for 2 minutes to allow the cell membranes to stabilise after which 500 µl of LB was added and incubated at 37°C, shaking, for 45 minutes to allow the cells to grow and multiply. After incubation the cells were spun down at 6000 rpm for 45 seconds and the supernatant was removed leaving behind approximately 100 µl, in which the cells were resuspended. The resuspended plasmid containing cells were then plated onto blue white screening plates made by spread plating 40 µl of X-gal at a concentration of 20 mg/ml (0.0113 g of X-Gal (Thermo Scientific R0401) and 565 µl of dimethylformamide (Sigma-Aldrich 227056)), 40 µl of IPTG at a concentration of 20 mg/ml (0.01 g IPTG (Thermo Science R0392) and 500 µl of sterile water) and 20 µl of ampicillin (Sigma A9518-5G) at a concentration of 100 mg/ml onto each luria agar (LA) plate. The LA plates were then incubated overnight at 37°C. After incubation a number of white colonies, between 5-6 per plate, were picked using a sterile toothpick and inoculated into 5 ml LB with ampicillin to a working concentration of 10 mg/ml, and incu-

bated, once again shaking, at 37°C overnight. The colonies were coded with their nursery code and colony number.

The plasmids were then extracted from the competent cells using the GeneJET Plasmid Miniprep Kit (ThermoFisher K0502) according to the manufacturer's protocol. All centrifugation steps were performed at 12 000 rpm. One ml from each culture was aliquoted into 1.5 ml micro-centrifuge tubes and centrifuged for 1 minute to pellet the cells, the supernatant was discarded and another 1 ml was added to each tube and recentrifuged to increased the amount of cells the extraction would be performed on. The cells were then resuspended in 250 µl of resuspension solution, which contained RNase A to begin the lysis process. Once resuspended, 250 µl of the lysis solution was added and mixed by inverting the tube gently, to avoid shearing the genetic material, a maximum of 5 times, until the solution became viscous and slightly clear. Then 350 µl of the neutralization solution was added and also mixed via inverting the tube a maximum of 5 times, until the lysate became cloudy, to halt the lysis process. The microcentrifuge tubes were then centrifuged for 5 minutes to pellet out the chromosomal DNA and cell debris. The supernatant was then transferred to the GeneJET spin column placed in a collection tube. The spin column was then centrifuged for 1 minute after which the flow through was discarded. Five hundred µl of the wash solution was then added to the spin column before centrifugation for 1 minute and the flow through discarded; this step was performed twice. The spin column was then centrifuged for 1 minute empty and incubated on the bench at room temperature for an additional minute, to remove residual ethanol left over from the wash steps. The spin column was then placed in a new sterile microcentrifuge tube and 25 µl of the elution buffer was added directly to the column membrane and incubated at room temperature for 2 minutes and centrifuged for 2 minutes. This step was performed twice to recover residual DNA, to produce a final volume of 50 µl of extracted plasmid.

To ensure successful insertion of the ITS gene the plasmids were digested with FastDigest *Eco*R1 (ThermoFisher FD0274). The digestion reaction contained 15 µl of sterile water, 2 µl of the 10x FastDigest green buffer, 2 µl of the plasmid and 1 µl of the FastDigest enzyme. The solution was gently mixed via pipetting up and down and then incubated at 37°C in a heating block (Labnet AccuBlock$^{TM}$) for 20 minutes. To ensure that selections made were distinct from one another, a further 2 digestions were performed on extracted plasmid samples.

The plasmids which contained an insert were then digested with the FastDigest *Taq*1 (ThermoFisher FD06772) and FastDigest *Hin*fI (ThermoFisher FD0804) (Anderson et al., 2014) to

discern the differences between samples. As the enzymes have different incubation temperatures a digestion reaction was set up as above with the primer *Hin*fI and incubated at 37°C for 5 minutes after which 1 µl of the *Taq*1 enzyme was added and incubated at 65°C for an additional 5 minutes. The samples were also digested separately with each enzyme as per above. The digests were then visualised on a 1% ethidium bromide agarose gel and analysed for differences and similarities. Two representative samples of each digestion profile were then sent to Inqaba Biotechnology Industries (Pty) Ltd. Pretoria for Sanger sequencing, forward and reverse, with M13 primers (Yun et al., 2000). The resulting Sanger sequences were analysed using Mega 7 (Kumar et al., 2015) and identified to genus level using BLAST GenBank (www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). The sequences were then submitted to GenBank and accession numbers received.

#### 2.2.4.2 Molecular identification via next-generation sequencing

##### 2.2.4.2.1 DNA extraction

For Illumina sequencing all visibly ECM fungal colonised root tips were removed from 3 seedlings from 3 different nurseries - 9 seedlings in total. These seedlings when combined provided the best coverage of all fungal morphotypes, and include the following seedlings from nurseries A, G and D: A 53, A 25, A 613, G 523, G 533, G 462, D 13, D 533 and D 26. The root tips were then kept in RNAlater for 3 days at 4°C prior to DNA extraction. DNA extraction was performed as described above in section 2.2.4.1.1

##### 2.2.4.2.2 PCR amplification

A nested PCR was performed on the ITS region to reduce nonspecific binding and thus reduce the possibility of non fungal sequences being amplified (Figure 2.2). This PCR was firstly performed to amplify the entire Internal Transcribed Spacer (ITS) region of the rDNA gene using the fungal primers ITS1F and ITS4, as described in section 2.2.4.1.2

The amplified ITS rDNA genes were then gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega A9281) according to the manufacturers protocol, using sterile techniques. The bands were cut out of the agarose gel and placed into 1.5 ml microcentrifuge tubes, to which 10 µl of Membrane Binding solution per 10 mg was added. The tube was then vortexed and incubated at 50°C for 3-5 minutes until the gel slice was completely dissolved and vortexed

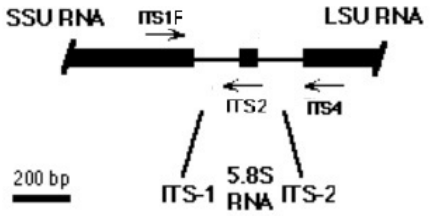



Figure 2.2: Internal Transcribed Regions (ITS) with the forward and reverse primers used to amplify the ITS region (Modified from Vilgalys, n.d)

again briefly to allow ensure the DNA was mixed. The solution was then transferred into a SV minicolumn and collection set up and incubated at room temperature for a minute to allow the DNA to bind with the membrane; after this the set up was centrifuged for 1 minute at 10 000 rmp. The flow through was then discarded and 700 µl of Membrane Wash Solution was added to the minicolumn and centrifuged at 13 400 rpm for a minute. This step was then repeated with 500 µl of the wash solution and centrifuged for 5 minutes to ensure the DNA was washed of any impurities. As the wash solution contains ethanol the flow through was discarded and the minicolumn set up was centrifuged empty at 13 400 rpm for one minute. The minicolumn was then placed in a new sterile 1.5 ml microcentrifuge tube and incubated empty at room temperature for 2 minutes to ensure all residual ethanol was evaporated off. Forty µl of nuclease-free water was then added directly to the membrane within the minicolumn and incubated at room temperature for 1 minute before centrifuging at 13 400 rpm for 1 minute to elute the DNA. The cleaned PCR product was then visualized to confirm success.

The second part of the nested PCR was then performed on the cleaned up ITS genes to amplify the ITS1 section of the gene. This was performed using the tagged primers Miseq-ITS1F and Miseq-ITS2 (Table 2.4) (White et al., 1990). The Miseq adapters (Earth Microbiome Project) were modified by Dr. Matcher, Rhodes University. These adapters provide a binding area for the Illumina primers used in the downstream processes. As above, 25 µl of the PCR product was used for the nested PCR amplification process which followed the protocol set out in table 2.5. The size of the amplified genes were determined via visualisation and gel purified as described above.

#### 2.2.4.2.3 Illumina sequencing

An additional PCR was undertaken to attach the MID tags to the PCR products produced above

Table 2.4: Nucleotide sequence of primers used to amplify ITS1 region for downstream Illumina sequencing.

| Primer | Sequence |
|---|---|
| Miseq-ITS1F | 5'- TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCTTGGTCATTTAGAGGAAGTA A-3' |
| Miseq-ITS2 | 5'- GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGGCTGCGTTCTTCATCGATGC-3' |

Table 2.5: Cycling parameters for PCR of ITS1 rDNA region.

| Conditions | Temperature (°C) | Time (seconds) | Cycles |
|---|---|---|---|
| Initial Denaturation | 95 | 300 | 1 |
| Denaturation | 98 | 30 | |
| Annealing | 45 | 30 | 15 |
| Extension | 68 | 30 | |
| Denaturation | 98 | 30 | |
| Annealing | 65 | 30 | 15 |
| Extension | 68 | 30 | |
| Final Extension | 72 | 420 | 1 |

using the Nextera XT index kit where 5µl of each adapter, N7XX and S5XX, was added. The reaction contained 25 µl KAPA Hifi Hotstart Ready Mix, 5 µl of each adapter, 5 µl of the PCR product and 10 µl of sterile water. The PCR conditions were initial denaturation at 95°C for 3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by the final extension step of 72°C for 5 minutes. The reactions were then cleaned up with AMPure XP beads and quantified using Picogreen, after which they were normalised and pooled together.

The samples were then sent to the University of the Western Cape for illumina Miseq sequencing using the Nextera XT version 3 where 200 cycles x2 were undertaken.

### 2.2.5 Statistical and bioinformatical analysis

A Shapiro test was performed on the percentage colonisation to determine normality. The null hypothesis was rejected showing the data was non-parametric. Thus, a Kruskal-Wallis Analysis of Variance (ANOVA) was performed and Tukey's Post-hoc test was used to determine

significant difference between treatments. All statistical analyses and rarefaction curve plotting were performed using RStudio Version 0.99.903 (RStudio team, 2015).

Analysis of Illumina results was undertaken with only the forward read sequences as the reverse reads were not usable. Analysis was performed using the WinSCP Version 5.11.2 (Build 7781) platform where Mothur v.1.38.1 (Schloss et al., 2009) was run from the CHPC servers (https://www.chpc.ac.za/). Sequences were analysed using the UNITE version 6 dynamic dataset. A complete log of the mothur commands can be found in Appendix D.

NMDS plots and heatmap were performed using Primer 7.

## 2.3 Results

### 2.3.1 Assessment of ectomycorrhizal colonisation

The percentage colonisation of each seedling from each nursery was calculated and averaged to produce a final percentage colonization for each of the 10 nurseries.

As can be seen from figure 2.3 the overall ECM percentage colonisation was low with only nursery G reaching above 20% colonisation. Nurseries B, F and J all had extremely low colonisation percentages of 3, 2 and 3%, respectively. The differences could be due to the different nursery practices, recorded below.

### 2.3.2 Nursery survey

Nurseries did not differ significantly in terms of management practices, especially when grouped into categories of levels of protection (Table 2.6). The one main and most important difference was the lack of any anti-microbial treatment used by the two nurseries which had above 15% ECM fungal colonisation. It is very likely that this lack of anti-microbial application, specifically the absence of chemical use, is the reason for these nurseries' higher ECM colonisation.

Pine bark was used by all 10 nurseries with 50% of the nurseries using it as their only substrate. The most favoured combination was found to be with coir, often in combination with vermiculite (Figure 2.4 ).

Table 2.6: Summary of the different nursery practices broken down into colonisation level categories.

| | Nursery practices | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Substrate type | | | | | | Seedling tray sanitation | | Anti-microbial treatments | | | | |
| Colonisation level | Pine Bark | Coir | Vermiculite | Peat | Perlite | Other | Steam | Chemical | Biological | Chlorine | Hard chemicals | Soft chemicals | None |
| Moderate >15% | X | X | | | | | X | X | | | | | X |
| Low 14 - 5% | X | X | X | X | X | | X | X | X | X | X | X | |
| Very low <5% | X | X | X | | | X | X | X | | X | X | X | |

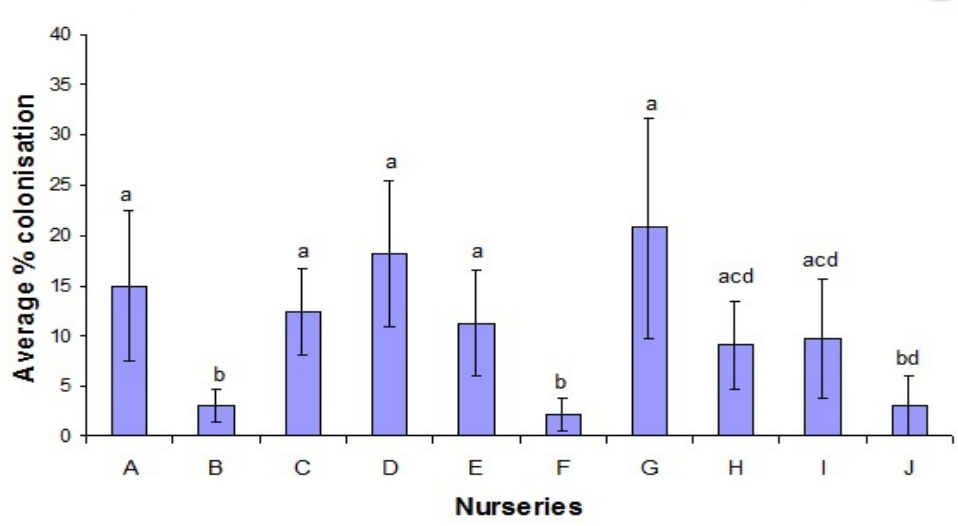



Figure 2.3: Summary of average percentage ectomycorrhizal colonization of *Pinus patula* seedlings for each of the ten South African nurseries. Kruskal-Wallis test; H: (9, N=180) =108.7233 p=0.00001. Different letters represent significant differences.

Half of the nurseries used a combination of steam and chemicals, such as Sporekill®, Everdip copper, chlorine or sodium hypochlorite (Figure 2.5) for the sanitation of seedling trays.

Many different anti-microbial treatments were used by some of the nurseries (Figure 2.6). The most commonly used anti-microbial treatment was chlorine, which was used by all the nurseries applying treatments. The nurseries which used chlorine in combination with chemicals did so usually with soft chemicals, such as Sporekill® and Celest® and less regularly with hard chemicals such as Benlate® and copper oxychloride. Nursery C combined all the above with the biological anti-microbials *Trichoderma* sp. and *Bacillus subtilus.* Nurseries G and D did not use any anti-microbial treatments and were the nurseries with the highest ECM fungal colonisation percentage (Table 2.3), 21 and 18% respectively.

None of the nurseries used any ECM fungal inoculum.

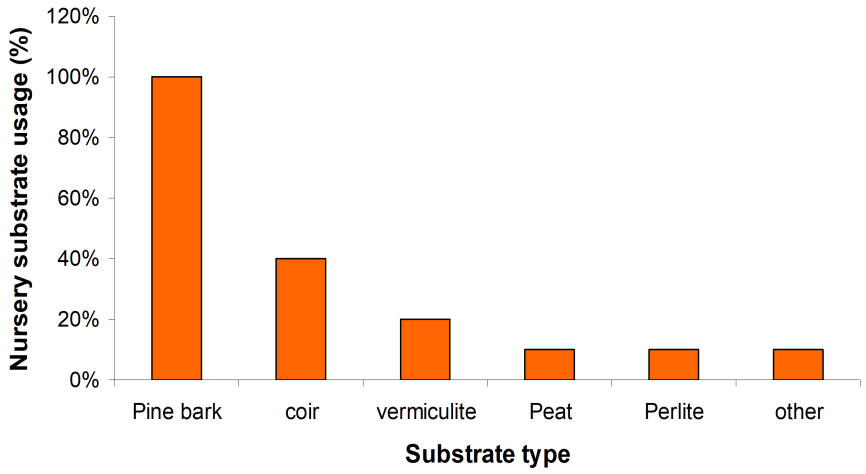



Figure 2.4: Summary and frequency of the different substrate types used in *P. patula* seedling production by the 10 South African nurseries.

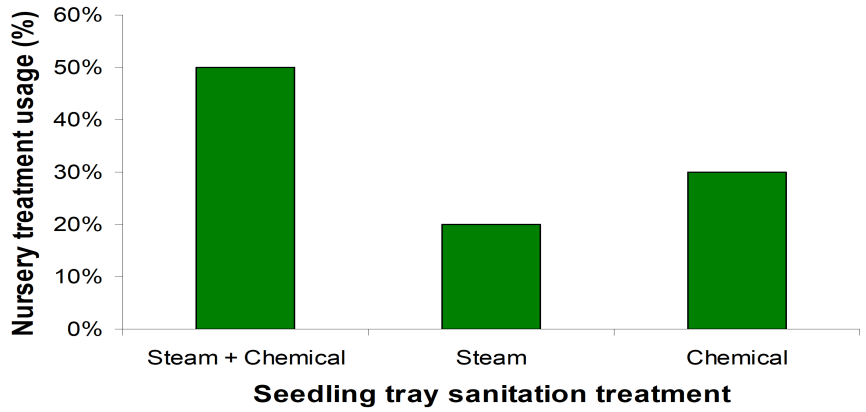



Figure 2.5: Summary and frequency of the different seedling tray sanitation treatments used in *P. patula* seedling production by the 10 South African nurseries.

### 2.3.3 Morphological identification

A total of 8 distinct morphotypes were identified from the 10 different nurseries and characterised. These morphotypes will be referred to as “Brown”, “Beige”, “White”, “Grey”, “Yellow”, “Yellow-brown”, “Black”, and “Yellow-orange”. The morphotype “Brown” was the most

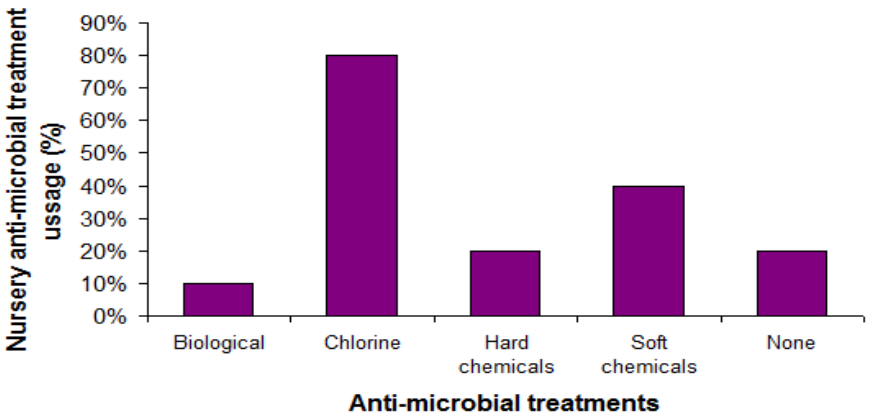



Figure 2.6: Summary and frequency of the different anti-microbial treatments used in *P. patula* seedling production by the 10 South African nurseries.

commonly found morphotype, in 8 of the 10 nurseries (Figure 2.7). It is believed that the majority of these ECM roots were in the early stages of colonisation, thus a number of defining characteristics such as rhizomorphs and hyphal mantles were not fully formed or as abundantly present.

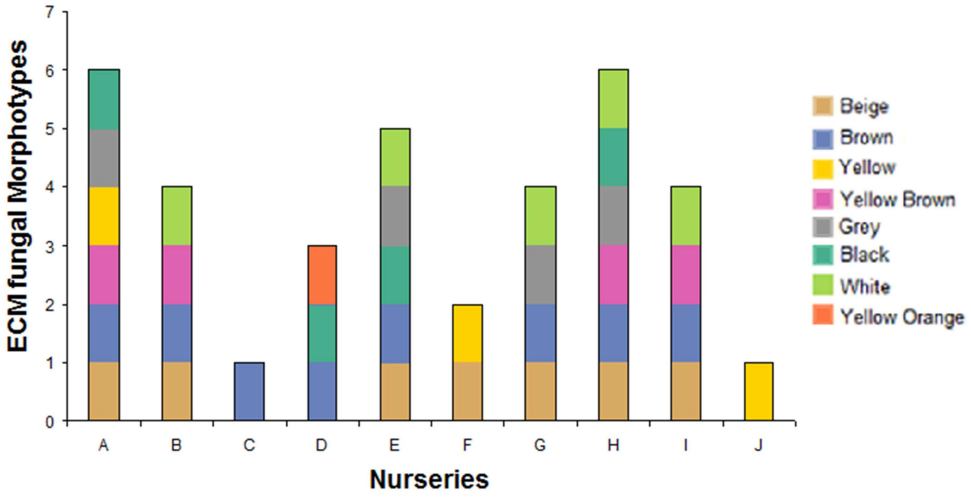
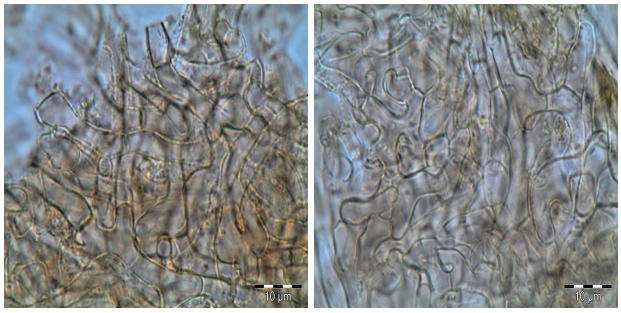



Figure 2.7: The ECM fungal diversity for the 10 *Pinus patula* nurseries, divided into the different morphological types.

While "Brown" was the most commonly found morphotype it was not possible to morphologically identify it. This morphotype had dichotomous ramifications with straight ramified ends.

The mantle surface was reticulate, brown, paler at the tips and fading to a darker brown as it aged (Figure 2.8a). Very few rhizomorphs were seen but those present did not have a defining colour (probably due to age), a restricted attachment, smooth and consisting of undifferentiated compactly arranged hyphae (Figure 2.8b). Both the inner and outer mantle layer were plectenchymatous, beginning with highly compacted inner mantle loosening in the outer mantle to the loosely arranged outer mantle surface where clamped hyphae were visible (Figures 2.8c,d,e).

(a) (b) (c)



(d) (e)

Figure 2.8: Morphotype "Brown" anatomical and morphological features a) ECM fungal root tips with a brown mantle sheath, paler at the tips due to age, and dichotomous branching, scale bar represents 200 μm. b) b) Rhizomorph showing undifferentiated compactly arranged hyphae, c) loosely arranged mantle surface with clamped hyphae indicated by the arrow, d) loosely arranged plectenchymatous hyphae of outer mantle layer, e) highly compacted plectenchymatous arrangement of the inner mantle, repeatedly branched and net like. Scale bar represents 10 μm.

The morphotype "Yellow-Brown" was very similar to "Brown" in appearance and colouring. Yet no rhizomorphs were found. This morphotype was found growing in large clusters with multiple branchings often stemming from a single offshoot. It had dichotomous short straight

(which may be due to young ECM) ramified ends and a reticulate mantle with yellow/beige root tips with a darker brown at the base (Figure 2.9a). The outer surface of the mantle was loosely arranged. The outer mantle had a plectenchymatous hyphal arrangement transitioning to a tighter plectenchymatous irregular arrangement in the inner mantle layer (Figures 2.9b,c). This morphotype was morphologically identified as *Thelephora terrestris*.

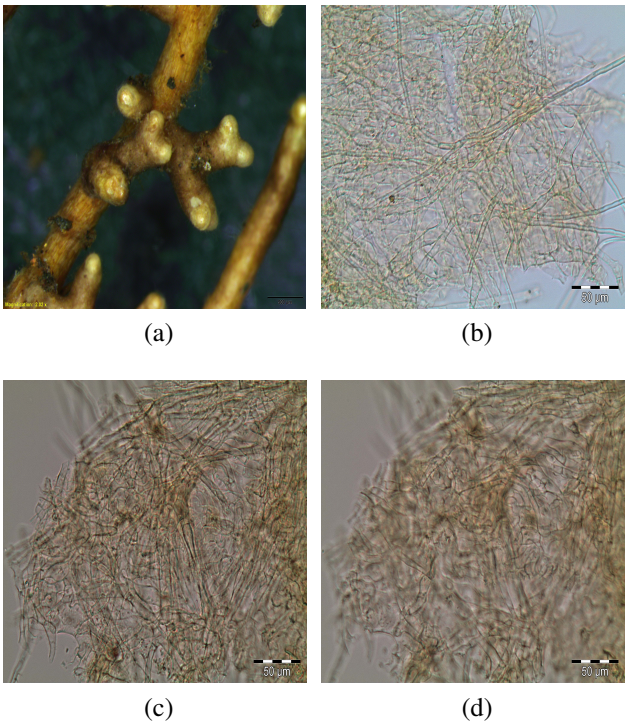



Figure 2.9: Morphotype "Yellow-Brown" anatomical and morphological features a) ECM fungal root tips with a brown going pale yellow at the tips, mantle sheath and dichotomous branching, scale bar represents 500 μm. b) Loosely arranged mantle surface c) plectenchymatous outer mantle hyphal arrangement transitioning to d) a tight plectenchymatous inner mantle layer. Scale bar represents 50 μm.

The "White" morphotype was tentatively morphologically identified as a *Boletus* species, species identification was not possible without more information. The morphotype was characterised by dichotomous ramifications with straight ramified ends. It also had a white shiny mantle with a reticulate, slightly fuzzy mantle appearance (Figure 2.10a). No rhizomorphs were seen, but this lack could have been as a result of young ECM age rather than morphological lack thereof. Unlike the "Brown" morphotype this morphotype did not have clamped hyphae, although a

loosely arranged outer mantle surface was observed along with plectenchymatous mantle layers of no discernable pattern (Figures 2.10c,d).

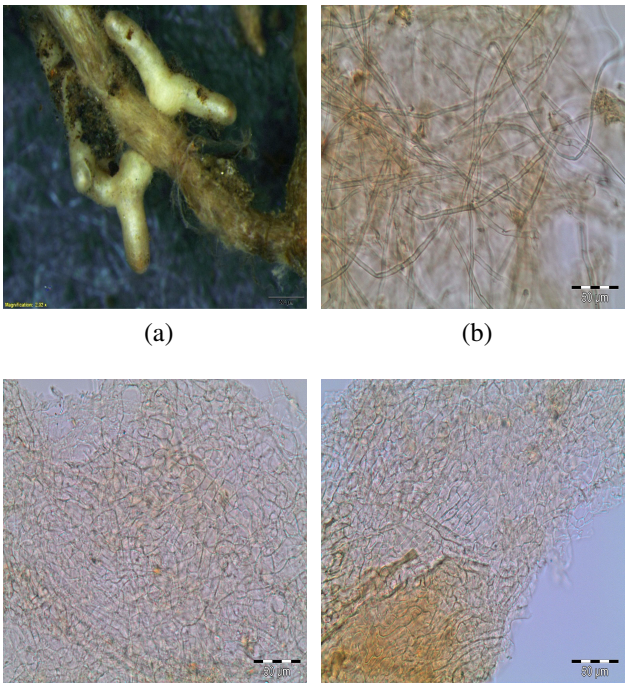
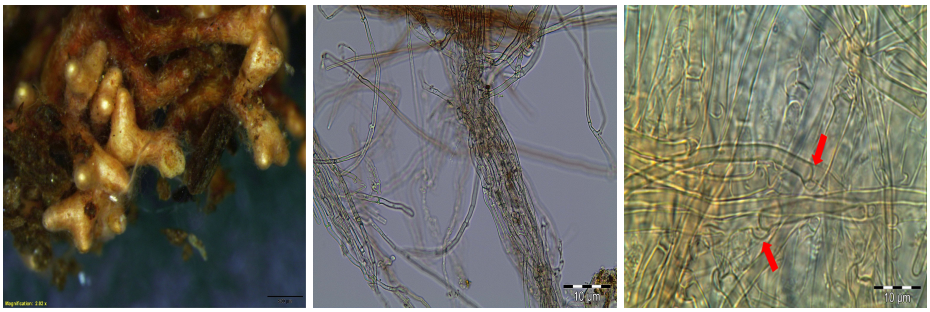
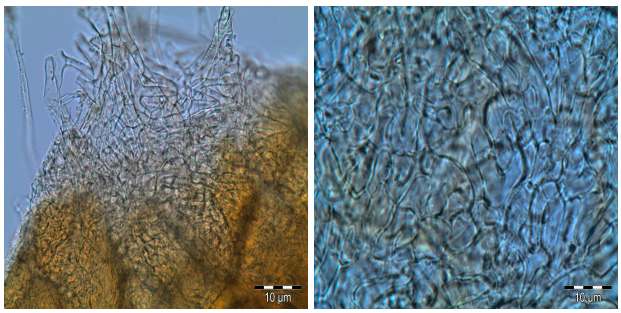


Figure 2.10: Morphotype "White" anatomical and morphological features a) ECM fungal root tips with a white mantle sheath, dichotomous branching, scale bar represents 500 μm. b) Loosely arranged mantle surface, c) outer plectenchymatous mantle hyphal layer, d) inner mantle plectenchymatous hyphal layer both with no discernible pattern. Scale bar represents 50 μm.

As with "Brown" and "White" the morphotype "Beige" had dichotomous ramifications with straight ramified ends although they grew more clustered together. It was also characterised by a beige, reticulate, shiny mantle which was paler at the end (Figure 2.11a). Multiple rhizomorphs were associated with this morphotype which were pale beige in colour, angled and slightly hairy. The hyphae within the rhizomorphs were undifferentiated and loosely woven (Figure 2.11b). The mantle consisted of a loose outer mantle surface with clamped hyphae, and an outer plectenchymatous layer transitioning to the inner pseudoparenchymatous layer (Figure 2.11c,d,e). This morphotype was morphologically identified as belonging to the *Russula* genus.



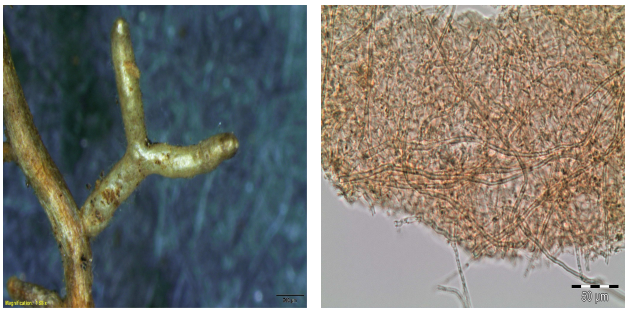
(a) (b) (c)



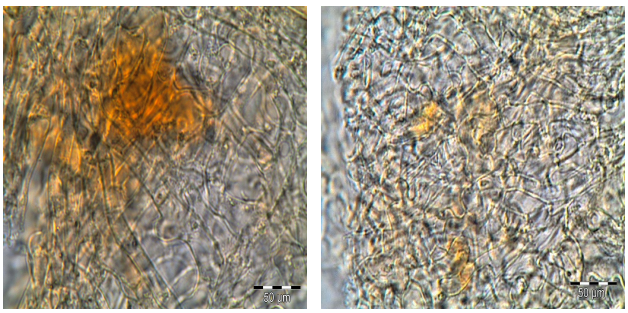
(d) (e)

Figure 2.11: Morphotype “Beige” anatomical and morphological features a) ECM fungal root tips with a beige mantle sheath, paler at the tips due to younger age, with multiple examples of dichotomous branching, scale bar represents 500 µm. b) Rhizomorph showing undifferentiated loosely woven hyphae, c) loosely arranged outer mantle surface with clamped hyphae indicated with arrows, d) outer plectenchymatous beginning to transition to pseudoparenchymatous mantle layer e) inner mantle showing a pseudoparenchymatous hyphal arrangement. Scale bar represents 10 µm.

The “Grey” morphotype had dichotomous ramifications with slightly bent ramified ends and a grey/silvery, reticulate mantle (Figure 2.12a). No rhizomorphs nor clamped hyphae were observed. The mantle consisted of an inner and outer layer of plectenchymatous hyphal arrangements tightening from outer to inner (Figures 2.12b,c). It could not be morphologically identified to a specific genus.



(a) (b)

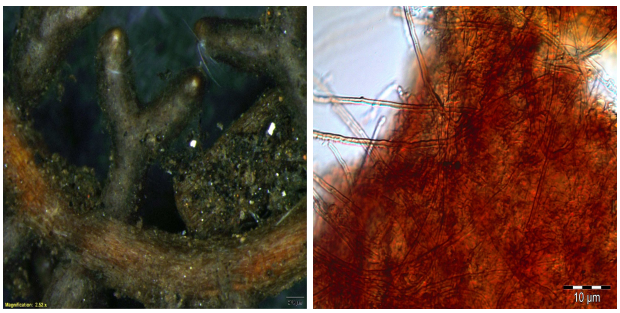


(c) (d)

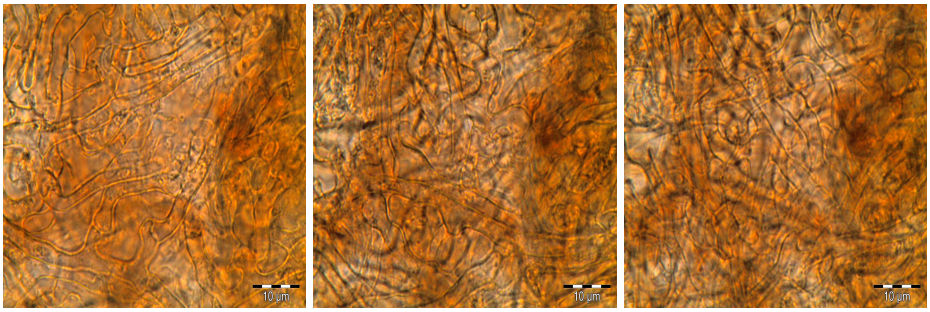
Figure 2.12: Morphotype "Grey" anatomical and morphological features a) ECM fungal root tips with a grey, silvery mantle sheath and dichotomous branching, scale bar represents 500 μm. b) Loosely arranged mantle surface, c) plectenchymatous outer mantle hyphal arrangement, d) compacted plectenchymatous inner mantle layer. Scale bar represents 50 μm.

The "Black" morphotype, while found in multiple nurseries, produced only a few examples per nursery. The root tips were dichotomously ramified with straight ramified ends with a darkly black reticulate mantle (Figure 2.13a). No rhizomorphs were produced. The outermost mantle consisted of loosely arranged hyphae; the outer, middle and inner layers of the mantle all consisted of plectenchymatously arranged hyphae with no discernable pattern, with both the outer and middle layers set into a gelatinous matrix (Figure 2.13b,c,d,e). This morphotype was morphologically identified as a *Pseudotomentella.*

The "Yellow-Orange" morphotype was the only morphotype found which presented a tuber like form of ramifications with a thick yellowy-beige reticulate mantle and lump like growth pattern (Figure 2.14a). The rhizomorphs produced were restricted with distinct, smooth margins and a slight "fluffy" halo under the dissecting microscope. The hyphae forming the rhizomorphs were differentiated with thick hyphae forming a central core. The mantle surface consisted of



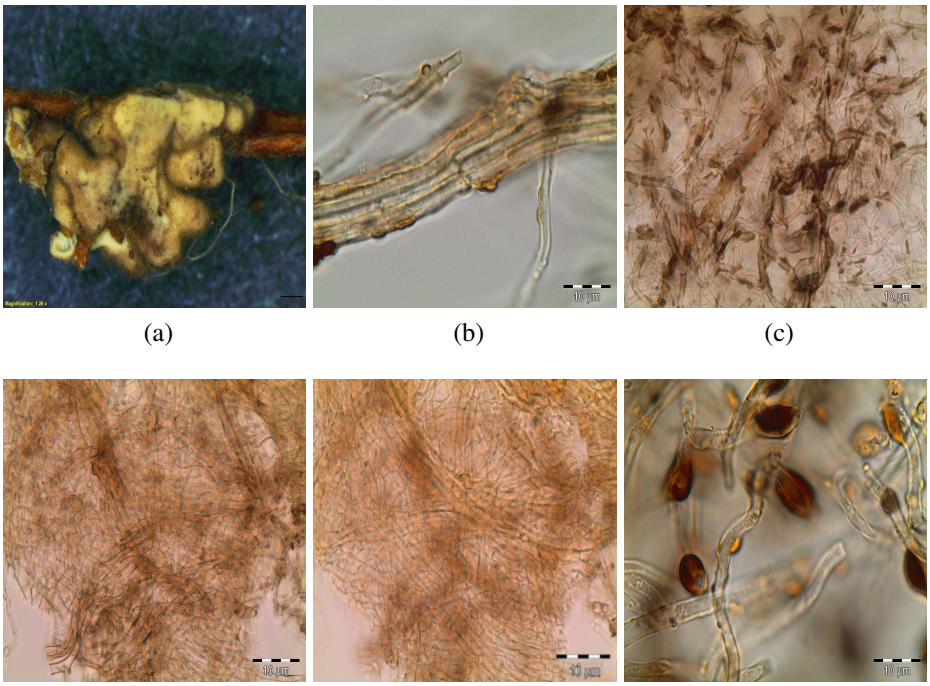

(a) (b)




(c) (d) (e)

Figure 2.13: Morphotype “Black” anatomical and morphological features a) ECM fungal root tips with a black mantle sheath, pale at the very tips and dichotomous branching, scale bar represents 200 µm. b) Loosely arranged mantle surface, c) plectenchymatous outer mantle with no discernible pattern and a slightly gelatinous matrix, d) plectenchymatous middle mantle layer in a gelatinous matrix with hyphae in no discernable pattern e) inner mantle hyphal arrangement also with no discernable pattern. Scale bar represents 10 µm.

many loosely arranged hyphae, tightening to a plectenchymatous outer and inner mantle layers in a star-like arrangement, especially visible in the inner mantle (Figure 2.14c,d,e). Figure 2.14f shows the oil-like droplets observed on the outer layers of the mantle and rhizomorphs of this morphotype. This morphotype was morphologically identified as belonging to the *Suillus* genus, possibly *Suillus sibiricus*.



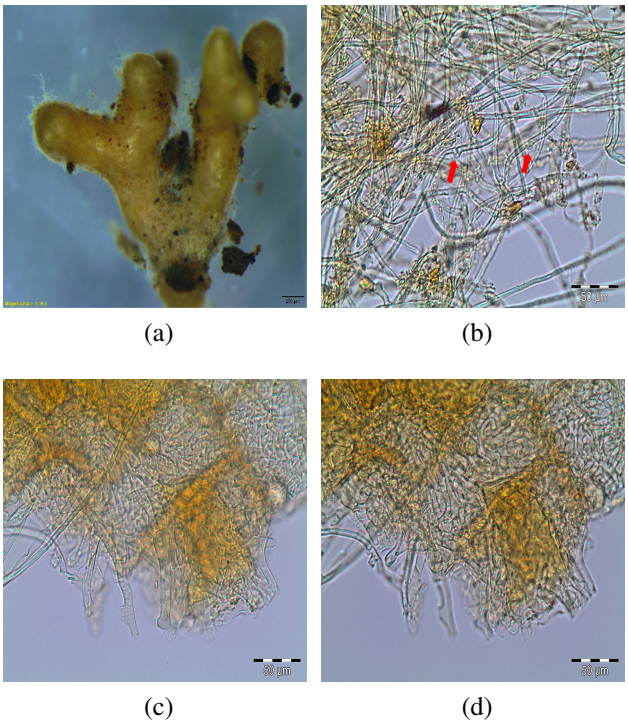
(a) (b) (c)

(d) (e) (f)

Figure 2.14: Morphotype "Yellow-Orange" anatomical and morphological features a) ECM fungal root tips with a thick yellow-orange mantle and tubercle-like ramification, scale bar represents 500 μm. b) Rhizomorph showing differentiated, thick hypahe forming a central core, c) Loosely arranged mantle surface with extruded pigments, d) plectenchymatous outer mantle hyphal layer with no discernible pattern, e) plectenchymatous inner mantle hyphae in a star-like arrangement, f) close up of drops of exuded pigment present on outer mantle surface. Scale bar represents 10 μm.

The "Yellow" morphotype appeared to be very early stage ECM fungal colonisation as the mantle on the straight dichotomous ramifications was extremely thin. Due to the thinness of the mantle it is likely that the yellow colour observed was actually the root and the mantle colour had not yet fully formed. The mantle surface was visibly reticulate though (Figure 2.15a). The

thin outer mantle consisted of loosely arranged, clamped hyphae. The outer and inner mantle layers consisted of plectenchymatous hyphae (Figure 2.15b,c,d). Morphological identification of this morphotype was not achieved.



(a) (b)

(c) (d)

Figure 2.15: Morphotype "Yellow" anatomical and morphological features a) ECM fungal root tips with a yellow mantle sheath and dichotomous branching, scale bar represents 200 μm. b) Loosely arranged mantle surface with clamped hyphae indicated by the arrows c) plectenchymatous outer mantle hyphal arrangement d) plectenchymatous inner mantle hyphal arrangement with no discernible pattern. Scale bar represents 50 μm.

## 2.3.4 Molecular identification

### 2.3.4.1 Cloning

The DNA was successfully extracted from the ECM fungal colonised root tips from the selected nursery seedlings. The first set of plasmids, digested with *Eco*R1, were all identified as uncultured *Thelephora* clones; the highest query cover was 95%, E-value 0.0 and percentage identity of 99%. Alignment in Appendix E.

A total of 18 plasmids were sequenced after digestion with the restriction enzymes *Hin*fI and *Taq*1. Of those 18, 12 samples were not successfully sequenced by Inqaba Biotechnology Industries. The remaining 6 samples were identified as either *Thelephora terrestris* or other common soil fungi (Table 2.7). This demonstrates the need for the comprehensive coverage provided by Illumina sequencing to determine the identity of the remaining non-dominant ECM fungal community suppressed by the dominate *T. terrestris* sequences.

Table 2.7: Summary of BLAST results of the aligned sequences for the plasmids digested with *Hin*fI and *Taq*1

| Isolate | Accession Number | Species | Aligned with | % Identification | E-value | Query coverage |
|---|---|---|---|---|---|---|
| A2 | MG786682 | *Thelephora terrestris* | GU931704 | 87 | 0.0 | 99% |
| I23 | MG789986 | *Thelephora terrestris* | JQ711902 | 100 | 0.0 | 88% |
| I12 | MG786683 | *Rasamsonia emersonii* | KP412244 | 100 | 0.0 | 81% |
| I14 | MG786684 | *Aspergillus chlamydosporus* | KY980617 | 92 | 0.0 | 86% |
| I21 | MG786685 | *Rasamsonia emersonii* | KP412244 | 99 | 0.0 | 82% |
| D2 | MG786687 | *Penicillium chrysogenum* | KP216986 | 100 | 0.0 | 89% |

#### 2.3.4.2 Illumina sequencing

The nested PCR produced products of approximately 700 (figure 2.16) and 330 bps (Figure 2.17), including the primers, as is expected for these areas of the ITS rDNA gene. Some non-specific binding can be seen in figure 2.16. To prevent product loss of the PCR product both bands were gel purified before PCR amplification for the ITS2 region.

It can be seen from figure 2.18 that there is a difference in the occurrence of different fungal families depending on the nursery. Roots from nurseries D and G were predominately colonised by saprotrophic fungi while nursery A is most commonly colonised by the ECM fungus *Thelephora* in the family Theleporaceae. The saprotrophic fungi were identified as belonging to the

Trichocomaceae family where species belonging to the genera, in order of highest occurrence, were *Rasamsonia, Penicillium, Talaromyces, Byssochlamys, Sagenomella, Aspergillus* and *Paecilomyces.* The species identified most frequently were the ECM fungus *Thelephora terrestris* and saprotrophic *Rasmasonia emersonii.*

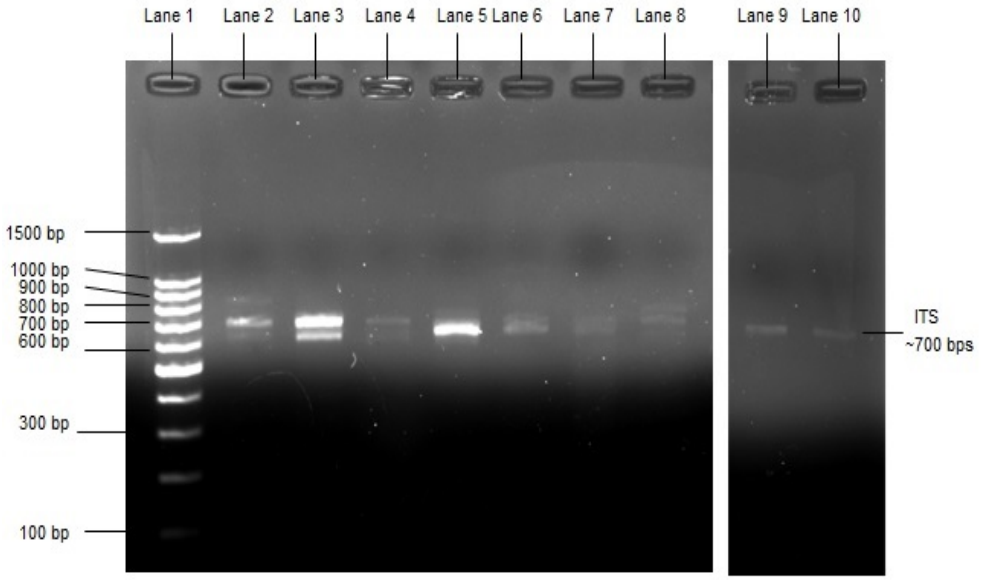



Figure 2.16: 1% ethidium bromide stained agarose gel contained the amplified ITS region of the different fungal isolates. Lane 1: 100 bp ladder, Lane 2: A 613, Lane 3: D 553, Lane 4: A 25, Lane 5: D 26, Lane 6: G 533, Lane 7: G 462, Lane 8: A 53, Lane 9: G 523, Lane 10: D 13. Lane 9 and 10 are from a separate gel due to space constraints.

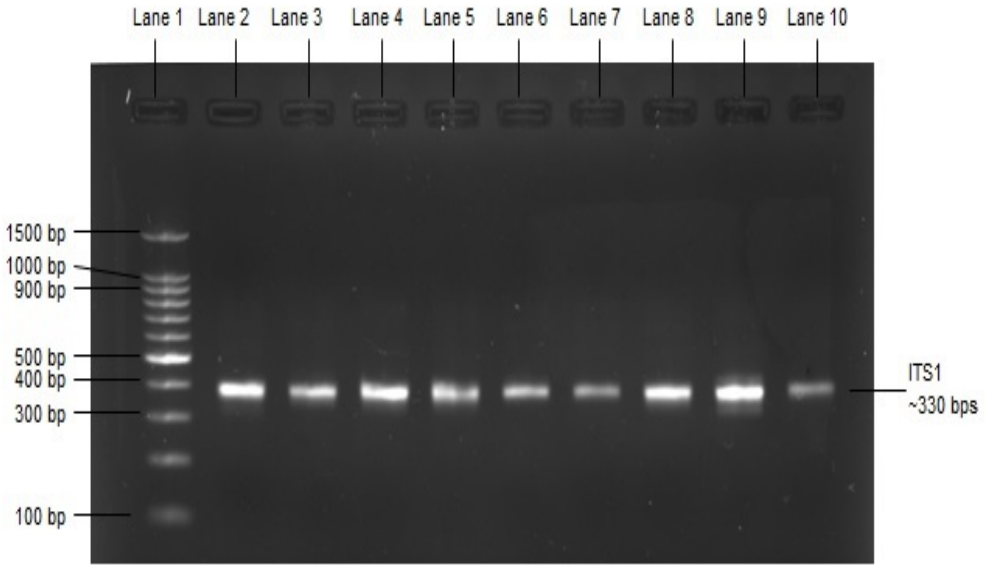



Figure 2.17: 1% ethidium bromide stained agarose gel contained the amplified ITS1 region of the different fungal isolates. Lane 1: 100 bp ladder, Lane 2: G 462, Lane 3: A 53, Lane 4 G 533, Lane 5: D 26, Lane 6: A 25, Lane 7: D 13, Lane 8: D 553, Lane 9: A 613, Lane 10: G 523.

Yeast families were also identified, but listed in additional families due to their low frequency. These included: Cystofilobasidium, Tremellales, Malassezia, Sporidiobolales and Debaryomycetaceae. Many commonly identified in association with ECM fungi. The family to which the pathogen *Fusarium,* and its sexual state *Gibberella,* belong were also present. *Gibberella zeae* was identified, on 9 separate occasions, all originating from the seedling G523. *Cladosporium ramotenellum,* a saprotrophic species in the family Davidiellaceae was only identified from nurseries D and G, neither of which used antimicrobial treatments of any kind. Species from the family Annulatascaceae and Dothrioraceae were only present in nursery A, which used chlorine as an antimicrobial treatment (Figure 2.21c).

The majority of the ECM fungal families had too low a frequency to be visible in figure 2.18. Therefore the heat map for ECM fungi only was generated to discern dominant species (Figure 2.19). As expected, the most commonly identified family was Thelephoraceae. Of the classified families, Pyronemataceae was the second most commonly identified, although it was only observed in nursery D. From this family only one genus, *Sphaerosporella,* was identified. While the specific species was not identified, it is likely that some of the fungi were the

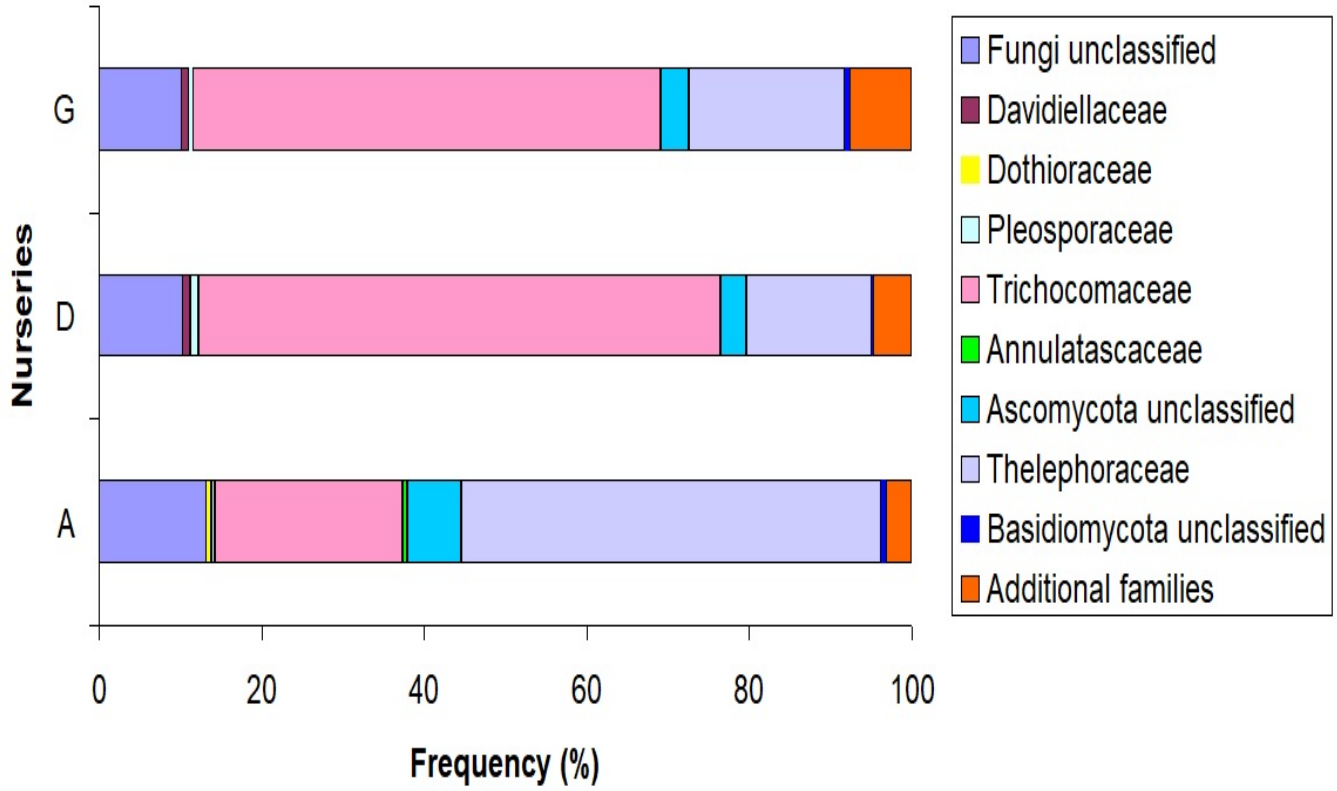



Figure 2.18: Family fungal diversity of *Pinus patula* roots of South African nurseries A, D and G. Identified with UNITE v6 dynamic dataset.

ECM *Sphaerosporella brunnea.* In the same class Pezizales, the ECM fungus *Wilcoxina,* while not identified, could possibly also be present in nursery D. *Inocybe jacobi* was only identified in nursery A. It can be seen from the similarity trees (Figure 2.19) that nursery D was more distinct from nurseries A and G, and contained more ECM fungal families.

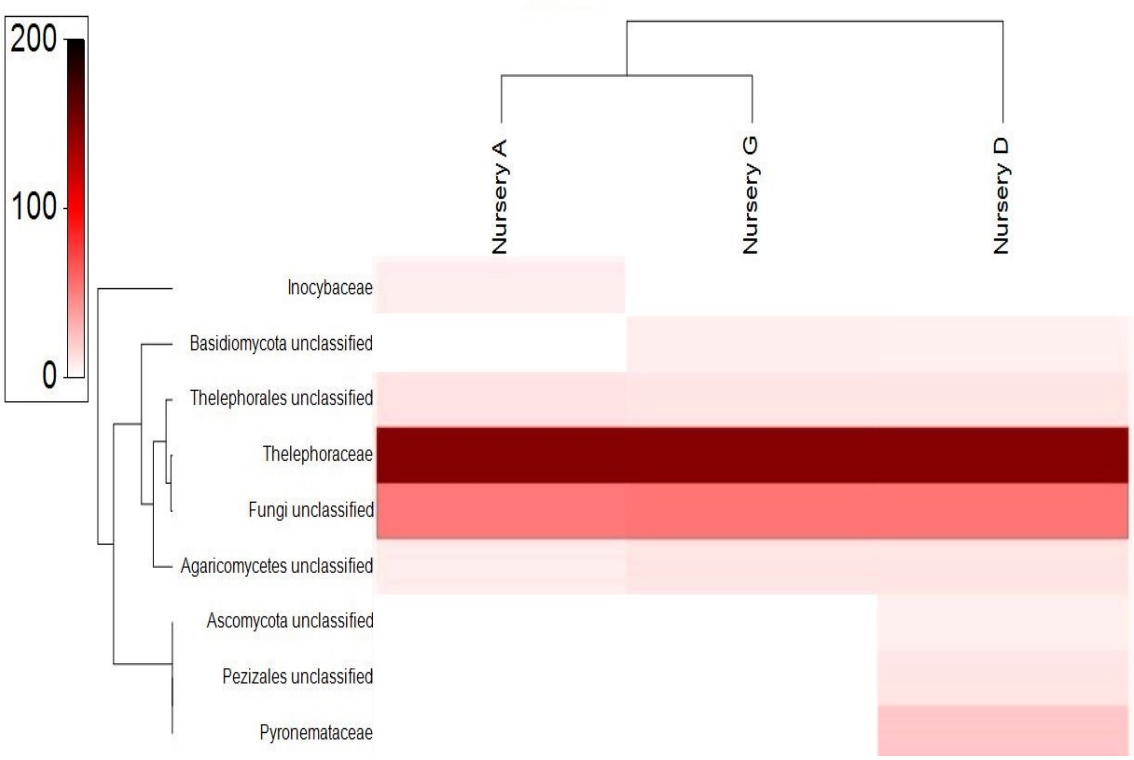



Figure 2.19: Abundance heatmap of ECM fungal containing families identified over the 3 nurseries sampled. Similarity trees standardised by total with a resemblance: index of association. Nurseries underwent a square root transformation and have a S17 Bray-Curtis similarity.

Interestingly, the differences in fungal diversity between the nurseries is also reflected in the nursery practices as shown in the non-metric multidimensional scaling (NMDS) plots (figure 2.21). In all 4 plots it can be seen how the seedlings from nurseries D and G clump together in comparison to nursery A. As mentioned in section 2.3.2 all nurseries used composted pine bark as their main substrate; D as its only substrate; G combined it with coconut coir; and nursery A used 5 different substrates: composted pine bark, coir, peat, perlite and vermiculite. Visually the clustering is the same for all 4 treatments, but based on the R values, only the different anti-microbial treatments and seed treatments had an effect on the type of fungi present on the seedling roots, and were one of the more likely causes of the fungal diversity seen in the figures

2.18 and 2.19.

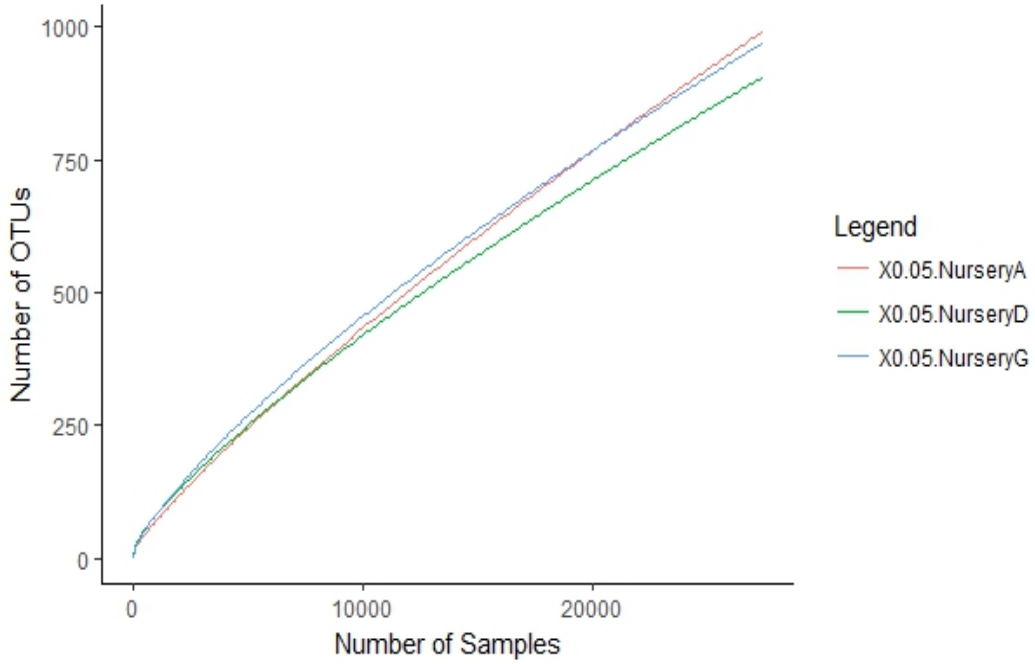



Figure 2.20: Alpha diversity rarefaction curve generated at 0.05 distance level for OTUs from the nine *P. patula* seedlings sampled, indicating the species richness of the root microbiome is larger then what is currently identified.

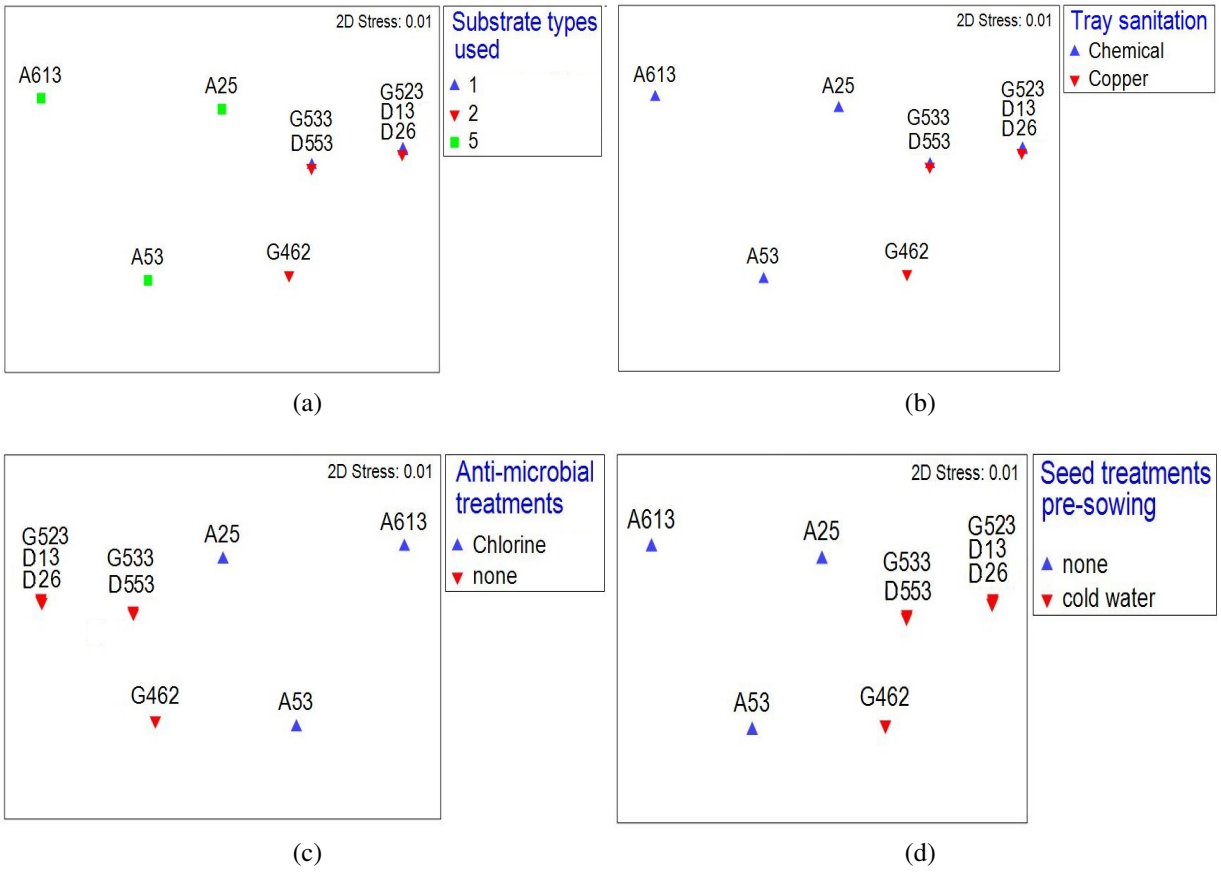



Figure 2.21: Non-metric multidimensional scaling plots using a square root transformation and S17 Bray-Curtis dissimilarity calculation, on the effect of different nurseries practices on fungal diversity. All graphs presented a 2D stress level of 0.01 a) The number of different planting substrates used for *P. patula* seedling germination and growth, ANOSIM one-way R: 0.366 p-value = 0.018, b) seedling tray sanitation, ANOSIM one-way R: -0.228 p-value = 0.929, c) preventative and or curative anti-microbial treatments used on the seedlings ANOSIM one-way R: 0.815 p-value = 0.012, d) pre-sowing treatments of *P. patula* seeds, ANOSIM one-way R: 0.815 p-value = 0.012.

The rarefaction curve (Figure 2.20) indicates that the species richness of the *P. patula* seedlings is larger still than what the identified Illumina results currently present and that larger sample sets would be suggested for future work. This shows that there is still so much to learn from the microbiome of seedlings. The figure also shows that the species richness for nursery D is slightly lower than that of nursery A and G.

## 2.4 Discussion

As South African nurseries do not inoculate their *Pinus* seedlings with ECM fungi, the overall levels detected were low, ranging from 2-21%. All seedlings originated from nurseries found within or near forested areas and thus, their levels of colonisation were expected to be higher. ECM fungal spores are distributed via wind dispersal, water erosion and animals, depending on whether the fruiting bodies are epigeous or hypogeous. It is likely that the seedlings were inoculated via one of these methods, most likely wind dispersal (Brundrett et al., 1996; Lilleskov and Bruns, 2005). The low overall ECM fungal colonisation percentages of the *P. patula* seedlings in the 10 nurseries could be due to a number of different factors, but are most significantly influenced by nursery management practices.

One such factor could be the substrate in which the seedlings are grown. For example, pine bark is used as the main or only substrate by all 10 of the nurseries. Pine bark produces a basic environment which can be detrimental to ECM fungal development and which are known to be acidophilous (Rincón et al., 2005). Oliveira et al., (2010) compared the ECM fungal colonisation on *Quercus ilex* seedlings and found that composted pine bark was not suitable for the best growth of seedlings nor ECM fungal growth. The composted pine bark substrate had the highest pH of the 3 substrates tested at pH 7.30. Similar results were found by Rincón et al., (2005) who found that ECM fungal colonisation of *Pinus pinea* L. seedlings was significantly reduced in the combination substrate of peat and composted pine bark, with a pH of 7.7. Both the development and germination of ECM fungi can be severely affected by pH levels over 6 (Hung and Trappe,1983).

Pine bark also contains phenols which have been found to be detrimental to ECM fungal ability to develop and form an association with their host plants (Oliveria et al., 2010). The phenolic content found in woody roots, which can accumulate in cortical cells, is one of the barriers ECM fungi must overcome when colonising host plant roots. In conifer ecosystems the phenolic levels can reach toxic levels and inhibit ECM fungal formation (Siqueira et al., 1991). Olsen et al., (1971) found that the phenols benzoic acid and catechol, isolated from aqueous extracts of aspen leaves, had an inhibitory effect on mycorrhizal growth. Similarly Yun and Choi, (2002) also found that mycorrhizal colonisation levels decreased in greenhouse trials using increasing concentrations of aqueous extracts from *Artemisia princeps var. orientalis.* Chu-Chou (1978) found that root bark exudates inhibited the growth of *Rhizopogon* species and also on *P. radiata*

seedlings, although the effect that these phenols have on ECM fungi differed between species. Some ECM fungi are able to produce and resist the inhibitory effect of phenols via the production of phenoloxidases (Siqueira et al., 1991). While the substrates could have been the cause for low ECM colonisation rates overall, when analysed as a whole they were found to have no significant effect on the diversity of the fungi present on the seedlings (Figure 2.21a).

It is more likely that the anti-microbial treatments used by the nurseries were the causes of the low and varied colonisation levels, although not all nurseries used anti-microbial treatments. Chlorine was used by all 8 nurseries which applied anti-microbial treatments, of which 4 used it exclusively as their only anti-microbial product. Most South African nurseries use chlorine to sterilise irrigation water before use and as a treatment against *F. circinatum* and other common nursery pathogens (Mitchell et al., 2011; Mitchell et al., 2012). It is commonly applied repeatedly to soil during the seedling's growth period (Mitchell et al., 2012). Chlorine in water is usually found in the form of hypochlorous acid (HOCl); in this form it is a fast acting oxidiser against the most potent fungi. It is able to control and prevent the growth of fungi by entering into the fungal cells and reacting with the proteins, thus disrupting the cell's essential metabolism (Stewart-Wade, 2011; Mitchell et al., 2012).

Chlorine may affect ECM fungi in 2 different ways: killing spores once they enter the soil medium and to prevent/reduce colonisation due to cell disruption. No research to my knowledge has been published on the effect of chlorine on ECM fungi, but it is likely that they would be at risk. Many ECM fungal species are prolific distributors and produce spore loads up to trillions of spores per $km^2$ (Peay et al., 2012). Due to this high spore production it is likely that these spores are produced with a "quantity over quality" approach and would thus likely have lowered resistance to the effects of chlorine. Spores with thinner walls are at more risk from chlorine than those with thicker walls (Stewart-Wade, 2011)

The most commonly used hard chemical by nurseries was Benlate or Benomyl. Thiazoles such as Benomyl have been found to have a slight enhancery effect on basidio- and ascomycetes at field recommended doses, although it is suppressive to zygomycetes and this varies amongst different strains (Trappe et al., 1984; Summerbell, 1988; Laatikainen and Heininen-Tanski, 2002). This stimulatory effect is believed to be due to the inhibitory effect that this fungicide has on other fungi with which ECM fungi would normally have to compete for nutrients (Summerbell, 1988). Chakravarty et al., (1990) found that the colonisation of *Paxillus involutus* on *Pinus resinosa* was significantly reduced when exposed to concentrations of Benomyl at 5000

and 10000 parts per million (ppm). Due to the limited enhancery effect of thiazoles on ECM fungi, it is considered by some to be suitable for the control of pathogens in nurseries where ECM fungal hosts such as Pinaceae are produced as long as care is taken with the dosage and frequency of application (Trappe et al., 1984: Brundrett et al., 1996).

Other hard chemicals were also used in the nurseries, included Cu oxychloride, and fungicides with the active ingredients propiconazole, prochloraz and tebuconazole. Copper was used by the nurseries as a dip for seedlings trays and as a fungicide in the form of Cu oxychloride. This fungicide has been shown to have an inhibitory effect on ECM fungal development and colonisation, in both field trials and axenic cultures, although this effect is species and dose-dependent (Trappe et al., 1984; Manninen et al., 1998; Laatikainen and Heininen-Tanski, 2002). Propiconazole was found to have a clearly toxic effect on ECM fungi on agar and reduced ECM fungal colonisation in pot trials. Teste et al. (2006) found that ECM fungal colonisation was reduced by approximately 55% with the application of propiconazole at the recommended dose of 0.5 g $l^{-1}$ on *Pseudotsuga menziesii* seedlings. Not all ECM fungi were inhibited by this fungicide as the basidiomycete species were less affected. Similar results were recorded by Manninen et al. (1998), who found that ascomycete ECM fungal growth was reduced significantly more than basidiomycetes species tested on *P. sylvestris* seedlings when exposed to propiconazole. Prochloraz has been tested against ECM fungi only in combination with other fungicides by Smaill and Walbert (2013) who found that certain ECM fungal species, such as *Rhizopogon rubescens,* had reduced abundance. No research on the effect of tebuconazole on ECM fungal growth and colonisation has been published.

Morphologically 8 distinct ECM fungal groups were identified on the roots of the *P. patula* seedlings. The tentative morphological identifications of the morphotypes “Yellow-Brown”, “White”, “Beige”, “Black” and “Yellow-Orange” were made according to the Colour Atlas key (Agerer, 1987-2012) and Agerer and Rambold (2004–2018). The immaturity of the majority of the colonised root tips made identification unreliable, showcasing the importance of molecular identification as has been found by previous studies (Quoreshi, 2008). The 9 seedlings selected for molecular analysis represented all 8 morphotypes. Yet, when compared, there was very little correlation between morphological identification and molecular identification. From the Illumina analysis it can be seen that the most common ECM fungus present and identified was *Thelephora terrestris,* belonging to the family Thelephoraceae (Figures 2.18 and 2.19). *Thelephora* is well known to be a vigorous pioneer or “early-stage” fungus species as it is

well adapted to the conditions present in forestry nurseries (Colpaert, 2013). The frequent turnover of seedlings in these nurseries creates favourable conditions for such pioneer species (Smith et al., 2015). *Thelephora terrestris* is one of the most common fungi found in forestry nurseries worldwide (Menkis et al., 2016), often occurring spontaneously in glasshouses, bare-root and containerised nurseries (Rudawska et al., 2017), especially on *Pinus* species (Colpaert, 2013). In a study by Menkis and Vasaitis (2011) on *P. sylvestris* it was the most common of 9 morphotypes and was identified on 92% of the seedlings.

*Thelephora terrestris* is able to colonise and maintain such high abundance because of the large quantities of spores it produces, quantities of up to 200 spores per $m^3$ of air have previously been recorded (Karst et al., 2008; Colpeart, 2013). *T. terrestris* has the ability to outcompete other ECM fungi and often lowers morphotype diversity as a result. Yet, despite its dominance in the nurseries once outplanted, *T. terrestris* is not considered a good competitor, especially against a wider naturalised ECM fungal community. It often fails to support seedlings in the field and is often completely replaced by the end of the 3rd year in the field (Menkis and Vasaitis, 2011; Colpaert, 2013; Menkis et al., 2016). *T. terrestris* does not survive well in soils of high acidity, temperature nor in the presence of heavy metals (Colpaert, 2013).

Historically *T. terrestris* was considered a weak parasite (Colpaert, 2013). The nutritional benefits of ECM fungi such as *T. terrestris* depend on the availability of nutrients and in environments of high fertility, such as nurseries. These ECM associates can act as conditional parasites, even leading to reduced growth and/or nutrient uptake (Karst et al., 2008; Smith et al., 2015). Smith et al., (2015) found that under fertilised conditions *T. terrestris* had a negative effect on the foliar P concentration, and plant nitrogen, magnesium, boron, manganese and zinc. Although these negative effects were not seen under conditions of low fertility, neither was there an increase in plant growth nor the mentioned nutrients detected, showing the little value that this species has as mutualistic partners under any fertility conditions. Thus, it is not surprising that *T. terrestris* was the most commonly identified ECM fungus. The difference in its levels of colonisation from nursery A to D and G are discussed below.

The second most commonly identified ECM fungal family was Pyronemataceae, which contains two mycorrhizal genera, *Geopora* and *Sphaerosporella,* although the majority of this family are considered root endophytes or saprotrophic (Tedersoo and Smith, 2013; Flores-Rentería et al., 2014). Only the genus *Sphaerosporella* was identified, and within this genus only the species *S. brunnea* is ectomycorrhizal (Danielson, 1984). This species has been found to be highly

similar to *T. terrestris* in both morphology and function. *S. brunnea* is rarely identified outside of nurseries and other natural habitats. It is also an early-stage coloniser, which produces a large number of spores for dispersal (Sánchez et al., 2014; Ángeles-argáiz et al., 2016). It also produces mycorrhizal root tips highly similar to those of the *T. terrestri* (Ángeles-argáiz et al., 2016). In nursery A, sequences identified as *Inocybe* species were identified, this ECM genus is another common "early-stage" coloniser found in forestry nurseries and other often-disturbed sites (Reddy and Natarajan, 1997; Nara, 2006)

Based on analysis by Ángeles-argáiz et al., 2016 and Tedersoo et al., (2006) it is likely that the "Yellow-Brown" morphotype is *T. terrestris* and/or *S. brunnea* (Figure 2.9), but due to their similarity in morphological characteristics they are commonly morphologically confused. These two EMC fungi were assigned to same morphotype. Both species form dichotomous branching on *Pinus* seedlings, have smooth brown mantles with few emanating hyphae, transparent tips, no rhyzomorphs and no hyphal clamp connections (Agerer and Rambold, 2004; Ángeles-argáiz et al., 2016). The morphotype "Brown" with the exception of a darker brown colouring and the presence of rhizomorphs, is highly similar to "Yellow-Brown" and could possibly be a more mature version of this morphotype, although this was not confirmed.

Not all of the designated morphotypes could be morphologically identified nor molecularly tied to an ECM species. The lack of correlation between morphological identifications and molecular results, with the exception of *T. terrestris* was due to the extreme dominance of *T. terrestris,* and/or the high possibility that during molecular analysis non-ECM secondary coloniser, present on the mycorrhizal root tips without affecting ECM morphology, amplified more strongly than the desired ECM coloniser (Rosling et al., 2013). This would explain the results of the cloning and original Sanger sequencing attempts.

It is important to know which ECM fungi are present within South African *Pinus* nurseries. But it is also important that they are found in the root microbiomes as a whole as it is this environment which dictates the health of plants. These organisms assist the plant to acquire nutrients, defend against predators and tolerate abiotic stresses. These attributes are required as these plants are not able to move to more ideal environments like other mobile organisms (Doty, 2017). The Illumina sequencing of the fungal inhabitants of the root microbiomes of South African *P. patula* seedlings provides a glimpse of the fungal constituents of that environment (Figure 2.18). While the rarefaction curve indicates that the species richness of the fungi in the different nurseries has not been completely sampled, and thus not examined to its fullest extent,

the information gathered from the Illumina analysis does give a very clear and important initial assessment of the microbiome of *P. patula* nursery cultivated seedlings.

The most commonly identified fungi belonged to the fungal family Trichocomaceae, with species such as *Penicillium, Aspergillus, Rasamsonia* and *Talaromyces.* The majority of the fungi in this family are classified as saprotrophic (Houbraken and Samson, 2011). Saprotrophic and ECM fungi, especially the most aggressive of each, are similar in structure and function, both producing rhizomorphs as a way to forage and acquire nutrients in the soil (Leake et al., 2002). In natural habitats ECM fungi and saprotrophic fungi dominate the microbial communities and rhizospheres of organic forest floors (Smith and Read, 2008). Saprotrophic fungi have been found to be more active under ECM fungal trees than arbuscular mycorrhizal (AM) dominated herbaceous environments. Yet when ECM fungi are excluded from an area of pine forest it was found that saprotrophic fungi decomposed much faster (Leake et al., 2002), illustrating the antagonistic and competitive effect these two fungi have on each other in natural environments (Lindahl et al., 2001; Smith and Read, 2008).

In plantation nurseries the environments are different to those found in natural habitats as the environment is altered with the use of fertilizers, regular irrigation and mechanical and chemical weed and pest control (Menkis and Vasaitis, 2011). Yet, seedlings are still associated with diverse communities of fungi. The most commonly identified non- ECM fungus found on all the seedlings was specifically *Ramasonia emersonii* and a number of species of *Penicillium.* There has been little research published on the associations between *Pinus* seedlings and *R. emersonii,* previously identified as *Talaromyces emersonii.* Literature indicates that *R. emersonii* is an endophyte producing potential enzymes useful to industry, such as cellulases, chitinases etc. (Mahfooz et al., 2017). Species of *Penicillium* alternatively have been identified as both saprotrophic and endophytic fungi. It is known to be one of the most commonly identified fungi in a wide range of habitats (Nicoletti et al., 2014; Visagie et al., 2014). Tannin-degrading species of fungi, such as *Penicillium* species, are commonly found in plantations around mycorrhizal roots. This is due to the increased tannin production which occurs as a result of ECM fungal colonisation. This tannin-rich environment is produced as a result of ECM fungal priming and increased plant defenses (Summerbell, 2005). Therefore it is not surprising that such a high percentage of fungi identified in association with ECM fungal roots were identified as species of *Penicillium* and the family Trichocomaceae. Menkis and Vasaitis (2011) found similar results on nursery grown *P. sylvestris* seedlings.

In addition, mycorrhizal helper yeasts were also identified, but at smaller frequencies. While less studied, different yeasts have been shown to increase the colonisation of ECM fungi (Garbaye and Bowen, 1989), and have been shown to be successful plant growth promoters and agents of biological control (Mestre et al., 2011; Elsharkawy et al., 2015). Some of the species identified in this study are amongst the most commonly identified yeast genera on mushrooms *Rhodotorula, Cryptococcus* and *Cystofilobasidium* (Yurkov et al., 2012). The species *Guehomyces pullulans* and *Cryptococcus* have been shown to have low levels of pathogen inhibition and plant growth promoting properties by Mestre et al., (2016). The association between mycorrhizal helper yeasts and AM fungi have been better researched, and species such as *Rhodotorula mucilaginosa* have been found to significantly increase the growth of multiple *Glomus* species (Fracchia et al., 2003).

Of the 3 nurseries that were assessed molecularly for overall fungal diversity, only nursery A applied chlorine as anti-microbial treatments. This application was shown to have a significant and visible effect on the fungal diversity of this nursery in comparison to nurseries D and G which did not apply anti-microbial treatments (Figure 2.21c). As stated, chlorine is commonly used by South African nurseries for the control of pathogens (van Wyk et al., 2012), and as a sterilant by scientists sterilizing external leaf surfaces when isolating endophytic fungi (Hyde and Soytong, 2008). Thus it can be assumed that if chlorine is so effective in the control of pathogens and external sterilization of plant material, its application as an anti-microbial agent would result in the reduction of the number of saprotrophic and epiphytic fungi present on and in the seedling roots. The reduction of saprotrophic and epiphytic fungal communities then creates opportunities for opportunistic colonisers such as *T. terrestris* because the population levels of their direct competitors have been reduced. As direct research for the effect of chlorine on ECM fungi is not known, this effect is best exemplified by the use of Benomyl in nurseries as reported by Summerbell, (1988).

Cold water soaking seed pre-sowing treatments had a similar effect on fungal diversity. Nursery A, which did not soak its seeds prior to sowing, had significantly different fungal diversity in comparison to the two nurseries which did soak their seeds, nurseries D and G (Figure 2.21d). Seeds are typically soaked in cold water before sowing so as to break the dormancy period and induce faster germination (Fernández-Pascual et al., 2013). No research has investigated the effect of cold water treatments on *Pinus* fungal diversity, yet it is likely that the use of this treatment would produced seedlings quicker. This would benefit the establishment of aggressive

opportunistic fungi which colonise the roots rapidly, dominating the available space. Slower germinating seeds permit other fungi such as ECM *T. terrestris* to achieve a foothold in the root environment of the seedling. The number of substrates used and tray sanitation practices did not have a significant effect on fungal diversity, although the same distribution was seen between nursery A and nurseries D and G.

In conclusion it can be seen that South African nurseries have low levels of ECM fungi associated with their seedlings along with a varied range of additional fungi. Fungal diversity varied according to the different nurseries practices. While difficulties were encountered with morphological and early molecular ECM fungal analysis, a good working knowledge of *P. patula* seedlings root microbiome under nursery conditions was achieved. All of the ECM fungal species identified via Illumina sequencing and preliminary morphological identification show that nurseries are ideal environments for "early-stage"colonisers. This emphasizes the need to inoculate seedlings with a variety of ECM fungi identified as the most beneficial for their growth and development, to prevent colonisation by less helpful "weed" mycorrhizal fungi.

# Chapter 3

# Assessing biocontrol potential of ectomycorrhizal fungi against *Fusarium circinatum* on *Pinus patula* seedlings

## 3.1 Introduction

Since the first detection of *Fusarium circinatum* (teleomorph= *Gibberella circinata*) in South Africa on *Pinus patula* seedlings in 1990 (Wingfield et al., 2008) this pathogen has become a major problem in production nurseries. It causes damping off, root and collar rot, and tip dieback, often resulting in large scale seedling mortality (Steenkamp et al., 2014). Due to these significant losses in yield and productivity this pathogen was characterised as the largest limitation to commercial forestry (Wingfield et al., 2008; Mitchell et al., 2011; Steenkamp et al., 2014). In South Africa, the most susceptible of the *Pinus* species is *P. patula* (Viljoen et al. 1995) but it is also the most commonly planted species as more resistant species such as *P. radiata* and *P. elliottii* produce wood of a poorer quality (Mitchell et al. 2011; DAFF 2014/2015).

The impact of *F. circinatum* is not only felt within the nurseries but also in plantations, where significant losses are experienced after outplanting the seedlings. Crous, (2005) determined that approximately 42% of all recently planted seedlings over 16 sites died due to infection with *F. circinatum*. The majority of the mortalities occurred within the first 30-140 days after planting,

with a mean seedling survival of 36-53% after 1 year. This implies that at least half of all field seedling death is due to infection. The *F. circinatum* related mortalities of *P. patula* result in losses in excess of R11 million per year to the industry, and in excess of R12 million a year when *P. radiata*, the second most susceptible *Pinus* species, is included (Viljoen et al., 1995; Mitchell et al., 2011).

The high levels of *F. circinatum* occurrence in South African nurseries is attributed to contaminated nursery containers, irrigation and in some cases growing media and plants (Mitchell et al., 2011; van Wyk et al., 2012). Infection rates have also been recorded to rise with an increase in the amount of nitrogen (N) given to seedlings (Mitchell et al., 2011). An increase in N also decreases the ectomycorrhizal (ECM) fungal ability to produce their fine-hyphal biomass or EMM. Multiple studies have found that the addition of N-rich fertilizers resulted in reduced or even inhibited growth of EMM and caused changes in community structures up to 2 years after the addition of N (Wallander and Nyland, 1992; Kårén and Nyland, 1997; Peter et al.,, 2001). The current approaches for control of *F. circinatum* include improved nursery sanitation and long-term strategies such as cloning, hybridisation breeding and selection to produce hybrids and trees of increased resistance to the pathogen. While the increased nursery sanitation strategy is showing promise (Van Wyk et al., 2012) there are a number of downsides to the cloning and hybrid programs. Cloning of resistant trees, while the quickest of the long term solutions, produces forestry stock with little genetic variability. Clones develop rooting problems due to the propagation techniques used and producing clones is also labour intensive (Bayle and Blakeway, 2002; Mitchell et al., 2011). There is therefore a need for a more economical and less time-consuming alternative for the control of this pathogen. One solution may lie in the early establishment of ECM fungi. The importance of ECM associations for seedlings are outlined in chapter 2 but their presence may also result in increased resistance to soil-borne pathogens via direct and indirect methods.

Most ECM fungi will inhibit non-mycorrhizal fungal growth via indirect means or via direct competitive exclusion for both space and nutrients. The presence of ECM fungi decreases the rate of pathogenic infection as a barrier (the ECM mantle) along the root tip is formed, preventing pathogenic root infections (Marx, 1972; Branzanti et al 1999). Non-mycorrhizal roots are more prone to infection (Ramachela and Theron, 2010; Mohan et al., 2015), especially succulent root growth which is predisposed to infection from *F. circinatum* (Mitchell et al., 2011). Competition for nutrients has been demonstrated between non-pathogenic strains of

*Fusarium* against their pathogenic counterparts. It is highly probable that the ECM fungi would similarly compete for nutrients and therefore act as effective biological control agents of root pathogens (Whipps, 2001).

ECM fungi may also function as an indirect biocontrol agent against pathogenic infection by enhancing the plants' own defense system, commonly referred to as induced systemic resistance (ISR). This occurs as a result of all mycorrhizal colonisation, not only ectomycorrhizas. This ISR results in broad-spectrum resistance to a wide range of pathogens, especially necrotrophic pathogens. A plant's immune system is activated when microbe-associated molecular patterns (MAMPs), conserved across a range of microbial organisms, are detected. Their detection triggers the long-lasting priming of the plant's salicylic acid dependent defenses and systemic acquired resistance. To facilitate complete mycorrhizal colonisation, mycorrhizal fungi locally suppress the MAMP induced immune system via the production of abscisic acid (ABA), which can also prime cell wall defenses (Cameron et al., 2013; Beardon et al., 2014). Due to this priming accumulated levels of phenols (Sylvia and Sinclair, 1983) fungistatic terpenes, sesquiterpenes (Marx, 1972), chitinases and other antipathogenic compounds (Whipps, 2001) are produced and often detected in the cortical cells of the host plant. This contributes to the overall resistance potential of the host plant (Marx, 1972).

Along with increasing the plant's own immune system, mycorrhizal fungi stimulate and provide habitats for beneficial bacteria and other microorganisms within the mycorrhizosphere, an extension of the plant's own rhizosphere. The bacteria found in the mycorrhizospheres are distinct from the bacteria associated with uncolonised roots (Frey-Klett, 2007). These bacteria are known to enhance the colonisation of ECM fungal formation, aid in nitrogen fixation, and the production of low-molecular weight organic acids which in turn increase the ECM fungal ability to weather minerals (Pool et al., 2001; Smith and Read 2008). These mycorrhizal helper bacteria (MHB) also have the potential to produce toxins, which influence other microbial interactions. The presence of ECM fungi alter a plant's rhizosphere due to the presence of these MHB (Smith and Read 2008).

More direct inhibition methods involve the production of antagonistic antifungal compounds such as chitinases or phenols (Suh et al., 1991; Yamajii et al., 2005; Mohan et al., 2015). ECM fungi have also been found to be effective against different strains of bacterial wilt in nurseries, decreasing disease rates from 72 to 39% (Gong et al., 1999). Suppressive effects such as these are considered to be important to the survival of *Pinus* seedlings in nursery and

forestry environments (Smith and Read, 2008).

The aims of this chapter are to (1) locate, culture and identify local ECM fungi, (2) isolate and identify MHB from said ECM fungi and, (3) determine whether ECM fungal inoculum and potential MHB - alone or in combination - can increase *P. patula* seedling growth when challenged with the pathogen *F. circinatum*.

## 3.2 Methods

### 3.2.1 Isolation of ECM fungi from selected fruiting bodies

ECM fruiting bodies were collected from *Pinus* stands in the highlands of Grahamstown in April 2015. The collected fruiting bodies were visually identified using field guides (van der Westhuizen and Eicker, 1994; Gryzenhout, 2010). Explants from within the fertile cap of the fruiting bodies were extracted using sterile technique and placed onto Modified Melin-Norkrans (MMN) (Marx, 1969) (modified by Bizabani, 2015) (Appendix A1) agar and incubated at 28°C.

#### 3.2.1.1 Molecular identification

From the sporocarp material collected above, molecular identifications were performed to confirm the visual identifications. This was achieved using the ZR Fungal/Bacterial DNA MiniPrep kit according to the manufacturer's protocol (see section 2.2.4.1.1). The successful extraction of DNA was confirmed by visualisation on a 1% agarose gel stained with ethidium bromide and photographed using a UV Transilluminator.

The Internal Transcribed Spacer (ITS) region of rDNA gene was amplified via PCR using the primers ITS1F and ITS4 (Chapter2, Table2.2). A 25 µl PCR reaction was set up as per section 2.2.4.1.2. The sizes of the amplified genes were determined via visualisation. The amplified ITS rDNA genes were then gel purified using the Wizard SV Gel and PCR Clean-Up System according to the manufacturer's protocol, using sterile technique (section 2.2.4.2.2). As multiple bands were present for each sample, with the exception of the isolate coded Salmon Suillus, all bands were gel excised and purified. These again were visualized on an agarose gel for confirmation of success and sent for Sanger sequencing at Inqaba Biotechnology Industries

(Pty) Ltd. Pretoria. The resulting Sanger sequences were analysed using Mega 7 (Kumar et al., 2015) and submitted to the GenBank and UNITE databases (www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997) once identified to genus level using BLAST.

### 3.2.2 ECM fungal associated bacteria

Bacteria found associated with the sporocarp material and isolated in section 3.2.1 were assumed to be interacting with the ECM fungus, due to the sterile technique used to isolate the material. A pure culture of each bacteria was obtained by selecting single colonies from discontinuous streaks on Nutrient Agar (NA) (Merck HG0000C1.500). Glycerol stocks of each isolate were made using 50% glycerol (Merck SAAR2676520LC) and stored at -80°C.

#### 3.2.2.1 Morphological identification

The MHB isolated above were grown in Nutrient Broth (NB) (Merck HG00024.500) overnight at 28°C. Gram stains of each isolate were performed. Two µls of broth were diluted and heat fixed onto a slide in 10 µl of sterile water. Once fixed, the bacteria were stained with crystal violet (Merck 169 80 00 DC) for 30 seconds, rinsed with water, and stained with iodine (Merck 322 28 00EM) for 30 seconds. The bacteria were then washed with absolute ethanol until the ethanol ran clear, indicating the removal of excess crystal violet dye. The bacteria were then stained with safranine (Merck 1017056) for an additional 30 seconds and rinsed off with water. The slides were left to air dry after which they were examined under a light microscope (Leica CME). The colour and shape of the bacteria of each isolate was recorded.

#### 3.2.2.2 Molecular identification

DNA from each bacterial isolate was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit according the manufacturer's protocol, with some adaptations. The bacteria were grown in NB for 2 days at 28°C prior to extraction; culture purity was confirmed before DNA extraction via gram staining. Bacteria from each isolate was pelleted out from 3 ml of broth via centrifugation and resuspended in 200 µl of sterile water. The remainder of the DNA extraction followed the same protocol outlined in section 2.2.4.1.1.

Table 3.1: Nucleotide sequence of primers used to identify isolated bacteria

| Primer | Sequence |
|---|---|
| FD1 | 5'- AGAGTTTGATCCTGGCTCAG -3' |
| RP2 | 5'- ACGGCTACCTTGTTACGACTT -3' |

Table 3.2: Cycling parameters for PCR of 16s rDNA region.

| Conditions | Temperature (°C) | Time (seconds) | Cycles |
|---|---|---|---|
| Initial Denaturation | 98 | 300 | 1 |
| Denaturation | 95 | 45 | 25 |
| Annealing | 55 | 15 | |
| Extension | 72 | 30 | |
| Final Extension | 72 | 300 | 1 |

The 16s rDNA gene was amplified via PCR using the primers FD1 and RP2 (Table 3.1) (Weisburg et al., 1991). A 25 µl reaction was set up as per section 2.2.4.2.2 and the PCR amplification process followed the parameters described in table 3.2. The sizes of the amplified genes were determined via visualisation on a 1% agarose gel and sized against a Lambda DNA/*EcoR1* +*Hin*d III markers ladder (Promeg a G1731).

The amplified 16s rDNA genes were then gel purified using the Wizard SV Gel and PCR Clean-Up System (Section 2.2.4.2.2) and visualized to confirm success. Products were sent to Inqaba Biotechnology Industries (Pty) Ltd. Pretoria for Sanger sequencing. The resulting sequences were analysed using Mega 7 (Kumar et al., 2015) and identified to genus level using BLAST GenBank and submitted to GenBank (www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997).

### 3.2.3 Identification of mycorrhizal helper bacteria plant growth promoting properties

For all of the following experiments 3 replicates, per MHB isolate, were used.

#### 3.2.3.1 Indole acetic acid (IAA) production

MHB cultures were grown for 2-3 hours in NB at 28°C on a shaker, after which their Optical Density (OD) was measured spectrophotometrically (UV mini-1240, Shimadzu) at 600 nm. The

cultures were then adjusted with sterile broth to ensure consistent colony forming units (CFU) densities. From each adjusted broth 100 µl was inoculated into 5 ml of DEV-Tryptophan Broth (TB) (Merck 1.10694.0500) and further incubated overnight at 28°C. After incubation the OD was measured at 530 nm and readjusted using sterile TB. Then, from each culture 1 ml was added to 2 ml of Salkowski reagent and left in the dark for 25 minutes at 25°C to allow for colour change. Their OD was then again measured at 530 nm and recorded. A standard curve of known concentrations of IAA (5, 10, 20, 50 and 100 µg/ml) was then created to allow for the calculation of the concentration of IAA produced by each of the MHB isolates (Appendix B) (adapted from Mestre et al., 2016 and Internet 1).

#### 3.2.3.2 Siderophore production

Bacteria were tested for their ability to produce iron-chelating siderophores using solid Chrome Azurol S (CAS) media (Milagres et al., 1999; Appendix A2).

Each MHB isolate was streaked onto a plate in single lines, with sterile uninoculated broth as the control. The plates were incubated at 28°C overnight and any colour change of the media from blue to yellow, indicating a positive response for siderophore production, was recorded (Pérez-Miranda et al., 2007). As L1 and S1 are both gram positive and thus not able to grow on CAS media the overlay method was also used for these isolates. They were streaked onto NA and grown overnight at room temperature. Cooled CAS media was poured over the top into the plates to produce an overlay. The plates were left overnight at 25°C to allow a reaction to develop (Louden et at., 2011).

#### 3.2.3.3 Phosphate solubility

The phosphate solubilising activity of the MHB was also assessed. MHB isolates were streaked onto multiple replicate plates of phosphate media (Sagervanshi et al., 2012; Appendix A3) with sterile uninoculated broth as the control and incubated at 28°C overnight. A change in the media from opaque to transparent indicated positive phosphate solubilisation, and was recorded.

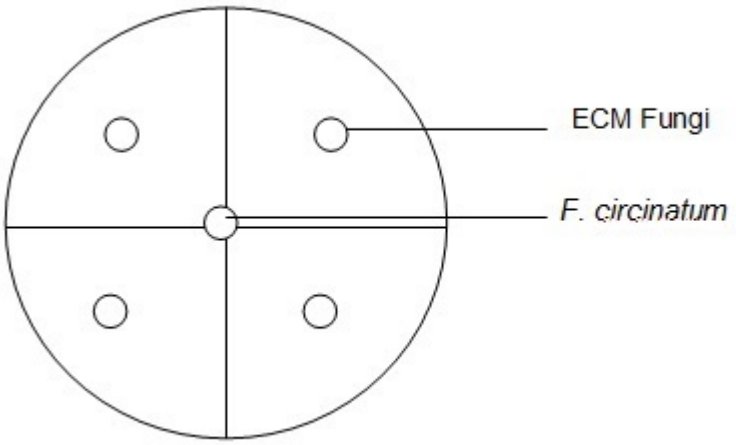



Figure 3.1: Illustration of experimental dual assay design

### 3.2.4 *Fusarium circinatum* isolates

Isolates of *F. circinatum* were provided by the Forestry and Agricultural Biotechnology Institute, University of Pretoria. The 5 isolates (CMWF 666 (VCO 21), CMWF 594 (VC08), CMWF 623 (VCO25), CMWF 701 (VCO30), CMWF 621 (VCO6)) provided, were originally collected from local South African *Pinus radiata* nurseries (Steenkamp et al., 2014). These isolates were cultured on Potato Dextrose Agar (PDA) (Merck HG00C100.500) at 28°C. For brevity the isolates will be abbreviated to FC 666, FC 594, FC 623, FC 701, FC 621, for the remainder of the thesis.

#### 3.2.4.1 Antifungal activity assay

To visualise the interaction between the ECM fungal isolates and the *F. circinatum* isolates an antifungal activity dual assay was conducted. Plates were divided into 4 sections and inoculated with plugs of the different *F. circinatum* isolates, above the central intersecting lines. The remaining 4 quadrants were inoculated with one of the ECM fungal isolates as illustrated in figure 3.1. Two replicates per ECM fungal isolate per *F. circinatum* strain were used.

### 3.2.5 Greenhouse trials to determine the effect on *P. patula* seedlings inoculated with ECM fungi, MHB and *F. circinatum*

#### 3.2.5.1 *Pinus patula* seedlings

The seedlings were grown from *P. patula* seeds provided by the Institute for Commercial Forestry Research (ICFR). Seeds were surface sterilised in 2% sodium hypochlorite (commercial bleach) and rinsed in sterile water. They were grown under controlled conditions in a 1:1 mix of sterile perlite and vermiculite for a period of 2 months before use in either trial.

#### 3.2.5.2 Inoculum preparation

The different ECM isolates were grown in 250 ml of MMN broth. The ECM fungi were incubated on a rotary shaker, at room temperature for a month to allow for maximum growth. On the day of the pot trial the ECM fungal cultures were homogenized using an Ultraturex. To standardize the concentration of each ECM fungal inoculum the homogenate was measured spectrophotometrically at a wavelength 600 nm and adjusted with sterile water to match the lowest OD reading. The homogenized fungal mixtures were then added to 0.3% water agar to form 1 L of a gel inoculum each.

The MHB isolates were grown in NB for 2 days on a rotary shaker at 28°C and similarly standardized with sterile broth.

The *F. circinatum* isolates were grown on PDA for a minimum of 2 weeks to allow for maximum spore production. The spores were harvested by placing 1 ml of sterile water onto the PDA plates and scraping with a glass rod. The spore suspension was added to 20 ml of sterile 15% glycerol in McCartney bottles. The bottles were vortexed for 30 seconds to produce a homogenous solution. From this initial suspension a 1:100 dilution with sterile water was made; and spore concentrations were then determined using a Neubauer hemocytometer (Thoma CE). The concentrations were determined by aliquoting 5 µl of the dilution onto the hemocytometer grid. The grid was then examined under a light microscope using the dark field light setting and the conidia/spores in 5 squares (Figure 3.2) were counted (top left, top right, bottom right, bottom left, and a middle square). This count represented the final amount of conidia. The count was repeated 4 times for each isolate. From these 4 sets of counts an average conidial count was determined (Equation 2) (Coombs, 2012).

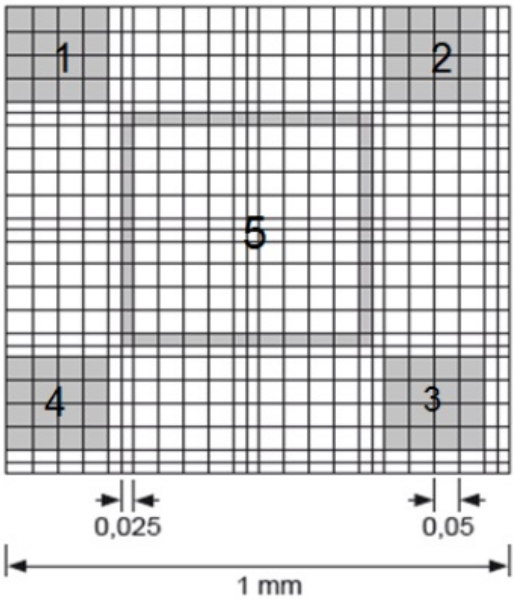



Figure 3.2: Hemocytometer grid layout indicating the 5 squares (shaded) that were counted to determine the average conidial concentration (square 5 contains 4 squares, of which one was randomly counted). (Modified from Lasec Product leaflet)

Equation 2

$$\text{Conidia/ml} = \text{DF} \times \text{D} \times \text{C}$$

Where: DF = Dilution factor; D = dilution volume; C = average number of conidia counted

For each conidial preparation made the viability of the spores was confirmed by spread plating 100 µl onto PDA plates. The percentage of spores which germinated were determined after 48 hours' incubation at 25°C.

#### 3.2.5.3 Greenhouse trial designs

Plastic 500 ml pots were sterilised with a 2% solution of sodium hypochlorite. The bottom of which were covered with surface sterilised small stones to prevent soil loss and increase drainage. Each pot was filled half-way with a sterilised 2:1:1 mixture of compost, perlite and vermiculite. Seedlings were placed in the appropriate ECM fungal inoculum mixture for approximately 10 minutes before planting, and an additional 8 ml of this same mixture was added to the roots of each seedling once planted to ensure colonisation. The seedlings of the negative

Table 3.3: Summary of the different treatments used in the plant growth promotion trial

| Ectomycorrhizal Isolates | Replicas per group | | | |
|---|---|---|---|---|
| | Bacterial Isolates | | | |
| | None | S1 | L1 | R2 |
| Control | 5 | 5 | 5 | 5 |
| Lactarius | 5 | 5 | 5 | 5 |
| Boletus | 5 | 5 | 5 | 5 |
| Salmon Suillus | 5 | 5 | 5 | 5 |
| Suillus | 5 | 5 | 5 | 5 |

control were soaked and inoculated with a sterile sloopy agar and water mix. Two pot trials were performed.

The first pot trial was designed to determine plant growth promotion, influenced by ECM and MHB treatments. The MHB broth was applied to the soil, just above the roots; 5 ml per plant. The number of replicates and treatment configuration is illustrated in table 3.3. The initial height of the seedlings was measured and recorded, after which seedling height was measured and recorded weekly for 20 weeks. The ECM fungal treatments were named after their field identification.

The bacterial isolates were not used for the second greenhouse trial, as statistical analysis showed they did not have a significant effect on the growth of the seedlings. The seedlings for this trial were planted as above with freshly prepared ECM fungal inoculum. One week into the trial, 1 ml *F. circinatum* spores at a concentration of 1 x $10^6 ml^{-1}$ were added to the soil above the roots. The number of replicates and treatment configurations are illustrated in table 3.4. The initial heights were measured upon planting and subsequently measured and recorded weekly for 9 weeks, due to time constraints.

Seedlings in both trials were placed in a mycorrhizal research tunnel having an average temperature range of 25/35°C, pots were irrigated daily with UV treated water and grown under natural lighting.

Table 3.4: Summary of treatments and replicates for biological control trial

| **Ectomycorrhizal Isolates** | **Replicas per group** | | | | | |
|---|---|---|---|---|---|---|
| | ***Fusarium circinatum* strains** | | | | | |
| | **None** | **FC 594** | **FC 621** | **FC 623** | **FC 666** | **FC 701** |
| Control | 10 | - | - | - | - | - |
| Positive controls | - | 10 | 10 | 10 | 10 | 10 |
| Lactarius | 10 | 10 | 10 | 10 | 10 | 10 |
| Boletus | 10 | 10 | 10 | 10 | 10 | 10 |
| Salmon Suillus | 10 | 10 | 10 | 10 | 10 | 10 |
| Suillus | 10 | 10 | 10 | 10 | 10 | 10 |

#### 3.2.5.4 Trial parameters recorded

After the greenhouse trials the seedlings were carefully removed from their containers and transported to the laboratory for further analysis. The roots were severed from the shoots and wet weights recorded. The roots were then placed in a glass dish over a 2 x 2 cm grid and covered with sterile water and photographed (as per Chapter 2, Section 2.2.1) to determine percentage colonisation. The roots were then dried in an oven at 60°C for 3-4 days and the resulting dry biomass was recorded.

### 3.2.6 Statistical analysis

A Shapiro test was performed on the percentage colonisation and root dry weight data to determine normality. The null hypothesis was rejected for both showing the data was non-parametric. Thus, a Kruskal-Wallis Analysis of Variance (ANOVA) was performed and a pairwise Wilcox test was performed to determine significant difference between treatments.

Seedling growth was analysed using a repeated measures ANOVA. A least-squares means pairwise comparison with a Tukey adjustment was performed to compare treatments to one another and determine significant differences. All statistical analyses were performed using RStudio Version 0.99.903 (RStudio team, 2015).

## 3.3 Results

### 3.3.1 Isolation and identification of ECM fungi from fruiting bodies

The fruiting body samples collected were field identified as *Boletus edulis, Suillus granulatus, Suillus salmonicolor* and *Lactarius deliciosus.* For the remainder of the chapter the isolates were thus coded by their field identified genus name: Boletus, Suillus, Salmon Suillus and Lactarius. Cultures were successfully grown on MMN agar from the ECM sporocarps collected.

When the DNA was amplified, multiple bands of very similar sizes were produced. To prevent loss of the correct sequence all were sequenced (Figure 3.3). The genera for which the ECM fungi were identified in the field was confirmed via molecular analysis, table 3.5. Salmon Suillus did not resolve to a satisfactory molecular identification. Unfortunately due to limited amounts of sample it was not possible to repeat the sample Salmon Suillus to resolve the specific species and provide a better sequencing result.

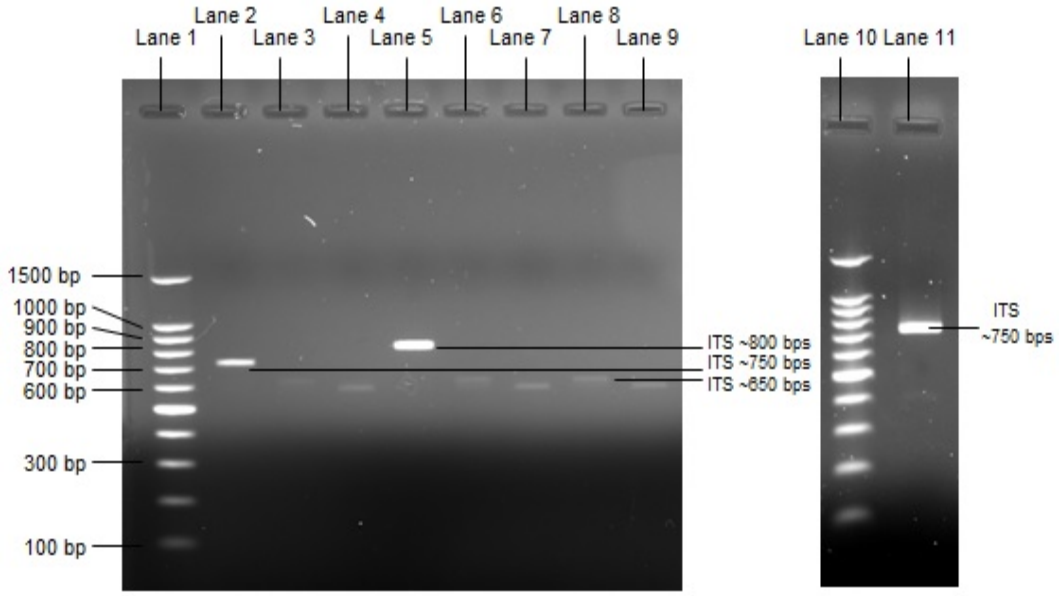



Figure 3.3: 1% ethidium bromide stained agarose gel containing amplified ITS gene from ECM sporocarps. Lane 1 + 10: 100 bp ladder, Lane 2 Suillus ITS, Lane 3+4: Suillus non-specific binding, Lane5: Boletus ITS, Lane 6+7: Boletus non-specific binding, Lane 8: Lactarius ITS, Lane 9: Lactarius non-specific binding, Lane 11: Salmon Suillus ITS.

Table 3.5: Summary of BLAST results for the ECM sporocarps.

| Sporocarp | Accession Number | Genera | Aligned with | % Identity | E-value | Query coverage |
|---|---|---|---|---|---|---|
| Boletus | MG806927 | *Boletus edulis f. reticulatus* | KY595992 | 95 | 0.0 | 100% |
| Suillus | MG806928 | *Suillus granulatus* | KU721244 | 99 | 0.0 | 100% |
| Salmon Suillus | MG806929 | *Suillus* | KX170996 | 91 | 1e-155 | 100% |
| Lactarius | MG833316 | *Lactarius quieticolor* | KX610696 | 100 | 0.0 | 100% |

### 3.3.2 ECM fungal associated bacteria

The 16s rDNA gene was amplified via PCR and produced the expected fragment size of approximately 1500 bp as can be seen in figure 3.4.

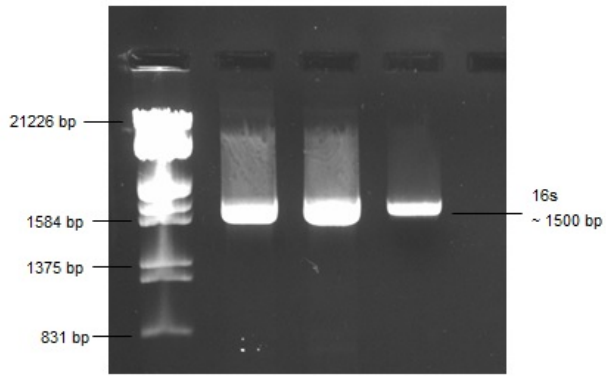



Figure 3.4: 1% ethidium bromide stained agarose gel containing the amplified 16s rDNA gene. Lane 1: Lambda DNA/*Eco*R 1 + *Hind* III ladder, Lane 2: S1, Lane 3: L1 and Lane 4: R2

From the BLAST analysis two of the bacteria, L1 and S1, were identified as *Bacillus* species. The third bacterial species, R2, was identified as *Stenotrophomas maltophilia* (Table 3.6).

Table 3.6: Summary of BLAST results for bacteria found associated with the ECM fungi sporocarps collected.

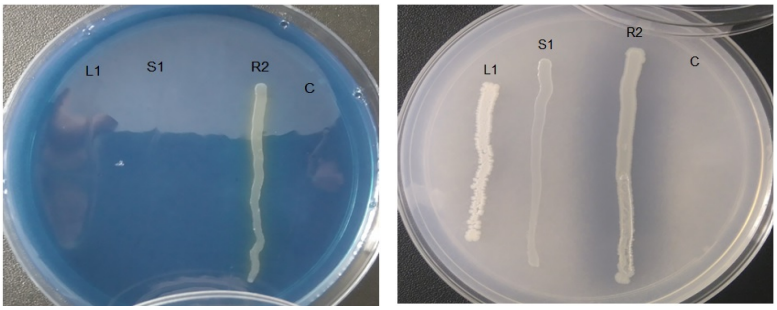
| Isolate | Accession number | Genera | Aligned with | % Identity | E-value | Query coverage |
|---|---|---|---|---|---|---|
| L1 | MG786679 | *Bacillus megaterium* | MG786679 | 99 | 0.0 | 100% |
| S1 | MG786680 | *Bacillus simplex* | MG693446 | 100 | 0.0 | 100% |
| R2 | MG786681 | *Stenotrophomas maltophilia* | MG571733 | 99 | 0.0 | 100% |

### 3.3.3 Identification of MHB plant growth promoting properties

It can be seen from table 3.7 that the only bacteria which exhibited all of the tested plant growth promoting properties was the isolate R2 (*S. maltophilia*). Its ability to solubilise phosphate and produce siderophores is highly visible in figure 3.5. The isolate L1 also tested positive for the production of siderophores, using the overlay method. While all isolates did produce IAA, R2, produced it in the largest quantities equaling a concentration of just over 20 µg/ml of IAA, in comparison to the much lower amounts produced by S1 and L1, between 10 and 20 µg/ml, respectively when compared to the standard curve (Appendix B).

Table 3.7: Summary of morphological characteristics and plant growth promoting properties of the MHB

| Isolate | Morphological characteristics | IAA production (µg/ml) | Phosphate solubilisation | Siderophore production |
|---|---|---|---|---|
| S1 | Gram positive rods | 12,36 | - | - |
| L1 | Gram positive rods | 13,89 | - | + |
| R2 | Gram negative rods | 26,79 | + | + |




(a) (b)

Figure 3.5: Phosphate and CAS media plates used for determining plant growth promoting properties of the bacterial isolates L1, S1 and R2; a) Siderophore producing MHB results. The colour change of streak R2 indicates positive result. b) Phosphate solubilising MHB results. The area of increased transparency around the streak of R2 indicates positive ability to utilise phosphate.

### 3.3.4 *F. circinatum* antifungal activity assay

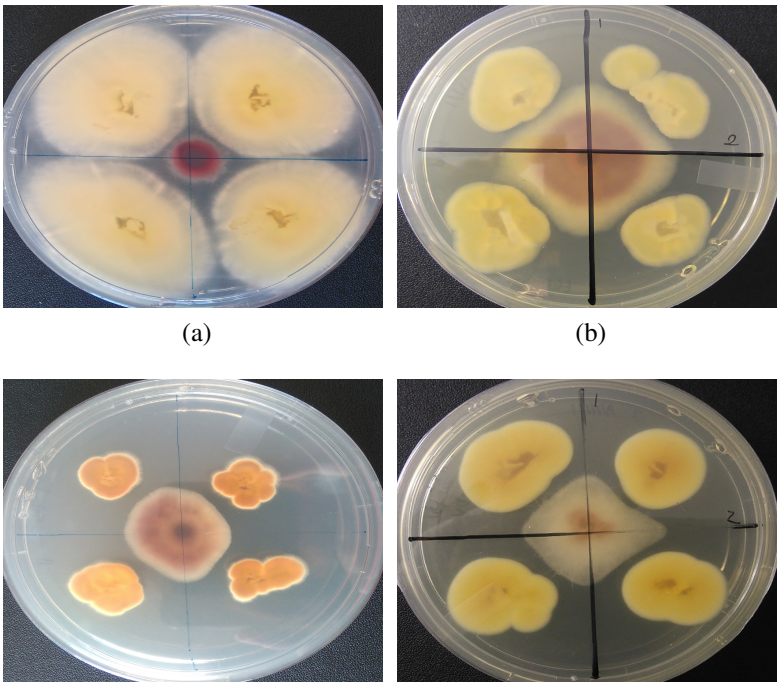
The *F. circinatum* growth was visibly inhibited and the pathogen actively avoided the ECM fungi most notably in the presence of Lactarius and Suillus (Figure 3.6a,d). While the inhibition is not as dramatically visible, both Salmon Suillus and Boletus also resulted in growth avoidance of the pitch canker isolates.

### 3.3.5 Greenhouse trials

#### 3.3.5.1 Plant growth promotion trial

The height of each seedling was adjusted with its initial measurement before analysis. Thus, these graphs represent the amount each seedling had grown over 20 weeks rather than their actual height; this was also performed for the biological control trial. To allow for easier comparison, the different MHB treatments in combination with the ECM fungi are presented separately and compared to the control.

From figure 3.7 it can be seen that the ECM fungal treatments Boletus and Salmon Suillus improved the growth of *P. patula* seedlings in comparison to the control. Of all the ECM



(a) (b)

(c) (d)

Figure 3.6: ECM fungal inhibition of *Fusarium circinatum* strains; a) Lactarius against *F. circinatum* 666, b) Salmon Suillus against *F. circinatum* 666, c) Boletus against *F. circinatum* 594, d) Suillus against *F. circinatum* 701.

fungi the seedlings inoculated with the isolate Salmon Suillus had the highest levels of growth, regardless of MHB (Figure 3.7a).

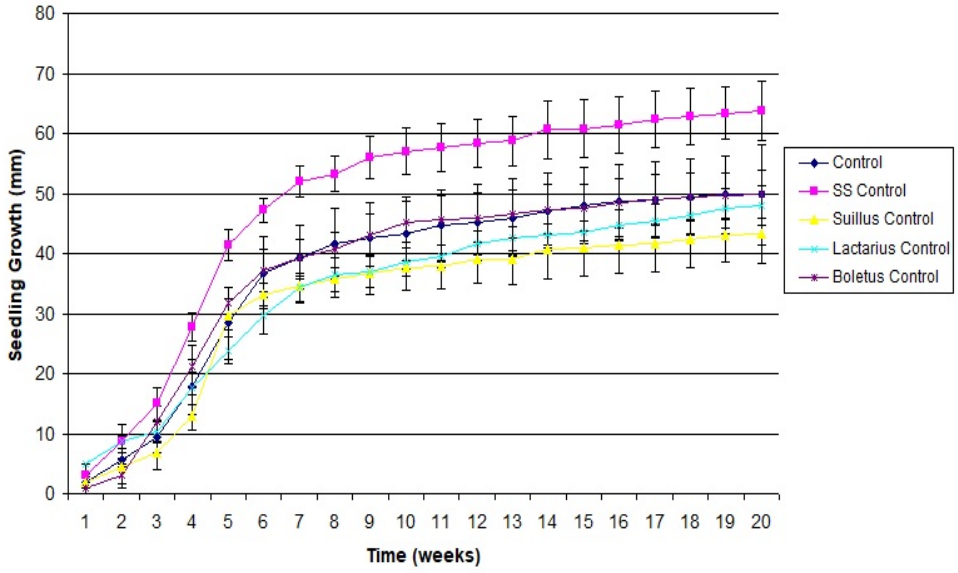
When the effect of each MHB was compared, only the treatment of Salmon Suillus was significantly different to the S1 control. The Suillus + S1 treatment had the lowest growth (Figure 3.7b). The significant differences are believed to be due to the effect of the ECM fungus rather than the MHB S1, especially as S1 had few MHB properties (Table 3.7). Conversely R2, which did show promising MHB properties produced no significant differences in seedling height. The MHB-only inoculated seedlings did show an increase in growth, but it was not significant. Overall only the ECM fungi were found to have a significant effect on the growth of the seedlings, and no significant effect was found as a result of the inoculation of the bacteria or the combination of the ECM fungi and bacteria (Table 3.8). Thus, the bacteria were omitted from the second greenhouse trial.

Table 3.8: ANOVA linear mixed effects model results for the weekly repeated seedling growth measurements for plant growth promotion trial

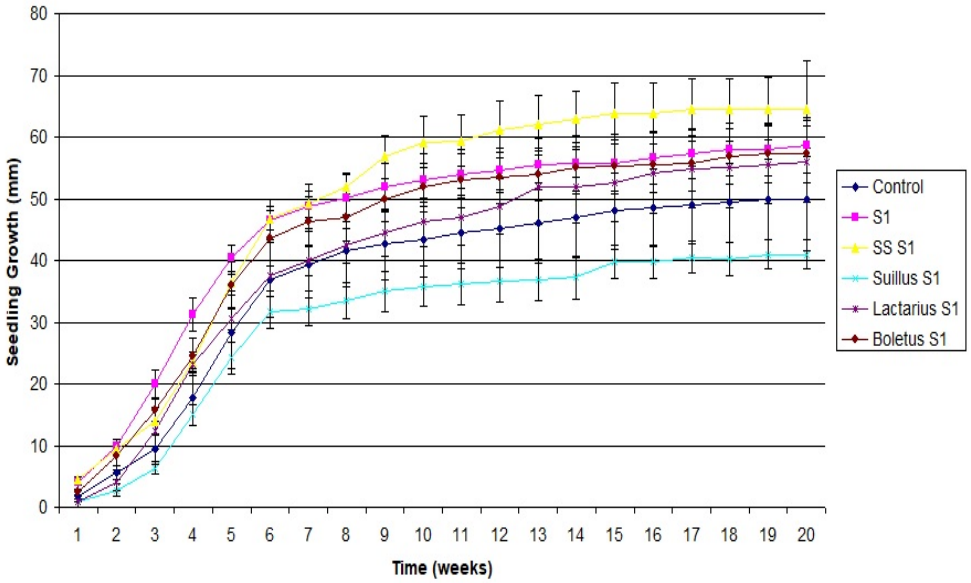
| **Treatment** | **F-value** | **P-value** |
|---|---|---|
| (Intercept) | 2876.5078 | <0.0001 |
| ECM fungi | 11.4919 | <0.0001 |
| MHB | 1.9094 | 0.1358 |
| ECM + MHB | 0.6070 | 0.8293 |

From figures 3.7 and 3.8 it can be seen that the treatment of Boletus not only improved seedling growth but also had the highest percentage colonisation. However, this was not reflected in root weight (Figure 3.9). The Boletus control treatments (i.e no MHB) had significantly higher levels of colonisation than the other treatments. In comparison, the other ECM inoculants did not significantly increase colonisation in response to MHB when compared to the control, indicating again that MHB did not play a key role in the early stages of ECM associations or plant growth in this study.

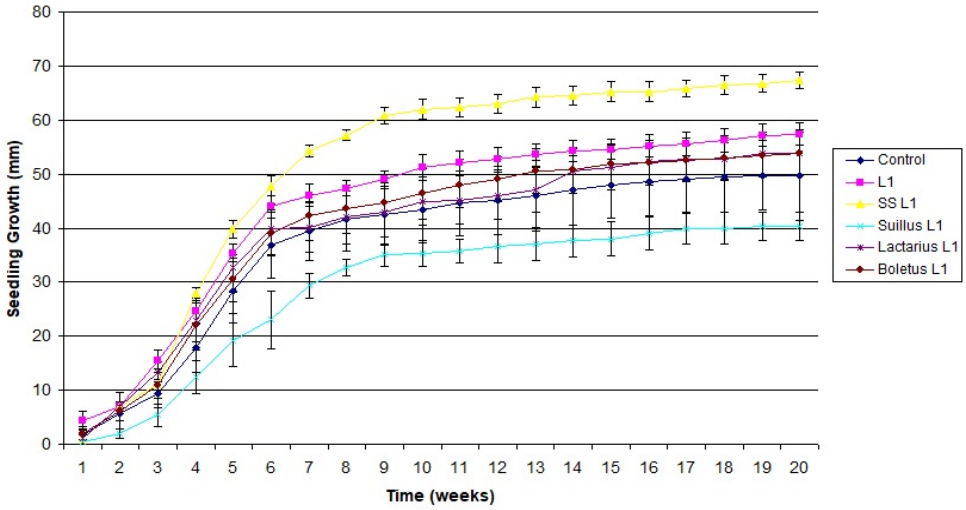
In comparison the Salmon Suillus treatment constituted some of the lowest colonisation levels, together with the Suillus inoculation (Figure 3.8) and average root biomass (Figure 3.9). The Lactarius treatments produced seedlings with the largest root biomass overall. This was significantly larger than Suillus + L1, the treatment which yielded the smallest root biomass, with the exception of the treatment Lactarius + R2 (Figure 3.9). Lactarius treatments also had


Seedling Growth (mm)
Time (weeks)
Control
SS Control
Suillus Control
Lactarius Control
Boletus Control

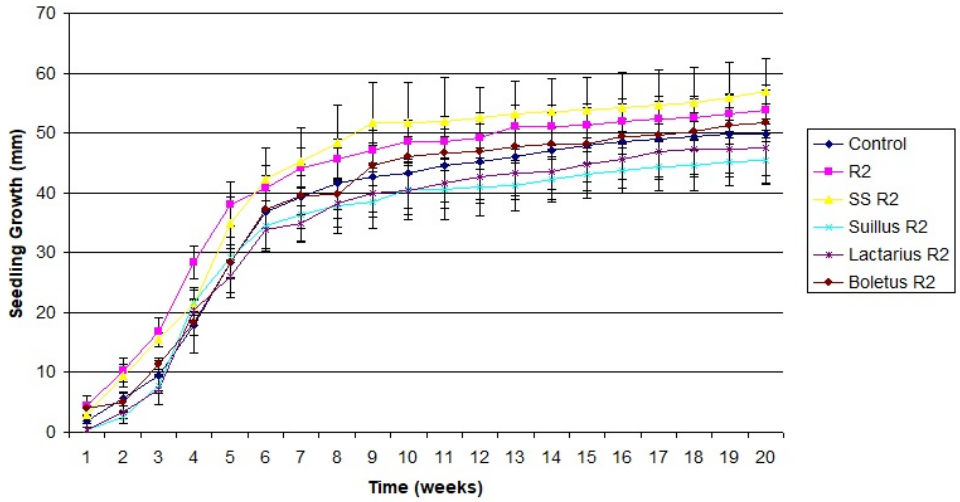
(a)


Seedling Growth (mm)
Time (weeks)
Control
S1
SS S1
Suillus S1
Lactarius S1
Boletus S1

(b)




(c)




(d)

Figure 3.7: Plant growth promotion trial *P. patula* seedling growth over 20 weeks in comparison to the negative control. a) Negative and ECM controls, b) S1 + ECM treatments and control, c) L1 + ECM treatments and control, d) R2 + ECM treatments and control. Salmon Suillus abbreviated to SS for space constraints. Error bars represent ± standard error.

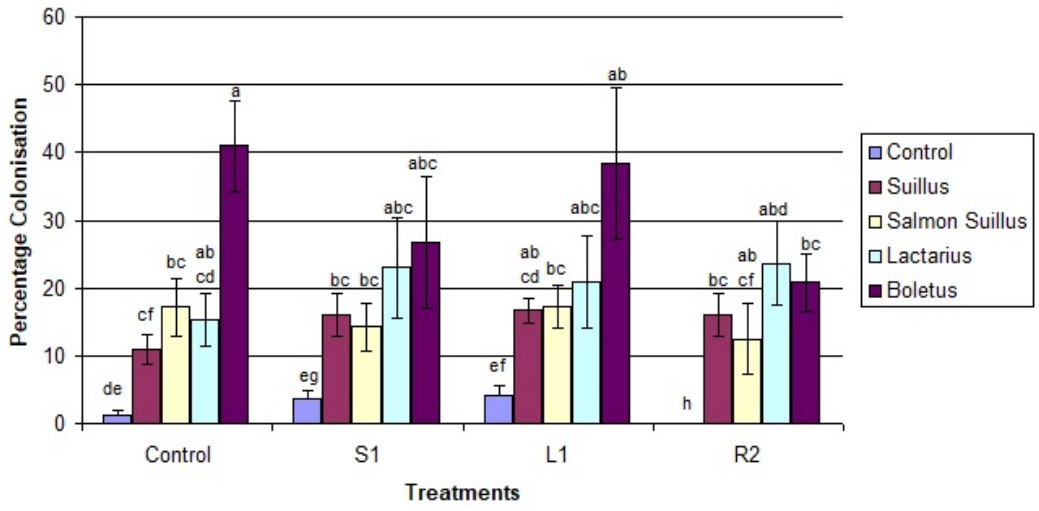



Figure 3.8: Average percentage colonisation for each treatment (Kruskal-Wallis H: (19, 87) 56.038, p = 1.61e-05). Error bars represent ± standard error. Columns with the same letters are not significantly different from one another.

the second highest levels of colonisation overall (Figure 3.8). Interestingly, while Lactarius + R2 had the highest level of Lactarius colonisation seedling growth was lower than the negative control (Figure 3.7d) and only just above the control for other treatment combinations.

In comparison to this improved growth the addition of the ECM fungus Suillus reduced the overall growth of the *P. patula* seedlings. This decrease was also reflected in colonisation levels, having the lowest levels of the ECM inoculated treatments (Figure 3.8). The same can be seen in the seedlings' root weight were Suillus also had the lowest root biomass, significantly so for Suillus + L1, which had the smallest root biomass in comparison to the control, bacterial controls and the majority of the Lactarius seedlings (Figure 3.9).

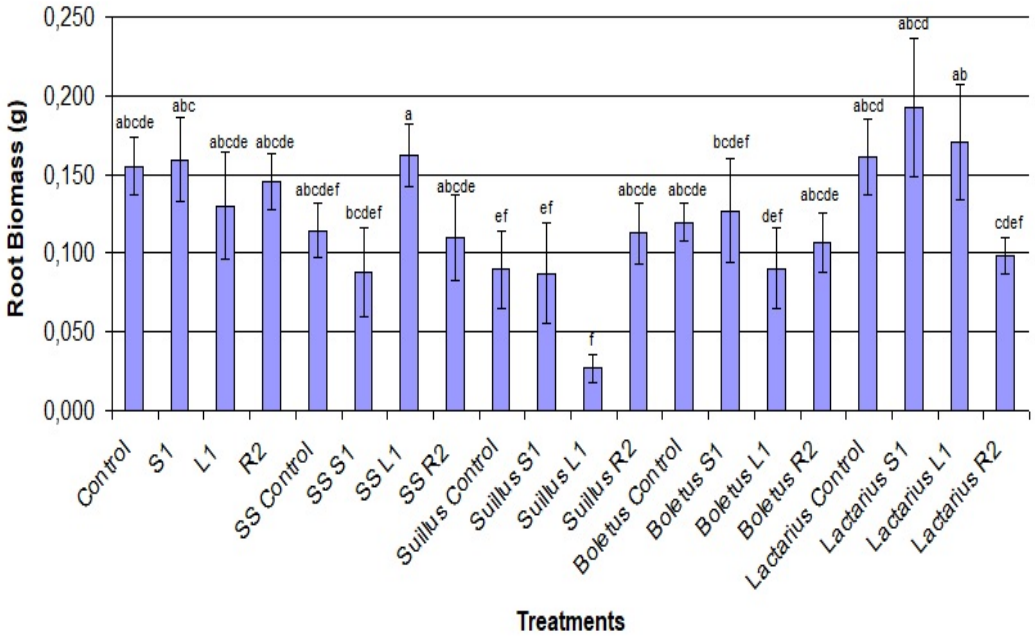
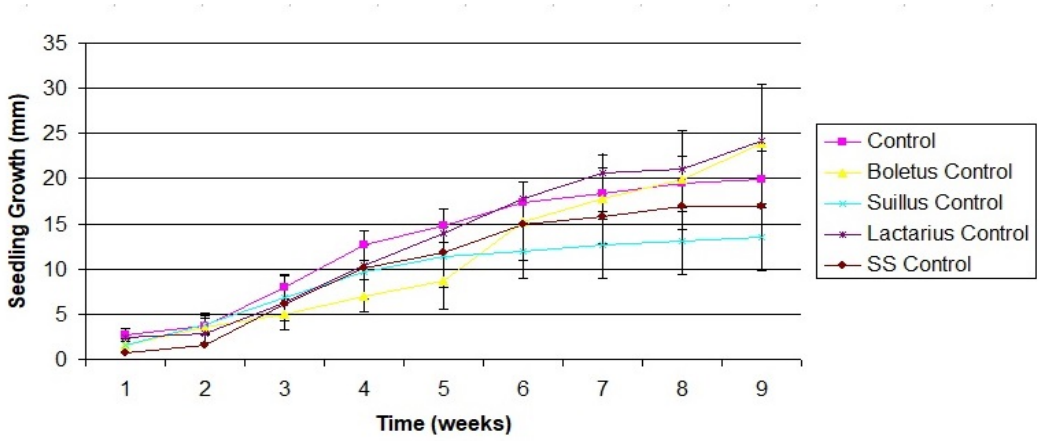



Figure 3.9: Average root dry biomass for *P. patula* seedlings for each treatment (Kruskal-Wallis H (19, 86) 30.103 , p = 0.05). Error bars represent ± standard error. For ease of interpretation the treatments Salmon Suillus was abbreviated to SS. Columns with the same letters are not significantly different from one another.

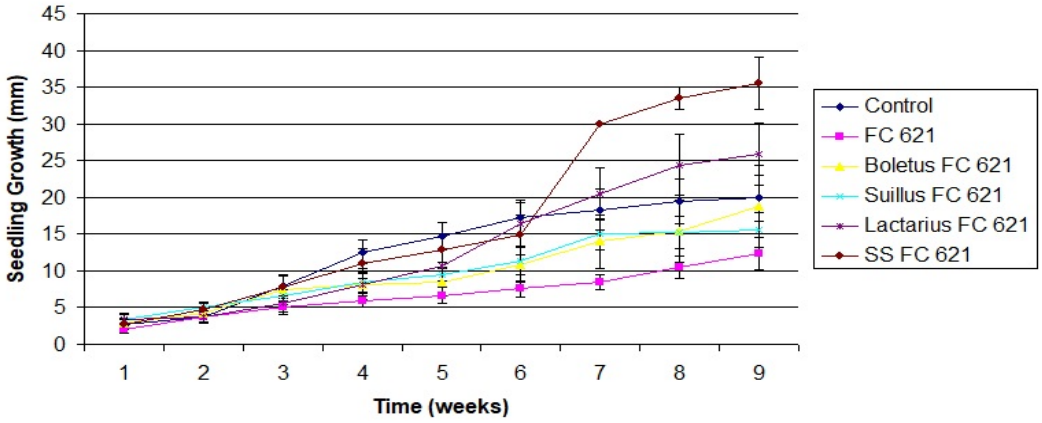
#### 3.3.5.2 Biological control trial

This greenhouse trial tested the efficiency of using ECM fungal inoculants on *P. patula* seedlings to increase tolerance to the pathogen *F. circinatum.* As the MHB used in the first greenhouse trial did not have a significant effect on seedling growth nor root weight, they were not used in this trial. The linear mixed effects model showed that the different treatments of ECM fungi and the combination of ECM fungi and *F. circinatum* had significant effects on the growth of the seedlings (Table 3.9).

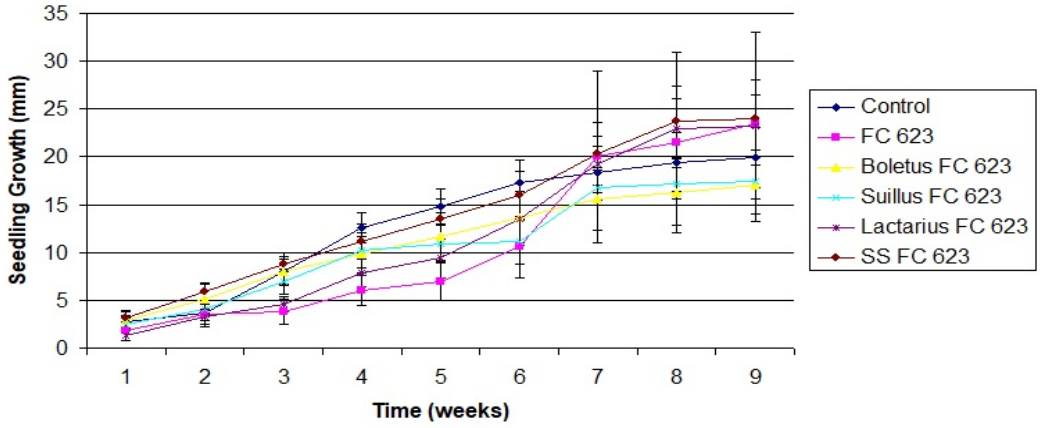
From figure 3.10 it is can be seen that inoculations with the fungus Lactarius produced the highest growth in comparison to the other ECM fungi and control treatments and more importantly continued to promote and increase the growth of seedlings inoculated with the different *F. circinatum* strains. It was the only ECM fungus to significantly increase the growth of the *P. patula* seedlings infected by the pitch canker strains; specifically, it was significant against


Seedling Growth (mm)
Time (weeks)
Control
Boletus Control
Suillus Control
Lactarius Control
SS Control

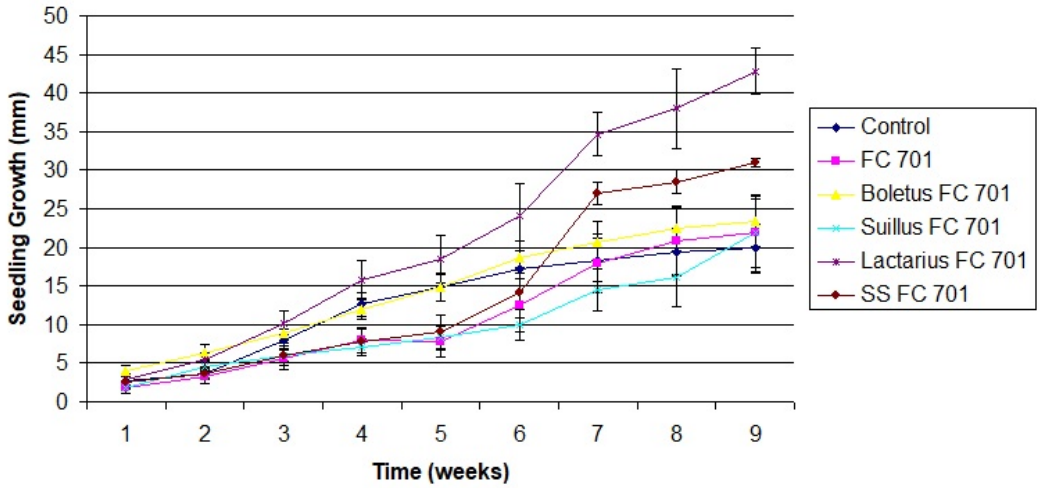
(a)


Seedling Growth (mm)
Time (weeks)
Control
FC 621
Boletus FC 621
Suillus FC 621
Lactarius FC 621
SS FC 621

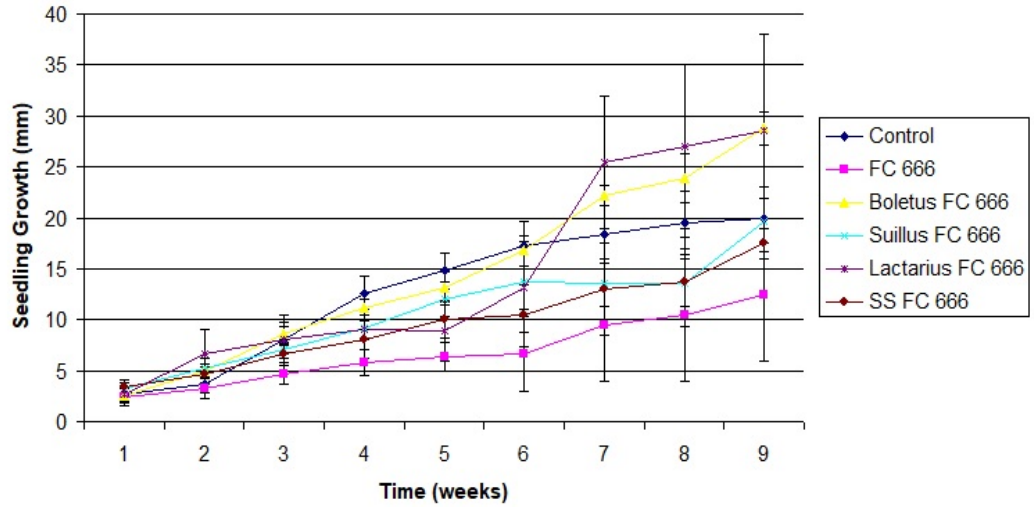
(b)


Seedling Growth (mm)
Time (weeks)
Control
FC 623
Boletus FC 623
Suillus FC 623
Lactarius FC 623
SS FC 623

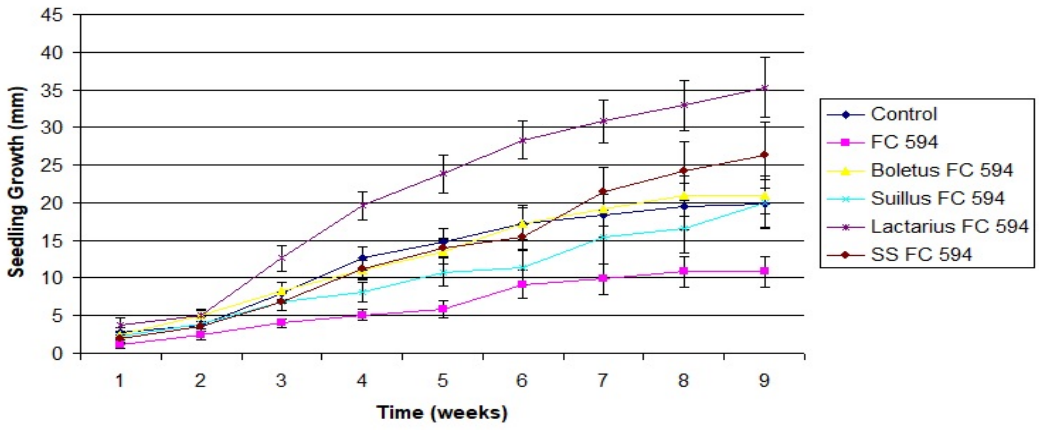
(c)


Seedling Growth (mm)
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
Time (weeks)
Control
FC 701
Boletus FC 701
Suillus FC 701
Lactarius FC 701
SS FC 701

(d)


Seedling Growth (mm)
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
Time (weeks)
Control
FC 666
Boletus FC 666
Suillus FC 666
Lactarius FC 666
SS FC 666

(e)




(f)

Figure 3.10: Biological control trial average weekly growth of the *P. patula* seedlings for each treatment over a period of 9 weeks. a) Negative and ECM controls, b) FC 621 + ECM fungi and control, c) FC 623 + ECM fungi and control, d) FC 701 + ECM fungi and control, e) FC 666 + ECM fungi and control, f) FC 594 + ECM fungi and control. For ease of interpretation; SS= Salmon Suillus and FC = *F. circinatum* were abbreviated. Error bars represent ± standard error.

the strain 594. No other ECM treatments produced significant increases in growth against both the *F. circinatum* control nor the negative control. This significant growth in the presence of *F. circinatum* is reflected in the antifungal assays (section 3.3.4), where Lactarius had the most visible inhibition of *F. circinatum* growth (Figure 3.6a).

For most treatments the application of Salmon Suillus produced the second highest average *P. patula* seedling growth (Figure 3.10). Since Salmon Suillus produced much less visible inhibition of *F. circinatum* (Figure 3.6b) *in vitro* it is highly likely that it increased seedling growth while inhibiting pathogenic infection using a different mechanism to Lactarius. Boletus and Suillus did not always increase the *P. patula* seedling's growth in comparison to the *F. circinatum* controls. Although both ECM fungi did demonstrate visible *in vitro F. circinatum* inhibition (section 3.3.4), it is proposed that even though plant growth is not being increased, pathogen inhibition is occurring.

The higher levels of growth and colonisation when exposed to the pathogen *F. circinatum* indicate the benefits of using ECM fungal inoculum on *Pinus* seedlings. Some very low levels of colonisation did occur in the controls, although this is likely due to splashing from surrounding

Table 3.9: ANOVA of linear mixed effects model for repeated measurements of seedling growth for biological control trial.

| Treatments | F-value | P-value |
|---|---|---|
| **(Intercept)** | 932.1432 | <0.0001 |
| **ECM fungi** | 12.1650 | <0.0001 |
| ***F. circinatum*** | 3.0175 | 0.0114 |
| **ECM + *F. circinatum*** | 20.441 | 0.0061 |

pots (Figure 3.11). While Lactarius had the highest seedling growth it had the lowest percentage colonisation, with significantly lower colonisation in the presence of some *F. circinatum* isolates, such as FC 666, 621 and 623 (Figure 3.11). On the other hand, Salmon Suillus had the highest percentage colonisation compared to other ECM fungi, especially with respect to treatments with FC 621 and FC 666. Boletus, which had the highest levels of colonisation in the first trial, had the second highest levels of colonisation on average in this trial, closely followed by Suillus with its much improved levels of colonisation in this experiment.

The colonisation levels for the most part did not correlate with root weight. Due to high variability, Suillus had the largest root biomass, which exceeded Lactarius and Salmon Suillus (Figure 3.12). Boletus had the second largest root biomass, as would be expected from its levels of colonisation. On the other hand Salmon Suillus had significantly smaller root biomass than either of these two treatments, on par with the *F. circinatum* control seedlings, while Lactarius gave variable results. Thus, while colonisation was high this did not correspond to higher root biomass, although this may be attributed to the shorter duration of this trial.

## 3.4 Discussion

*Bacillus* species are often isolated from soil and even ECM fungal sporocarps, and many have been identified as MHB in association with ECM fungi in the past (Garbaye, 1994; Frey-Klett et al., 1997; Poole et al., 2001). A strain of *B. megaterium* known as *var. phosphaticum* has been shown to solubilise phosphate (Han et al., 2006), but not all strains of this species or those of *B. simplex* are able to do so as evidenced by S1 (Schwartz et al., 2013). *Bacillus megaterium,* though, was shown to produce siderophores using the overlay method (Hu and Boyer, 1996). All bacterial strains identified in this study were shown to produce IAA although at varying

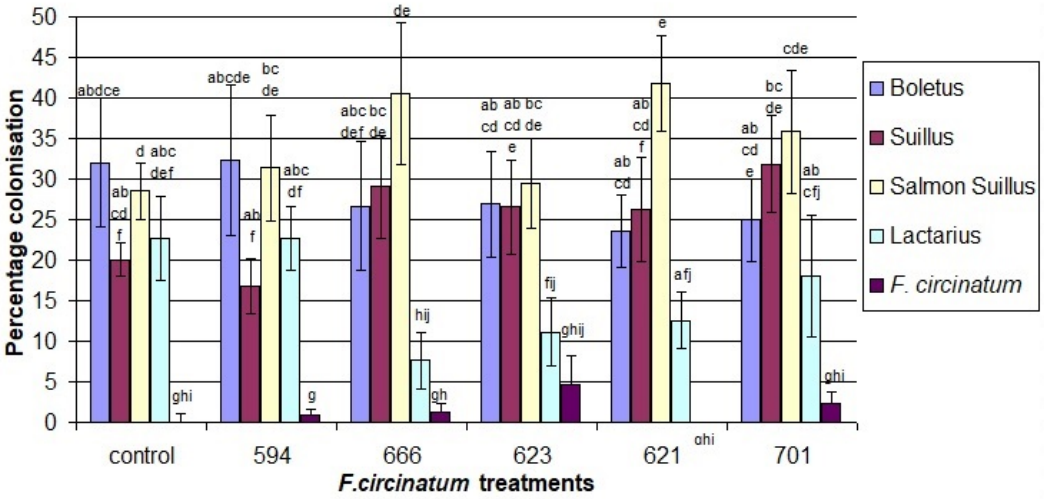



Figure 3.11: Average colonisation levels of *P. patula* seedlings after inoculation with ECM seedlings and exposure to *F. circinatum* (Kruskal-Wallis H (29, 270) = 130.02, p = 9.497e-15). Error bars represent ± standard error. Columns with the same letters are not significantly different from each other.

levels. In this study the bacterial isolate *Stenotrophomas maltophilia* showed the strongest reactions and exhibited positive results for all the plant growth promoting properties tested. It has a worldwide distribution, and is most commonly found in soil and plants. This common endophytic genus can enhance plant productivity by producing IAA, solubilising phosphate and producing siderophores (Ryan et al., 2009; Xiao et al., 2009; Collavino et al., 2010).

Despite these growth promoting properties no significant differences in growth could be attributed to bacterial applications in the plant growth promotion trial. Thus, it is probable that the isolated bacteria were not MHB despite being collected from the mycorrhizal sporocarps. Bacterial specificity is as important when selecting MHB as it is for selecting the correct ECM fungal partner for the host trees. MHB do not always exhibit positive and beneficial effects on mycorrhizal development and have been reported to be highly fungal specific (Garbaye, 1994; Frey-Klett et al., 2007; Rigamonte et al., 2010; Kurth et al., 2013).

The specificity of MHB has also been shown to be strain specific. Dunstan et al., (1998) determined that the MHB *P. fluorescens* BBc6 inhibited the growth of an Australian *Laccaria laccata* even though it had been shown previously to enhance the growth of an American *L. laccata* strain. This study demonstrated that the establishment of Lactarius ECM treatment and

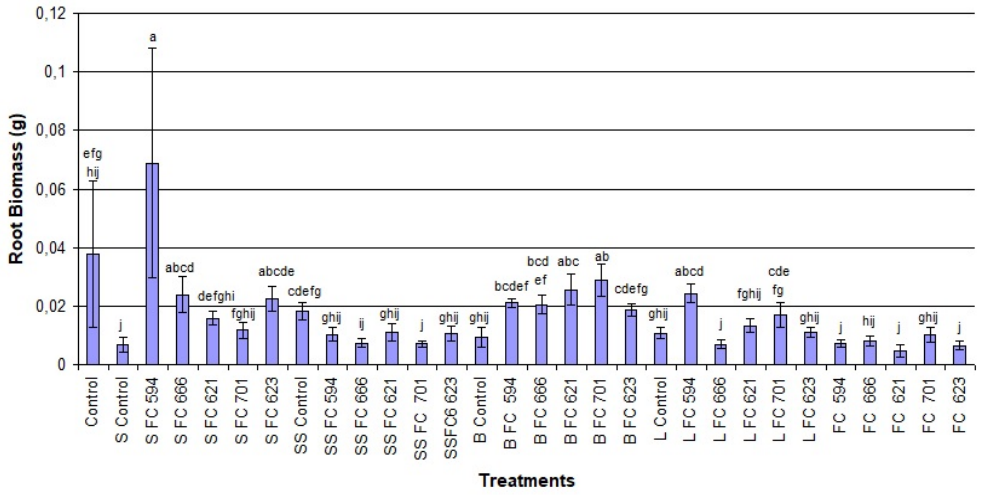



Figure 3.12: Average root dry biomass for each treatment (Kruskal-Wallis H (29, 270) = 117.38, p=1.36e-12). Error bars represent ± standard error. For ease of interpretation treatment names were shortened; S= Suillus, SS= Salmon Suillus, B= Boletus, L=Lactarius and FC= *F. circinatum.* Columns with the same letters are not significantly different from one another.

seedling growth was not enhanced by L1, nor were Salmon Suillus and Suillus ECM colonisation and plant growth improved by S1 collected from *Lactarius deliciosus* and *Suillus granulatus,* respectively. While the bacteria in this study were isolated from sporocarps collected in the same area as the ECM fungal strains, fungal species and strain must be taken into account. This specificity is especially evident in the MHB's ability to enhance mycorrhizal root tip formation or symbiosis promotion (Shishido et al., 1996; Kurth et al., 2013). Aspray et al., (2006) found that 5 different MHB strains did not have a significant effect on the colonisation of *Lactarius rufus* but 2 of the strains did have an effect on the dichotomous short root branching. Therefore it is possible that the bacteria used in this study may have altered the ECM root architecture, rather than increase seedling growth and colonization, which was not investigated.

Fungal species or strain may not be the only important and/or limiting factor to MHB interaction. Apart from ECM colonization responses, selected plant growth enhancing parameters are routinely investigated as a means to establish MHB interaction with ECM fungi and their hosts. But are these properties really indicative of these interactions? IAA production is believed to increase the growth and production of short lateral roots, thus in theory increasing the surface area available for mycorrhizal colonisation and increasing the chances for contact between ECM fungi and the host roots (Garbaye, 1994). But as can be seen from figure 3.8 this

effect was not observed, especially with the bacterial R2 isolate, which produced high levels of IAA. The levels of colonisation are evidently based on ECM fungal species (Sanchez-Zabala et al., 2013) alone rather than being because of the bacteria's presence. Aspray et al., (2006) found that colonisation enhancement was not associated with IAA production, collaborating the finding of this study.

It is likely that the role of IAA in mycorrhizal colonisation is overstated as the receptivity of the plant root to fungal colonisation is due to its nutritional nitrogen and phosphate status rather than the availability of colonisable root tips (Garbaye, 1994). A plants nutritional status alters the permeability of the cell membranes, resulting in decreased permeability and thus reducing the amount of exudates released. This results in altered and reduced signals required for the recognition between ECM fungi and hosts, as well as for effective colonisation (Garbaye, 1994). Similarly, phosphate solubilisation and siderophore production ability is an accepted means of improving nutrient acquisitions and pathogen protection (Schelkle and Peterson, 1997; Rodríguez and Fraga, 1999). In this study the growth of the seedlings was not significantly improved by the presence of the bacteria, displaying these properties.

One potential promising alternative trait that could be used to test and select for MHB is the production of trehalose. The exact role of this fungal derived sugar is not known. But its exclusive use has been shown to be a characteristic for bacteria which are responsible for increased mycorrhizal fungal growth (Duponnois and Kisa, 2006). The growth of the ECM fungus *Pisolithus albus* was significantly increased *in vitro* by the MHB *Pseudomonas monteilii* on media with trehalose as the carbon source, while no significant effects were recorded with other carbon sources. Frey-Klett et al., (1997), Izumi et al., (2006) and Uroz et al., (2007) all showed that bacteria isolated from on and near mycorrhizal roots preferentially used the fungal derived sugar, trehalose, which differed from the bacteria isolated from the bulk soil. Thus, it is likely that trehalose is a strong determinant in the MHB-ECM fungal interaction, particularly in the asymbiotic or initial stage of mycorrhizal development. The ability of bacteria to utilise trehalose *in vitro* could be a useful step to confirm if bacteria isolated are MHB and would provide an alternative test for MHB properties. For increased plant growth promotion properties, testing for the ability to fix nitrogen would also be important due to it being a growth limiting factor for plant nutrition, especially of large trees (Frey-Klett et al., 2007).

In the first trial the seedlings inoculated with Suillus has the lowest growth, colonisation and root weight. This could be due to a number of different factors, such as instability, slower

colonisation rate or most likely a less than optimal relationship between the fungus and the host plant. While *Suillus* species are known to have high specificity to conifers, they usually have a narrow host range and associate with a single host genus, usually either *Pinus, Larix* or *Pseudotsuga* (Dahlberg and Finlay, 1999). This strain of Suillus while collected from a *Pinus* stand, was collected under Stone pines *P. pinea* and thus may be more adapted for interaction with this species. Similar results of negative seedling growth due to ECM fungal inoculation were observed by Ricon et al., (2007) on *Pinus halepensis* seedlings inoculated with *Rhizopogon roseolus.* In some cases ECM fungi can act more like parasites rather than symbionts, especially in the presence of host plants they do not typically associate with (Kipfer et al., 2012). This differs from the Salmon Suillus inoculum, which produced the tallest *P. patula* seedlings and thus is the most suitable inoculum for the seedlings. This is especially true if the objective of the nursery is to produce large, strong, superior seedlings, able to withstand transplantation.

An initial decrease in growth is sometimes seen in systems where host plants and fungal symbiont rely solely on each other for carbon compounds and nutrients to support growth (Smith and Read, 2008), which is the case with outplanted forestry seedlings. This could also explain the low plant growth associated with the Lactarius + R2 and the Lactarius control treatments, although the Lactarius treatments had the second highest levels of colonisation in the first trial. High levels of colonisation do not always translate into increased seedling growth or biomass as found by Onwuchekwa et al., (2014). The relationship between colonisation levels and seedling growth is believed to be a result of ECM fungi exerting greater energy demands on the plant for the support of its hyphal mass and/or due to environmental conditions such as moisture (Kipfer et al., 2012; Onwuchekwa et al., 2014).

An increase in root biomass associated with ECM inoculation would be expected, but was not recorded in this study. Sanchez-Zabala et al., (2013) found similar results where *P. pinaster* seedlings showed increased growth after ECM fungal inoculation. However no significant differences were recorded on the root biomass of the seedlings. It is highly possible that the carbon allocated to the roots was used to increase ECM colonisation rather than increase hyphal extension and root growth into the soil for nutrient acquisition (Egerton-Warburton and Allen, 2001). The short duration of the experiment must also be taken into account. Once sufficient colonisation had developed it is possible that carbon allocation may have shifted to hyphal extension into the rhizosphere overtime. This requires a more detailed investigation.

*Lactarius* species have also been shown to prefer soil horizons with more abundant mineral nu-

trients, or inorganic nitrogen levels, in comparison to soils with high organic material content for nutrient acquisition (Baier et al., 2006; Corrales et al., 2017). The mineral conditions in this study were predominantly organic. Thus, the preference of Lactarius for inorganic nutrient sources may be the cause of the results observed in this study, where it is likely the Lactarius inoculum placed energy into root colonisation and was therefore not able to promote growth. Due to its preference for inorganic nutrients this Lactarius inoculum would be optimal for seedlings out-planted into compartments where the slash had been burned. Burning has been shown to significantly increase inorganic nitrogen levels in the soil for up to 205 days after medium to intense burning (Certini, 2005). Reduced growth also sometimes occurs in times of low irradiance, which can limit the levels of photosynthesis but not colonisation (Smith and Read, 2008). While this explanation is unlikely it is possible the replicates for those treatments were unknowingly placed in areas of increased shade compared to the other treatments, as the pots were not rotated on a regular basis.

*Suillus* species have evolved to respond only to certain chemical stimuli from their specific host roots (Dahlberg and Finlay, 1999). Diterpene resin acid or abietic acid have been found to induce spore germination in *Suillus* species, so the introduction of these chemicals into the inoculum may increase Suillus hyphal growth. This specific resinous acid has been found to stimulate germination of *Suillus granulatus* spores (Fries and Newman, 1990), the species identified for this treatment. This would require further investigation.

In the second trial, only the ECM fungus Lactarius significantly improved the growth of the *P. patula* seedlings inoculated with pitch canker, especially isolate 594. While the increase in growth of plants subjected to other ECM + FC treatments were not significant, an increase in growth was still observed for all Lactarius and Salmon Suillus + FC strains treatments. Salmon Suillus had the highest growth and corresponding high levels of root colonisation. Thus any future work on aspects of ECM fungal biological control of *F. circinatum* would need to focus on both the Lactarius and Salmon Suillus isolates.

The biological pot trial results, indicating that Salmon Suillus is a promising contender, are not reflected in the results of the dual anti-pathogenic *in vitro* assay that was conducted, where Salmon Suillus showed little to no visible levels of inhibition against *F. circinatum.* This indicates that it employs an indirect mode of action through colonisation of roots, thereby limiting pathogen infection sites. ECM fungi form a mantle around each and every root tip they colonize which can be anything from 1-2 hyphal diameters thick to 30-40 diameters. Thus in order

for a pathogen to enter the plant roots, it must now penetrate the ECM fungal mantle and the plant epidermal cell walls before infection can occur (Marx, 1972; Branzanti et al., 1999). It is also well known that non-mycorrhizal roots, especially non-lignified growth, are targeted by *F. circinatum.* Thus, mycorrhizal colonisation is a viable form of biological control, particularly at the seedling stage. In a study performed by Branzanti et al., (1999) it was found that spores of the pathogens *Phytophthora cinnamomi* and *P. cambivora* were only detected on non-ECM colonised roots. In natural environments ECM fungi not only competitively exclude pathogens from infection sites but also compete for nutrients, such as litter patches. The ECM fungi use the carbon acquired from the host plant to rapidly colonise and grow within these nutrient sources and deplete the available nutrients in advance of other competitor organisms (Leake et al., 2001).

Conversely Lactarius, despite producing a significant increase in seedling growth, had the lowest level of colonisation. Thus, it is proposed that this ECM fungus exhibits a more direct form of inhibition against *F. circinatum.* This was demonstrated by the strong growth inhibition of *F. circinatum* observed in the *in vitro* dual assay. Similar results of low colonisation levels and inhibited *Fusarium* infection were observed by Mateos et al., (2017). Their study found that mycorrhizal formation was significantly decreased when *P. sylvestris* and *P. pinea* seedlings were co-inoculated with *Suillus luteus* and *F. oxysporium* or *F. verticillioides* yet fungal infection was still inhibited. This indicates that the inhibition of the pathogenic fungus was not related to competition for space but was rather due to a direct form of inhibition (Mateos et al., 2017). Direct forms of inhibition include the production of antifungal enzymes like chitinases and ß-1,3-glucanases. Both of these are considered to be important enzymes in the lysis of fungal cell walls as chitin and ß-1,3-glucan are key fungal cell wall components and have been found in the cell walls of many plant pathogens (Mucha et al., 2006; Mohan et al., 2015). Mohan et al., (2015) found that while the amount of chitinase produced differed between ECM fungal species in dual culture *in vitro* experiments each species tested exhibited significant inhibition of different plant pathogens. ECM fungi also produce other anti-fungal compounds such as oxalic acids, phenolics, steroids and hydrogen peroxide (Suh et al., 1991; Yamajii et al., 2005; Soytong et al., 2014; Takakura, 2015). For example Duchnes et al., (1988a, 1988b) observed a six-fold reduction in the sporulation of the pathogen *Fusarium oxysporum* in the *Pinus resinosa's* rhizosphere after inoculation with the ECM fungus *Paxillus involutus.* This was found to be due to ethanol-soluble compounds with fungal-toxic effects found in the rhizosphere 3 days after

ECM fungal inoculation.

From the Suillus' anti-fungal assay it was highly likely that it too inhibited *F. circinatum* infection via direct means even though the Suillus treatment did not increase the growth of the *P. patula* seedlings. Mateos et al., (2017) also reported cases of levels of ECM fungal colonisation, high fungal pathogen inhibition and absence of improved seedling growth.

It appears though that Boletus uses a more indirect form of pathogen inhibition, similar to Salmon Suillus, by aggressively colonising plant roots and increasing root biomass. It is also possible that the inhibition observed in the trial was due to increased levels of plant nutrition attained via ECM association. This would have allowed the host plant to disproportionately allocate resources to its defense mechanisms (Bennet et al., 2005). Increased seedling growth seen as a result of ECM inoculation shows that overall the plant's tolerance and resistance to infection from the presence of *F. circinatum* in the potting soil was decreased due to the biological control properties, direct or indirect, of the ECM fungi.

Interestingly, despite the shorter length of the biological control trial the ECM fungal treatments overall had raised levels of colonisation in comparison to the plant growth promotion trial. This increase in mycorrhizal colonisation is believed to be a direct result of the co-inoculation with *F. circinatum*, as observed by Zampieri et al., (2017) who observed this phenomenon in the presence of the pathogenic fungi *Heterobasidion irregulare* and *H. annosum*. The cause of the increase in mycorrhizal colonisation is not know, but is either a response of the ECM fungi to the stress that the plant is experiencing due to pathogenic infection/recognition (Zampieri et al., 2017) or that the host plant's defenses become lowered due to pathogenic infection resulting in a more favourable environment for ECM colonisation process (Fitter and Garbaye, 1994). Similar increases in colonisation in the presence of pathogenic fungi are seen with arbuscular fungi as observed by Garcia-Romera et al., (1998) on soybean in the presence of *F. oxysporium* and Diedhou et al., (2003).

It is also possible that the conditions for the second trial were more conducive for mycorrhizal growth, because although the tunnel conditions are controlled to some extent the first pot trial was conducted in winter with lower tunnel temperatures, while the second trial was conducted in early summer. This change in temperature and season is likely to have had an effect on ECM growth and colonisation as ECM fungi are recorded as having higher growth levels in early summer (Smith and Read, 2008; Höberg et al., 2010).

ECM fungi also prevent fungal infection by cultivating bacteria which are able to produce anti-

fungal compounds or indirectly inhibit the growth of the pathogen (Frey-Klett, et al., 2005). All the MHB in this study were tested for the production of siderophores. Siderophores can be used in the prevention of pathogenic fungal growth as they remove available iron from the immediate area via chelation (Pérez-Miranda et al., 2001).

However the MHB were not utilised in the second trial and could be tested further to develop a more holistic approach to the control of the *Fusarium.* An interesting area of future study would be a combination of the two best performing ECM fungi, Salmon Suillus and Lactarius, with the bacteria L1 and R2 against the different *F. circinatum* strains.

In conclusion it can be seen from the two trials conducted that of the successfully isolated ECM fungi, the two best isolates for inoculation of *P. patula* seedlings in South African nurseries would be *Lactarius quieticolor* and the *Suillus* isolate Salmon Suillus. Optimisation of fungal growth and development of an inoculum would be the next step in reaching this goal.

# Chapter 4

# General Discussion

A plant's microbiome is responsible for its health, longevity and productivity (Shakya et al., 2013; Barnes et al., 2016; Gallart et al., 2018). The microbiome both in and around a plant's roots controls a large portion of a plant's ability to access nutrients such as nitrogen and phosphate, nutrient recycling and exchange, and carbon sequestration (Daguerre et al., 2017). It is made up of a large number of below-ground microbial communities of fungi, bacteria and archaea (Baldrian, 2017), the composition of which is driven by a number of different factors. These include the host plant, soil pH, soil nutrients and to a lesser extent seasonal changes (Barnes et al., 2016; Baldrian, 2017; Gallart et al., 2018). Within nurseries these conditions are more controlled and it is the host plant which most strongly dictates its microbiome. They play a defining role in dictating their microbiome through direct interaction and influence with the microbial taxa through its roots and root exudates, actively recruiting soil microorganisms for their rhizospheres (Baldrian, 2017; Gallart et al., 2018).

The most significant of these factors for the control of the fungal microbiome is the host plant (Peršoh, 2015). Within forestry nurseries conditions are different from those found in forests and plantations, due to activities such as fertilization, irrigation, microbial and weed pest control and growth conditions, thus affecting the microbial biome (Iwanski et al., 2006; Menkis and Vasaitis, 2011; Gallart et al., 2018). *Pinus* seedlings, in nurseries as in natural environments, associate with a diverse set of fungi ranging from saprotophs, endophytes and ECM fungi, which play a unique role within a plant's microbiome and are often the point of contact between the root and the surrounding environment (Daguerre et al., 2017). In forestry plantations and natural forest ecosystems nearly all fine roots are associated with ECM fungi; however the same

can not be said of nursery seedlings. The way in which these seedlings are grown has been found to have a significant effect on the levels and type of ECM fungi colonisation (Menkis and Vasaitis, 2011). Menkis et al., (2005) found that bare-root seedlings of *Pinus sylvestris* had the highest levels of ECM fungal colonisations, followed by those grown in plastic trays and then greenhouse grown seedlings. Iwanski et al., (2006) and Menkis and Vasaitis (2011) both found that the nursery conditions resulted in significantly lower levels of ECM colonization overall and that the majority of the mycorrhizal fungi present were *Thelephora terrestris* and *Wilcoxina mikolae,* both "weed" mycorrhizal fungi.

Less is known about the relationship between plants and endophytic and saprotrophic fungi. Some endophytes offer their host plant some ecological or physiological advantages, while others are latent saprotrophs or pathogens, waiting for the plant to either die or become damaged (Nicoletti et al., 2014). Thus, it is important to begin research with the knowledge of a plant's microbiome in order to anticipate and interpret how additions onto the system such as inoculation with ECM fungi would interact with the microbial, specifically fungal community, including and especially naturally occurring ECM fungi. It is well established that colonization with ectomycorrhizal fungi increases a seedling's chance of survival and overall health once outplanted (Grossnickle, 2005; Quoreshi et al., 2008). In chapter 2 the microbiome of South African commercially produced *P. patula* seedling roots was investigated with a specific emphasis on naturally occurring ECM fungi. To date no research has been carried out on the microbiome of containerised *P. patula* seedlings in South Africa. The majority of the research within this area has focused on the associations and effects of bacterial communities or bacterial and fungal interactions.

One of the pitfalls experienced in this study was the poor correlation between the morphological and molecular ECM fungal identifications. In ECM fungal community research it is rare to find studies which identify ECM based only on morphological identifications without molecular confirmation. Both methods have their advantages and disadvantages. Morphological identification of colonized root tips or sporocarps are based on dichotomous keys which, while comprehensive, is a process that requires training and skill and often still results in mis or unidentification due to a lack or similarity of morphological features (Tedersoo et al., 2006; Rosling et al., 2003; Clasen et al., 2018), as was seen chapter 2. The advent of molecular techniques based on DNA and/or RNA analysis allowed for many of the gaps left from morphological identification, such as differentiation of phenotypic variation and a more comprehensive

view of fungal communities, to be filled (Jonsson et al., 1999; Tedersoo and Nilsson, 2016; Clasen et al., 2018). Molecular work has advantages such as high sensitivity, and is not subject to phenotypic variations, environmental actions, fungal developmental stage or other factors which affect ECM fungal morphology. The invention of Illumina and other next generation sequencing techniques has allowed for investigations of such root associated communities to be culture and/or clone library independent, thus reducing time and overall costs (Buée et al., 2009; Clasen et al., 2018).

For fungal research the ITS region of the genome has been the most widely targeted for molecular identification (Johnson et al., 2012), for which many variations of universal fungal primers ITS1 and ITS4 have been developed (White et al., 1990). Over time primers have been developed for specific fungal identification. Yet at this time there are no ECM fungi specific primers available, due to the wide and diverse range of ECM fungi belonging to different phyla and families (Clasen et al., 2018). Some primer pairs have been created for specific genera (Tedersoo and Nilsson, 2016), but this limits the identification process to ECM fungi which are known to be present. Thus, relying heavily once again on morphological identifications and is not a viable solution for ecological community diversity studies. Additionally a common problem experienced when analyzing ECM fungi is contamination by either secondary ECM fungal colonization of the same root tip, root endophytes or other fungi present in the soil, which have a greater affinity for the primers than the fungus being identified (Rosling et al., 2003; Clasen et al., 2018). This results in mixed sequences which only report the identification of the most dominant fungal sequence. While this problem can be overcome with next generation sequencing, levels of ECM fungal identification are still low as demonstrated in this study due to the low affinity of some ECM fungi to the universal ITS primers.

## 4.1 ECM fungi in commercial forestry

The majority of the ECM fungi identified on the *P. patula* seedlings analysed in this study were shown to be "weed" mycorrhizal fungi, such as *T. terrestris* or only tentatively morphologically identified as beneficial mycorrhizal fungi, as with the "Yellow-Orange" morphotype; tentatively identified as *Suillus.* These results, along with the overall low levels of ECM fungal colonisation found, emphasize the need for a beneficial ECM inoculum for use and application in South African forestry nurseries. In chapter 3 the locally harvested ECM fungi *Lactarius quieticolor*

Table 4.1: Commercial ectomycorrhizal fungi inoculants produced through different processes by different companies (Adapted from Rossi et al, 2007)

| Commercial product | Type/process | Company |
|---|---|---|
| BioGrow Blend® | Spores | Terra Tech, LLC |
| MycoApply®-Ecto | Spores | Mycorrhizal Applications Inc. |
| Mycorise Pro Reclaim® | Propagules ecto+endo | Symbio Technologies Inc. |
| Myke® Pro LF3 | Propagules | Premier Tech Biotechnologies |
| Mycor Tree® | Spores | Plant Health Care, Inc. |
| MycoRhiz® | Mycelium/SSF | Abbott Laboratories |
| Somycel PV | Mycelium/SSF | INRA – Somycel S.A. |
| Ectomycorrhiza Spawn | Mycelium/SSF | Sylvan Spawn Laboratory, Inc. |
| Mycobead® | Mycelium/Submerged | Biosynthetica Pty. Ltd. |

SSF = solid-state fermentation

and the *Suillus* species isolate Salmon Suillus were identified as beneficial ECM isolates which improved plant growth, increased pathogen resistance and provided protection against the economically important nursery pathogen *F. circinatum.* Both isolates demonstrated significant potential for development into a nursery inoculum in South Africa due to their beneficial properties and existing adaptation to the South African environment. In the case of Salmon Suillus and Lactarius there is an additional economic benefit of the production of edible fruiting bodies once the seedlings are outplanted in the forestry plantations. Currently no ECM fungi are used in South Africa as an inoculum, nor are there South African products available for inoculation (Table 4.1).

Overall very few ECM fungi have been developed into commercial inoculation products, partially due to the large gaps in the literature which pertain to the production and application processes of ECM fungi on larger scales (Rossi et al., 2007; Rossi et al., 2017). The majority of the isolates developed consist of the ECM fungi *Pisolithus tinctorius* or *Rhizopgon* (Sebastiana et al., 2013; Internet 2), shown to have great beneficial effects on *Eucalyptus* trees (Rossi et al., 2007). ECM fungi have become well known as a good practice for improving seedling nursery stock and for forest productivity. The production and marketing of ECM fungal inoculum for commercial use has increased over the years (Ricon et al., 2005; Rossi et al., 2007; Repáč, 2011). ECM fungal inoculation not only has potential in the nurseries but also for outplanting as they increase successful establishment in the field, but also as increased seedling growth in the nursery directly results in savings due to increased throughput (Smith and Read,

2008). Production of an ECM fungal inoculum consists of several steps, some of which have been achieved in this study. Firstly the appropriate ECM isolate must be selected, via trials that investigate compatibility and efficiency, such as those conducted in this study. Additionally the ECM fungal isolate must also be selected on the basis of fungal growth rate, as the slower the growth rate, the longer and more costly production becomes; viability during storage; maintenance of infectivity after the production of inoculum and in the environment it will experience once applied; applicability; and costs (Rossi et al., 2007; Repáč, 2011).

Ectomycorrhizal inoculum is usually produced in one of 3 ways; natural, such as forest soil or humus, basidiospores or vegetative mycelia. Natural inoculation is rarely used, not for the lack of ECM fungal spores present, but rather because it is not possible to control which ECM fungi are present, the colonisation rates are low and there is a high risk of pathogens being introduced (Rossi et al., 2007; Smith and Read, 2008; Repáč, 2011; Bassani et al., 2013). Commercial ECM inoculum commonly use either basidiospores or vegetative inoculum. Basidiospores, in comparison to vegetative inoculum, are significantly easier to collect. They are collected directly from the sporocarp and thus do not require extended growing time in sterile pure cultures and require less space for storage. Basidiospores are collected either from spore prints and dried crushed sporocarps or by homogenising the fruiting bodies in water. This does require the ECM fungus chosen to be a species known for producing large quantities of spores, to make the inoculum viable. In addition, as spores are collected directly from the sporocarps, the collection is subject to seasonal variabilities as well as genetic variabilities due to the collection from multiple sporocarps (Brundrett et al., 1996; Rossi et al., 2007; Smith and Read, 2008; Repáč, 2011; Bassani et al., 2013).

The third main form of ECM inoculum, and the most effective, is vegetative inoculum (Rossi et al., 2007; Repáč, 2011). But this form of inoculum is considered the most difficult and expensive to produce, due to the equipment and overall time required (Bassani et al., 2013). Vegetative inoculum is produced in one of two main ways, solid or liquid-state fermentation, as the vegetative slurry most commonly produced for laboratory studies is not a feasible option once production is up-scaled (Rossi et al., 2007; Repáč, 2011).

Solid-state fermentation of ECM inocula is the most commonly produced form of vegetative inoculum, although not the most effective of the two forms. Its main advantage is the relatively simple equipment and materiel required, and production is only limited by space needed for incubation. Additionally large amounts of inoculum can be produced from a single strain known

to be beneficial, unlike basidiospores (Brundrett et al., 1996). To produce solid inoculum, ECM fungi are grown on a substrate typically consisting of a mixture of peat-moss and vermiculite, usually in a 1:7 (v/v) ratio, depending on the preferred final pH, supplemented with a nutritive solution. The substrate is firstly sterilized and then inoculated with either plugs or a suspension of the mycelia (Brundrett et al., 1996; Rossi et al., 2007; Sebastiana et al., 2013). ECM fungi are slow growers and depending on the isolate can take 2-4 months to produce the final inoculum. There are a number of benefits of using solid ECM inoculum. Firstly the vermiculite substrate house and protect the mycelia until the colonization of host roots occurs. Secondly, it is a cheap material which facilitates good aeration in the soil and absorbs nutrient solutions well (Rossi et al., 2007; Smith and Read, 2008). Conversely, the nutrient solution which vermiculite absorbs diffuses into the pores of the substrate which result in increased chances of pathogenic contamination; and if the absorbed nutrient solution is not fully utilised the substrate must be washed before use (Rossi et al., 2007).

ECM fungi produced via liquid fermentation have been traditionally produced using submerged liquid bioreactors (Repáč, 2011), although research spearheaded by Rossi et al., (2002; 2007; 2017) show the potential of airlift bioreactors for commercial scale ECM fungal growth. While more expensive, depending on the bioreactor used, liquid fermentation requires less time and space to produce large quantities of inoculum as the contact between the mycelia and nutrient solutions is maximized (Brundrett et al., 1996; Rossi et al., 2007; Smith and Read, 2008). Once cultivated, the fungal mycelial broth is immobilized in polymeric matrices, most commonly within calcium alginate beads. This form of ECM inoculum has proven to be the most efficient as the fungi are better protected within the beads, and thus survive for a longer period and therefore is more likely to have higher colonization rates, especially in comparison to solid-state substrate carriers (Rossi et al., 2007; Repáč, 2011). Even though it is a more efficient form of inoculum, as mentioned above solid-state fermentation is more commonly used due to the difficulties associated with liquid fermentation. ECM fungi are slow growers, thus problems encountered in liquid fermentation are compounded. They can clog up nutrient and oxygen feeding tubes, they do not sporulate within this system requiring the mycelia to be mechanically dispersed before inoculation within the bioreactors. Additionally, shearing often occurs within the reactors themselves. This causes hyphal damage, thus potentially affecting viability (Rossi et al., 2007; 2017).

The time at which it is best for the ECM fungi to be applied depends on the inoculum type.

Spore inoculum can take one of 3 different forms:, dry, encapsulated or suspended in a liquid slurry. Bassani et al., (2013) investigated spore viability in different storage forms over time, for a number of different ECM fungal species and found that overall storage time negatively affected spore viability and mycorrhizal establishment regardless of storage type. Although variations were observed between ECM fungal species. It was found that the *Suillus* strain investigated performed best under both storage methods. Spores are the easiest to apply, as they can be introduced either before or when the seeds are sown into the planting material, directly onto the seeds via seed coat or even via the irrigation system. Yet compared to vegetative inoculum basidiospores have a much slower colonization rate, due to the fact that spores must first germinate before colonization and ECM formation can occur (Brundrett et al., 1996; Rossi et al., 2007; Repáč, 2011). Vegetative ECM inoculum can be applied in a number of different ways, such as mixed in with the potting material or directly into the planting hole when transplanting to larger pots to maximize contact with the roots, or introduced into the general rooting zone of seedlings (Rossi et al., 2007; Sebastiana et al., 2013). Liquid vegetative inoculum can also be produced into a slurry by the farmer, into which the seedling roots can be directly dipped, although this is less common on a large scale due to the labour involved (Brundrett et al., 1996).

The use of a solid vegetative mycorrhizal inoculum has been shown to be the most efficient of vegetative inocula for the downstream production of ECM fungal fruiting bodies (Wang et al., 2012). The harvesting of edible ECM fruiting bodies is often seen as an additional source of income for the local population, as these sporocarps are considered delicacies (Smith and Read, 2008; Diaz et al., 2009; Wang et al., 2012; Azul et al., 2014). The majority of the edible ECM fungi are harvested from natural forests. A decline in crops over the years has been reported due to social and environmental conditions (Yun and Hall, 2004; Smith and Read, 2008). Thus, the production of known edible ECM fungi from inoculated seedlings would be of great benefit to the local community, sporocarp industry and plantation owners. The first fruiting bodies of *L. deliciosus* were reported in New Zealand on inoculated outplanted *P. radiata* seedlings 1-2 years following inoculation by Yun and Hall (2004). The first successful production of *L. deliciosus* on inoculated seedlings was recorded by Poitou et al., (1984) in France and subsequent production of fruiting bodies has continued in this plantation for 20 years (Wang et al., 2012). High irrigation levels have been found to be crucial for the development and persistence of ECM fungal production of fruiting bodies (Wang et al., 2012). Possible

pitfalls which are often encountered, especially when outplanting seedlings into established plantations or forests, is that after 3-5 years the ECM fungi from the nursery are replaced with the ECM fungi found naturally within said plantations (Smith and Read, 2008). Yet as the ECM fungi used in this study were harvested from a well established *Pinus* population it is less likely that these identified beneficial ECM fungi would be replaced with time and the possibility of continued *Suillus* and *Lactarius* sporocarps for harvesting is high.

## 4.2 Future work

This study was undertaken with the main aims of determining and investigating naturally occurring ECM fungal colonisation in South African nurseries and to find potential isolates of South African ECM fungi for future development into ECM inoculum for *P. patula* seedlings which would increase seedling development and resistance to *F. circinatum*.

While Illumina analysis of the naturally occurring ECM fungi in the nursery seedlings provides initial insight into the fungal community associated with South African *P. patula* seedlings. As indicated by the rarefaction curve, additional sampling is required to give comprehensive and complete understanding of the microbiome of nursery seedlings. Future work would involve additional sampling of ECM rooting tips from seedlings representing all of the nurseries surveyed. The nurseries surveyed also grow other *Pinus* species also popular in the forestry industry such as *Pinus elliottii,* no work has been carried on the microbiome, ECM fungi and other microbe interactions of these seedlings and future work should involve these other *Pinus* species. The use of more specific primers, either analyzing via ECM fungal genera or by currently unavailable ECM specific primers which would contribute significantly towards identifying ECM fungi in microbiome communities.

As the length of the pot trials undertaken was so short, future research should involve longer trials, which additional ECM species. Significant additional work must also be undertaken to develop the beneficial ECM fungi identified in the pot trials into successful commercial South African ECM fungal inocula. Future work would involve the optimization of growth conditions required for the isolates *Lactarius quieticolor* and the *Suillus* isolate Salmon Suillus *in vitro*. Analysis on the productivity and survival rate of *P. patula* seedlings after inoculation and outplanting, and investigation into the most efficient methods of fungal inoculation to produce the highest levels of mycorrhizal colonisation need to be undertaken. Additional investigation into

the possibility of producing edible fruiting bodies from the Salmon Suillus isolate under nursery conditions and/or in plantations. As the ECM fungi used were isolated from well established South African *Pinus* stands, it is unlikely the isolate would be phased out by already established ECM fungi in the plantations.

Additional research to isolate and identify MHB and MHY for the ECM fungal strains *Lactarius quieticolor* and the *Suillus* isolate Salmon Suillus isolated in this study, should be undertaken. These potential MHB and yeasts would be subjected to a much wider range of tests for mycorrhizal helping properties which could potentially be more indicative of true ECM fungal associations.

## 4.3 Conclusion

This study was designed to identify and investigate ECM fungi and ECM fungal levels of root colonisation in South African nurseries and to determine whether selected ECM fungi and MHB were capable of promoting *P. patula* growth and reduce incidences *F. circinatum* fatalities. While positive identification of the ECM fungi present within the nurseries proved difficult, an overall initial view of the fungal root microbiome found in South African nurseries was achieved along with the determination that ECM fungal root colonisation levels are very low throughout South African nurseries. This demonstrates the need for ECM fungal use within these nurseries. From the two greenhouse trials conducted it was concluded that of the isolated ECM fungi, the two best performing isolates that showed promising potential for use as an inoculum of *P. patula* seedlings in South African nurseries were *Lactarius quieticolor* for its increased resistance to *F. circinatum* and the *Suillus* isolate Salmon Suillus for the enhancement of the growth of the seedlings. None of the MHB isolated were found to have an effect on the growth of the seedlings. Future investigations would focus on broadening the fungal root microbiome knowledge. More specific ECM fungal primers and longer field trials with the ECM fungal isolates shown to be beneficial to plant growth. Optimization of fungal growth and development of an inoculum would be the next step in reaching this goal.

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# Appendices

## Appendix A

### A1. Modified Melin Norkans (MMN)

To 990 ml of distillied $H_2O$ add:

- 20 g Glucose Anhydrous
- 1 g Potassium di-hydrogen orthophosphate
- 0.5 g Magnesium sulphate hexahydrate
- 0.5 g Ammonium nitrate
- 500 µl 1% Ferric citrate
- 500 µl 1% Zinc solution

Adjust to pH 5 (adding NaOH).

- Add 15 g Bacteriological agar (omit for broth)

Autoclave at 121℃ for 15 minutes.
Once cooled filter sterilise (0.45 µm filter) and add:

- 50 µg Thiamine in 10 ml sterile water
- 50 mg/L of chloramphenical
- 50 mg/L of ampicillin

## A2. Chrome Azurol S agar

A. Bacteriological agar PIPES ($L^{-1}$)

To 1 L of distillied $H_2O$ add:

- 5 g Malic Acid
- 0.5 g Dipotassum Phospahte
- 0.2 g Magnesium Sulphate Heptahydrate
- 0.1 g Sodium Chloride
- 0.2 g Calcium Chloride Dihydrate
- 5.5 g Potassium Hydroxide
- 1 g Ammonium Chloride
- 30 g Piperazine-1,4-bis(2-ethane-sulfonic acid) (PIPES)
- Add 15 g Bacteriological Agar

Adjust to pH 6.8 with NaOH pellets while stirring. Autoclave at 121ºC for 15 minutes.

B. CAS (Chrome Azurol S) solution

Solution 1 (CAS):

- Add 0.060g chrome azurol S (Sigma-Aldrich 199532-25G) in 25 ml of de-ionised water

Solution 2:

- Dissolve 0.0027 g of $FeCl_3.6H_2O$ in 10 ml of 10 mM HCl

Solution 3:

- Dissolve 0.073 g of hexadecyltrimethyl-ammonium bromide (HDTMA) in 40 ml of de-ionised water. In a container large enough to accomodate solution 1 and 2.

Combine solution 1 with 9 ml of solution 2 and add to solution 3. Solution should be purple-blue colour. Autoclave at 121ºC for 15 minutes.

C. Preperation of media

Allow to cool after autoclaving.

Add the CAS solution to the PIPES solution, gently inverting to mix.

Pour as normal or as overlay onto petri dishes.

Media should be dark blue.

## A3. Phosphate solubilising agar

**P solubilising**

To 1 L of distillied $H_2O$ add:

- 10 g Glucose Anhydrous
- 5 g Calcium Phosphate
- 5 g Magnesium Chloride Hexahydrate
- 0.25 g Magnesium Sulphate Heptahydrate
- 0.2 g Potassium Chloride
- 0.1 g Ammonium sulphate

Adjust to pH 7.

- Add 15 g of Bacteriological Agar

Autoclave at 121ºC for 15 minutes.

## A4. Luria media

To 1 L of distillied $H_2O$ add:

- 10 g Tryptone
- 5 g Yeast Extract Powder
- 5 g Sodium Chloride

Adjust to a pH of 7.2

- add 15 g Bacteriological Agar (omit for broth)

Autoclave at 121ºC for 15 minutes.

# Appendix B

## Preparation of Standard Curve for Estimation of IAA

1. Salkowski Reagent: Combine 2 ml 0.5M Iron(III) Chloride + 49 ml distilled $H_2O$ + 49 ml 70% Perchloric Acid

2. IAA Standards:

- 10 mg IAA was added to 10 ml Acetone. This was the 1000 µg/ml stock.
- 1 ml of 1000 µg/ml stock was added to 9 ml tryptophan broth (TB). This was the 100 µg/ml standard.
- 5 ml of 100 µg/ml standard was added to 5 ml TB. This was the 50 µg/ml standard.
- 2 ml of 100 µg/ml standard was added to 8 ml TB. This was the 20 µg/ml standard.
- 1 ml of 100 µg/ml standard was added to 9 ml TB. This was the 10 µg/ml standard.
- 1 ml of 50 µg/ml standard was added to 9 ml TB. This was the 5 µg/ml standard.

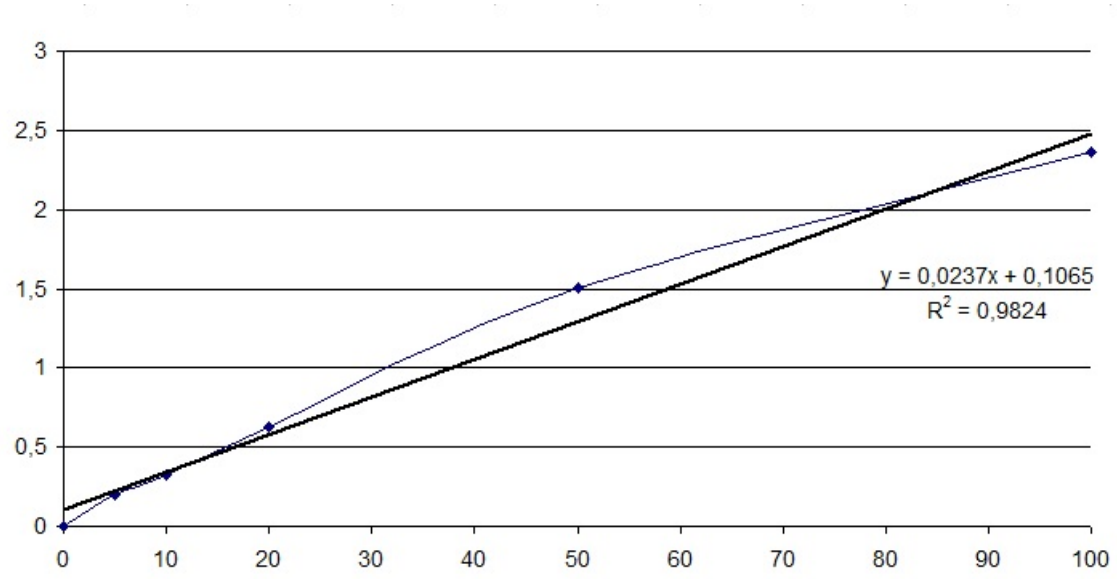


Figure 1. Standard curve prepared from IAA standards, 5, 10, 20, 50, 100 µg/ml in Tryptone Broth.

3. 2 ml of Salkowski Reagent per standard was added to labelled MacCartney bottles.

4. 1 ml of each standard was added to each bottle including pure TB as a negative control.

5. Vials were incubated in the dark at room temperature for 25 mins to allow colour development and spectrophotometrically analysed at 530 nm.

6. Absorbance readings were used to create a standard curve (Figure 1)

7. The equation (y = 0.0237x + 0.1065), produced by the correlation graph (Figure 1), was used to calculate approximate concentrations of IAA in culture supernatants (Table 1)

Table 1. Summary of IAA standard concentrations returned by the equation y = 0.0237x + 0.1065.

| IAA concentration (µg/ml) | $OD_{530}$ | IAA calculation |
|---|---|---|
| 0 | 0 | -4,4937 |
| 5 | 0.201 | 3,9873 |
| 10 | 0.329 | 9,3882 |
| 20 | 0.622 | 21,7511 |
| 50 | 1.506 | 59,0506 |
| 100 | 2.359 | 95,0422 |

# Appendix C

## Nursery Survey

1. Which substrates do you use for your *Pinus patula* seedlings?

| | |
|---|---|
| Pine Bark | |
| Coir | |
| Peat | |
| Perlite | |
| Vermiculite | |
| Other: Please specify | |

2. What sanitation practices do you employ for your P. patula seedling trays?

| | |
|---|---|
| Steam | |
| Chemical(s): Please specify | |
| Other: Please specify | |

3. What fungicides or anti-microbials do you employ on *P. patula* seedlings?

| | |
|---|---|
| Biological: Please specify the main products used | |
| Soft chemicals: Please specify main products used | |
| Hard chemicals: Please specify main products used | |
| Chloride | |
| I do not use chemicals | |
| Other: Please specify | |

4. Is your fungicide use preventative or curative?

| | |
|---|---|
| Preventative | |
| Curative | |
| I do not use fungicides | |

5. Is a seed dressing applied to the *P. patula* seedlings?

| | |
|---|---|
| Yes | |
| No | |

6. Do you treat *P. patula* seeds before sowing?

| | |
|---|---|
| No | |
| Hot water | |
| Cold water | |
| Chemical: Please specify chemical(s) used | |

7. Do you apply an ectomycorrhizal innoculum?

| | |
|---|---|
| Yes | |
| No | |

8. Do you grow other Pinus species?

| | |
|---|---|
| Yes: Please specify | |
| No | |

# Appendix D

```
mothur > fastq.info(fastq=VEE_ASS25_S70_L001_R1_001.fastq)
10000
20000
30000
40000
50000
58201
Output File Names:
VEE_ASS25_S70_L001_R1_001.fasta
VEE_ASS25_S70_L001_R1_001.qual

mothur > summary.seqs(fasta=VEE_ASS25_S70_L001_R1_001.fasta,
    processors=24)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       35      35      0       3       1
2.5%-tile:      1       292     292     0       4       1456
25%-tile:       1       300     300     0       5       14551
Median:         1       301     301     0       5       29101
75%-tile:       1       301     301     0       6       43651
```

```
97.5%-tile:     1       301     301     0               10      56746
Maximum:        1       301     301     35              35      58201
Mean:           1       299.821 299.821 0.00774901      5.68956
# of Seqs: 58201
Output File Names: VEE_ASS25_S70_L001_R1_001.summary
It took 0 secs to summarize 58201 sequences.

mothur > trim.seqs(fasta=VEE_ASS25_S70_L001_R1_001.fasta, qfile
   =VEE_ASS25_S70_L001_R1_001.qual, qwindowaverage=20,
   minlength=180)
Using 24 processors.
Output File Names:
VEE_ASS25_S70_L001_R1_001.trim.fasta
VEE_ASS25_S70_L001_R1_001.scrap.fasta
VEE_ASS25_S70_L001_R1_001.trim.qual
VEE_ASS25_S70_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_ASS25_S70_L001_R1_001.trim.
   fasta)
                Start   End     NBases  Ambigs          Polymer NumSeqs
Minimum:        1       35      35      0               3       1
2.5%-tile:      1       292     292     0               4       1456
25%-tile:       1       300     300     0               5       14551
Median:         1       301     301     0               5       29101
75%-tile:       1       301     301     0               6       43651
97.5%-tile:     1       301     301     0               10      56746
Maximum:        1       301     301     35              35      58201
Mean:           1       299.821 299.821 0.00774901      5.68956
# of Seqs: 58201
Output File Names: VEE_ASS25_S70_L001_R1_001.trim.summary
It took 0 secs to summarize 56724 sequences.

mothur > make.group(fasta=VEE_ASS25_S70_L001_R1_001.trim.fasta,
```

```
    groups=NurseryA)
Output File Names:
VEE_ASS25_S70_L001_R1_001.trim.groups

mothur > fastq.info(fastq=VEE_ASS53_S34_L001_R1_001.fastq)
10000
20000
30000
35207
Output File Names:
VEE_ASS53_S34_L001_R1_001.fasta
VEE_ASS53_S34_L001_R1_001.qual

mothur > summary.seqs(fasta=VEE_ASS53_S34_L001_R1_001.fasta,
    processors=24)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       35      35      0       3       1
2.5%-tile:      1       283     283     0       5       881
25%-tile:       1       300     300     0       5       8802
Median:         1       301     301     0       5       17604
75%-tile:       1       301     301     0       6       26406
97.5%-tile:     1       301     301     0       10      34327
Maximum:        1       301     301     35      35      35207
Mean:           1       297.958 297.958 0.0255347       5.89812
# of Seqs:  35207
Output File Names:  VEE_ASS53_S34_L001_R1_001.summary
It took 1 secs to summarize 35207 sequences.

mothur > trim.seqs(fasta=VEE_ASS53_S34_L001_R1_001.fasta, qfile
    =VEE_ASS53_S34_L001_R1_001.qual, qwindowaverage=20,
    minlength=180)
Output File Names:
VEE_ASS53_S34_L001_R1_001.trim.fasta
```

```
VEE_ASS53_S34_L001_R1_001.scrap.fasta
VEE_ASS53_S34_L001_R1_001.trim.qual
VEE_ASS53_S34_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_ASS53_S34_L001_R1_001.trim.
    fasta)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       180     180     0       3       1
2.5%-tile:      1       208     208     0       5       860
25%-tile:       1       283     283     0       5       8593
Median:         1       294     294     0       5       17185
75%-tile:       1       301     301     0       6       25777
97.5%-tile:     1       301     301     0       9       33510
Maximum:        1       301     301     0       29      34369
Mean:           1       286.452 286.452 0       5.76898
# of Seqs: 34369
Output File Names: VEE_ASS53_S34_L001_R1_001.trim.summary
It took 1 secs to summarize 34369 sequences.

mothur > make.group(fasta=VEE_ASS53_S34_L001_R1_001.trim.fasta,
     groups=NurseryA)
Output File Names: VEE_ASS53_S34_L001_R1_001.trim.groups

mothur > fastq.info(fastq=VEE_ASS613_S11_L001_R1_001.fastq)
10000
20000
30000
40000
50000
60000
70000
80000
90000
```

```
98897
Output File Names:
VEE_ASS613_S11_L001_R1_001.fasta
VEE_ASS613_S11_L001_R1_001.qual

mothur > summary.seqs(fasta=VEE_ASS613_S11_L001_R1_001.fasta,
    processors=24)
                Start   End     NBases  Ambigs      Polymer     NumSeqs
Minimum:        1       35      35      0           3           1
2.5%-tile:      1       143     143     0           4           2473
25%-tile:       1       168     168     0           4           24725
Median:         1       263     263     0           5           49449
75%-tile:       1       301     301     0           6           74173
97.5%-tile:     1       301     301     0           14          96425
Maximum:        1       301     301     35          68          98897
Mean:           1       234.159 234.159 0.00374127  5.63833
# of Seqs: 98897
Output File Names: VEE_ASS613_S11_L001_R1_001.summary
It took 0 secs to summarize 98897 sequences.

mothur > trim.seqs(fasta=VEE_ASS613_S11_L001_R1_001.fasta,
    qfile=VEE_ASS613_S11_L001_R1_001.qual, qwindowaverage=20,
    minlength=180)
Output File Names:
VEE_ASS613_S11_L001_R1_001.trim.fasta
VEE_ASS613_S11_L001_R1_001.scrap.fasta
VEE_ASS613_S11_L001_R1_001.trim.qual
VEE_ASS613_S11_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_ASS613_S11_L001_R1_001.trim.
    fasta)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       180     180     0       3       1
```

```
2.5%-tile:      1 201     201     0    4       1250
25%-tile:       1 278     278     0    5       12498
Median:         1 293     293     0    5       24995
75%-tile:       1 301     301     0    6       37492
97.5%-tile:     1 301     301     0    16      48739
Maximum:        1 301     301     0    68      49988
Mean:           1 283.532 283.532 0    6.21305
# of Seqs: 49988
Output File Names: VEE_ASS613_S11_L001_R1_001.trim.summary
It took 1 secs to summarize 49988 sequences.

mothur > make.group(fasta=VEE_ASS613_S11_L001_R1_001.trim.fasta
    , groups=NurseryA)
Output File Names: VEE_ASS613_S11_L001_R1_001.trim.groups

mothur > fastq.info(fastq=VEE_DYT13_S82_L001_R1_001.fastq)
10000
20000
30000
33603
Output File Names:
VEE_DYT13_S82_L001_R1_001.fasta
VEE_DYT13_S82_L001_R1_001.qual

mothur > summary.seqs(fasta=VEE_DYT13_S82_L001_R1_001.fasta,
    processors=24)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       35      35      0       3       1
2.5%-tile:      1       282     282     0       4       841
25%-tile:       1       300     300     0       6       8401
Median:         1       301     301     0       6       16802
75%-tile:       1       301     301     0       6       25203
97.5%-tile:     1       301     301     0       10      32763
```

```
Maximum:        1 301      301      35          35       33603
Mean:           1 299.106  299.106  0.00351159  6.15377
# of Seqs: 33603
Output File Names: VEE_DYT13_S82_L001_R1_001.summary
It took 0 secs to summarize 33603 sequences.

mothur > trim.seqs(fasta=VEE_DYT13_S82_L001_R1_001.fasta, qfile
   =VEE_DYT13_S82_L001_R1_001.qual, qwindowaverage=20,
   minlength=180)
Output File Names:
VEE_DYT13_S82_L001_R1_001.trim.fasta
VEE_DYT13_S82_L001_R1_001.scrap.fasta
VEE_DYT13_S82_L001_R1_001.trim.qual
VEE_DYT13_S82_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_DYT13_S82_L001_R1_001.trim.
   fasta)
                Start  End      NBases  Ambigs  Polymer  NumSeqs
Minimum:        1      180      180     0       3        1
2.5%-tile:      1      202      202     0       4        811
25%-tile:       1      279      279     0       6        8105
Median:         1      296      296     0       6        16210
75%-tile:       1      301      301     0       6        24315
97.5%-tile:     1      301      301     0       10       31609
Maximum:        1      301      301     0       31       32419
Mean:           1      283.244  283.244 0       6.05413
# of Seqs: 32419
Output File Names: VEE_DYT13_S82_L001_R1_001.trim.summary
It took 0 secs to summarize 32419 sequences.

mothur > make.group(fasta=VEE_DYT13_S82_L001_R1_001.trim.fasta,
    groups=NurseryD)
Output File Names: VEE_DYT13_S82_L001_R1_001.trim.groups
```

```
mothur > fastq.info(fastq=VEE_DYT26_S58_L001_R1_001.fastq)
10000
20000
30000
40000
40797
Output File Names:
VEE_DYT26_S58_L001_R1_001.fasta
VEE_DYT26_S58_L001_R1_001.qual

mothur > summary.seqs(fasta=VEE_DYT26_S58_L001_R1_001.fasta,
    processors=24)
                Start   End NBases      Ambigs      Polymer NumSeqs
Minimum:        1       35      35      0           3       1
2.5%-tile:      1       271     271     0           4       1020
25%-tile:       1       300     300     0           5       10200
Median:         1       301     301     0           6       20399
75%-tile:       1       301     301     0           6       30598
97.5%-tile:     1       301     301     0           9       39778
Maximum:        1       301     301     35          35      40797
Mean:           1       298.185 298.185 0.00137265  5.9561
# of Seqs: 40797
Output File Names: VEE_DYT26_S58_L001_R1_001.summary
It took 0 secs to summarize 40797 sequences.

mothur > trim.seqs(fasta=VEE_DYT26_S58_L001_R1_001.fasta, qfile
    =VEE_DYT26_S58_L001_R1_001.qual, qwindowaverage=20,
    minlength=180)
Output File Names:
VEE_DYT26_S58_L001_R1_001.trim.fasta
VEE_DYT26_S58_L001_R1_001.scrap.fasta
VEE_DYT26_S58_L001_R1_001.trim.qual
```

```
VEE_DYT26_S58_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_DYT26_S58_L001_R1_001.trim.
    fasta)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       180     180     0       3       1
2.5%-tile:      1       204     204     0       4       992
25%-tile:       1       279     279     0       5       9915
Median:         1       295     295     0       6       19830
75%-tile:       1       301     301     0       6       29744
97.5%-tile:     1       301     301     0       9       38667
Maximum:        1       301     301     0       32      39658
Mean:           1       283.862 283.862 0       5.8709
# of Seqs: 39658
Output File Names: VEE_DYT26_S58_L001_R1_001.trim.summary
It took 0 secs to summarize 39658 sequences.

mothur > make.group(fasta=VEE_DYT26_S58_L001_R1_001.trim.fasta,
     groups=NurseryD)
Output File Names: VEE_DYT26_S58_L001_R1_001.trim.groups

mothur > fastq.info(fastq=VEE_DYT553_S94_L001_R1_001.fastq)
10000
20000
30000
36165
Output File Names:
VEE_DYT553_S94_L001_R1_001.fasta
VEE_DYT553_S94_L001_R1_001.qual

mothur > summary.seqs(fasta=VEE_DYT553_S94_L001_R1_001.fasta,
    processors=24)
                Start   End     NBases  Ambigs      Polymer NumSeqs
```

```
Minimum:        1  35       35       0           3        1
2.5%-tile:      1  273      273      0           4        905
25%-tile:       1  300      300      0           5        9042
Median:         1  301      301      0           6        18083
75%-tile:       1  301      301      0           6        27124
97.5%-tile:     1  301      301      0           10       35261
Maximum:        1  301      301      35          35       36165
Mean:           1  297.837  297.837  0.0241394   5.93765
# of Seqs: 36165
Output File Names: VEE_DYT553_S94_L001_R1_001.summary
It took 0 secs to summarize 36165 sequences.

mothur > trim.seqs(VEE_DYT553_S94_L001_R1_001.fasta, qfile=
    VEE_DYT553_S94_L001_R1_001.qual, qwindowaverage=20,
    minlength=180)
Output File Names:
VEE_DYT553_S94_L001_R1_001.trim.fasta
VEE_DYT553_S94_L001_R1_001.scrap.fasta
VEE_DYT553_S94_L001_R1_001.trim.qual
VEE_DYT553_S94_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_DYT553_S94_L001_R1_001.trim.
    fasta)
                Start  End    NBases   Ambigs  Polymer  NumSeqs
Minimum:        1  180      180      0       3        1
2.5%-tile:      1  204      204      0       4        866
25%-tile:       1  280      280      0       5        8654
Median:         1  293      293      0       6        17307
75%-tile:       1  301      301      0       6        25960
97.5%-tile:     1  301      301      0       9        33747
Maximum:        1  301      301      0       31       34612
Mean:           1  283.866  283.866  0       5.81816
# of Seqs: 34612
```

```
Output File Names: VEE_DYT553_S94_L001_R1_001.trim.summary
It took 1 secs to summarize 34612 sequences.

mothur > make.group(fasta=VEE_DYT553_S94_L001_R1_001.trim.fasta
    , groups=NurseryD)
Output File Names: VEE_DYT553_S94_L001_R1_001.trim.groups

mothur > fastq.info(fastq=VEE_GEA462_S22_L001_R1_001.fastq)
10000
20000
30000
30402
Output File Names:
VEE_GEA462_S22_L001_R1_001.fasta
VEE_GEA462_S22_L001_R1_001.qual

mothur > summary.seqs(fasta=VEE_GEA462_S22_L001_R1_001.fasta,
    processors=24)
                Start   End     NBases  Ambigs  Polymer     NumSeqs
Minimum:        1       35      35      0       3           1
2.5%-tile:      1       254     254     0       5           761
25%-tile:       1       299     299     0       5           7601
Median:         1       301     301     0       6           15202
75%-tile:       1       301     301     0       6           22802
97.5%-tile:     1       301     301     0       10          29642
Maximum:        1       301     301     35      35          30402
Mean:           1       297.266 297.266 0.0145385 5.80084
# of Seqs: 30402
Output File Names: VEE_GEA462_S22_L001_R1_001.summary
It took 0 secs to summarize 30402 sequences.

mothur > trim.seqs(fasta=VEE_GEA462_S22_L001_R1_001.fasta,
    qfile=VEE_GEA462_S22_L001_R1_001.qual, qwindowaverage=20,
```

```
    minlength=180)
Output File Names:
VEE_GEA462_S22_L001_R1_001.trim.fasta
VEE_GEA462_S22_L001_R1_001.scrap.fasta
VEE_GEA462_S22_L001_R1_001.trim.qual
VEE_GEA462_S22_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_GEA462_S22_L001_R1_001.trim.
    fasta)
                Start   End     NBases  Ambigs  Polymer  NumSeqs
Minimum:        1       180     180     0       3        1
2.5%-tile:      1       205     205     0       5        732
25%-tile:       1       278     278     0       5        7312
Median:         1       294     294     0       5        14623
75%-tile:       1       300     300     0       6        21934
97.5%-tile:     1       301     301     0       9        28513
Maximum:        1       301     301     0       28       29244
Mean:           1       283.743 283.743 0       5.67737
# of Seqs: 29244
Output File Names: VEE_GEA462_S22_L001_R1_001.trim.summary
It took 0 secs to summarize 29244 sequences.

mothur > make.group(fasta=VEE_GEA462_S22_L001_R1_001.trim.fasta
    , groups=NurseryG)
Output File Names: VEE_GEA462_S22_L001_R1_001.trim.groups

mothur > fastq.info(fastq=VEE_GEA533_S46_L001_R1_001.fastq)
10000
20000
28294
Output File Names: VEE_GEA533_S46_L001_R1_001.fasta
    VEE_GEA533_S46_L001_R1_001.qual
```

```
mothur > summary.seqs(fasta=VEE_GEA533_S46_L001_R1_001.fasta,
    processors=24)
                Start   End     NBases  Ambigs          Polymer NumSeqs
Minimum:        1       35      35      0               3       1
2.5%-tile:      1       273     273     0               4       708
25%-tile:       1       300     300     0               5       7074
Median:         1       301     301     0               6       14148
75%-tile:       1       301     301     0               6       21221
97.5%-tile:     1       301     301     0               10      27587
Maximum:        1       301     301     35              35      28294
Mean:           1       298.371 298.371 0.00971938      5.85265
# of Seqs: 28294
Output File Names: VEE_GEA533_S46_L001_R1_001.summary
It took 0 secs to summarize 28294 sequences.

mothur > trim.seqs(fasta=VEE_GEA533_S46_L001_R1_001.fasta,
    qfile=VEE_GEA533_S46_L001_R1_001.qual, qwindowaverage=20,
    minlength=180)
Output File Names:
VEE_GEA533_S46_L001_R1_001.trim.fasta
VEE_GEA533_S46_L001_R1_001.scrap.fasta
VEE_GEA533_S46_L001_R1_001.trim.qual
VEE_GEA533_S46_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_GEA533_S46_L001_R1_001.trim.
    fasta)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       180     180     0       3       1
2.5%-tile:      1       207     207     0       4       689
25%-tile:       1       280     280     0       5       6886
Median:         1       295     295     0       6       13772
75%-tile:       1       301     301     0       6       20658
97.5%-tile:     1       301     301     0       9       26855
```

```
Maximum:        1 301       301       0     29        27543
Mean:           1 285.185   285.185   0     5.76324
# of Seqs: 27543
Output File Names: VEE_GEA533_S46_L001_R1_001.trim.summary
It took 0 secs to summarize 27543 sequences.

mothur > make.group(fasta=VEE_GEA533_S46_L001_R1_001.trim.fasta
    , groups=NurseryG)
Output File Names: VEE_GEA533_S46_L001_R1_001.trim.groups

mothur > fastq.info(fastq=VEE_GEAA523_S23_L001_R1_001.fastq)
10000
20000
30000
40000
50000
60000
61449
Output File Names:
VEE_GEAA523_S23_L001_R1_001.fasta
VEE_GEAA523_S23_L001_R1_001.qual

mothur > summary.seqs(fasta=VEE_GEAA523_S23_L001_R1_001.fasta,
    processors=24)
                Start  End  NBases      Ambigs       Polymer  NumSeqs
Minimum:        1      35   35          0            3        1
2.5%-tile:      1      282  282         0            5        1537
25%-tile:       1      300  300         0            6        15363
Median:         1      301  301         0            6        30725
75%-tile:       1      301  301         0            6        46087
97.5%-tile:     1      301  301         0            10       59913
Maximum:        1      301  301         75           44       61449
Mean:           1      299.301  299.301  0.00758352  6.20825
```

```
# of Seqs: 61449
Output File Names: VEE_GEAA523_S23_L001_R1_001.summary
It took 0 secs to summarize 61449 sequences.

mothur > trim.seqs(fasta=VEE_GEAA523_S23_L001_R1_001.fasta,
    qfile=VEE_GEAA523_S23_L001_R1_001.qual, qwindowaverage=20,
    minlength=180)
Output File Names:
VEE_GEAA523_S23_L001_R1_001.trim.fasta
VEE_GEAA523_S23_L001_R1_001.scrap.fasta
VEE_GEAA523_S23_L001_R1_001.trim.qual
VEE_GEAA523_S23_L001_R1_001.scrap.qual

mothur > summary.seqs(fasta=VEE_GEAA523_S23_L001_R1_001.trim.
    fasta)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       180     180     0       3       1
2.5%-tile:      1       200     200     0       4       1472
25%-tile:       1       276     276     0       6       14714
Median:         1       293     293     0       6       29428
75%-tile:       1       301     301     0       6       44141
97.5%-tile:     1       301     301     0       10      57383
Maximum:        1       301     301     0       44      58854
Mean:           1       281.532 281.532 0       6.09462
# of Seqs: 58854
Output File Names: VEE_GEAA523_S23_L001_R1_001.trim.summary
It took 0 secs to summarize 58854 sequences.

mothur > make.group(fasta=VEE_GEAA523_S23_L001_R1_001.trim.
    fasta, groups=NurseryG)
Output File Names: VEE_GEAA523_S23_L001_R1_001.trim.groups

mothur > merge.files(input=VEE_ASS25_S70_L001_R1_001.trim.fasta
```

```
-VEE_ASS53_S34_L001_R1_001.trim.fasta-
VEE_ASS613_S11_L001_R1_001.trim.fasta-
VEE_DYT13_S82_L001_R1_001.trim.fasta-
VEE_DYT26_S58_L001_R1_001.trim.fasta-
VEE_DYT553_S94_L001_R1_001.trim.fasta-
VEE_GEA462_S22_L001_R1_001.trim.fasta-
VEE_GEA533_S46_L001_R1_001.trim.fasta-
VEE_GEAA523_S23_L001_R1_001.trim.fasta, output=wholetrees4.
fasta)
Output File Names: wholetrees4.fasta

mothur > merge.files(input=VEE_ASS25_S70_L001_R1_001.trim.
groups-VEE_ASS53_S34_L001_R1_001.trim.groups-
VEE_ASS613_S11_L001_R1_001.trim.groups-
VEE_DYT13_S82_L001_R1_001.trim.groups-
VEE_DYT26_S58_L001_R1_001.trim.groups-
VEE_DYT553_S94_L001_R1_001.trim.groups-
VEE_GEA462_S22_L001_R1_001.trim.groups-
VEE_GEA533_S46_L001_R1_001.trim.groups-
VEE_GEAA523_S23_L001_R1_001.trim.groups, output=wholetrees4.
groups)
Output File Names: wholetrees4.groups

mothur > summary.seqs(fasta=wholetrees4.fasta)
              Start  End    NBases  Ambigs  Polymer  NumSeqs
Minimum:      1      180    180     0       3        1
2.5%-tile:    1      204    204     0       4        9086
25%-tile:     1      280    280     0       5        90853
Median:       1      294    294     0       6        181706
75%-tile:     1      301    301     0       6        272559
97.5%-tile:   1      301    301     0       10       354326
Maximum:      1      301    301     0       68       363411
Mean:         1      284.217 284.217 0      5.88815
```

```
# of Seqs: 363411
Output File Names: wholetrees4.summary
It took 2 secs to summarize 363411 sequences.

mothur > count.groups(group=wholetrees4.groups)
NurseryA contains 141081.
NurseryD contains 106689.
NurseryG contains 115641.
Total seqs: 363411.
Output File Names: wholetrees4.count.summary

mothur > screen.seqs(fasta=wholetrees4.fasta, group=wholetrees4
   .groups, summary=wholetrees4.summary , maxambig=0, maxlength
   =350)
Output File Names:
wholetrees4.good.summary
wholetrees4.good.fasta
wholetrees4.bad.accnos
wholetrees4.good.groups
It took 9 secs to screen 363411 sequences.

mothur > summary.seqs(fasta=wholetrees4.good.fasta, processors
   =24)
                Start  End    NBases   Ambigs  Polymer  NumSeqs
Minimum:        1      180    180      0       3        1
2.5%-tile:      1      204    204      0       4        9086
25%-tile:       1      280    280      0       5        90853
Median:         1      294    294      0       6        181706
75%-tile:       1      301    301      0       6        272559
97.5%-tile:     1      301    301      0       10       354326
Maximum:        1      301    301      0       68       363411
Mean:           1      284.217 284.217 0       5.88815
# of Seqs: 363411
```

```
Output File Names: wholetrees4.good.summary
It took 2 secs to summarize 363411 sequences.

mothur > unique.seqs(fasta=wholetrees4.good.fasta)
363411 293771
Output File Names: wholetrees4.good.names wholetrees4.good.
   unique.fasta

mothur > summary.seqs(fasta=wholetrees4.good.unique.fasta,
   processors=24)
                Start  End    NBases  Ambigs  Polymer  NumSeqs
Minimum:        1      180    180     0       3        1
2.5%-tile:      1      201    201     0       4        7345
25%-tile:       1      276    276     0       5        73443
Median:         1      292    292     0       6        146886
75%-tile:       1      300    300     0       6        220329
97.5%-tile:     1      301    301     0       10       286427
Maximum:        1      301    301     0       68       293771
Mean:           1      281.571 281.571 0      5.97573
# of Seqs: 293771
Output File Names: wholetrees4.good.unique.summary
It took 2 secs to summarize 293771 sequences.

mothur > count.seqs(name=wholetrees4.good.names, group=
   wholetrees4.good.groups)
It took 3 secs to create a table for 363411 sequences.
Total number of sequences: 363411
Output File Names: wholetrees4.good.count_table

mothur > chimera.vsearch(processors=24, fasta=wholetrees4.good.
   unique.fasta, count=wholetrees4.good.count_table,
   dereplicate=t)
It took 201 secs to check 0 sequences from group NurseryD.
```

```
It took 239 secs to check 0 sequences from group NurseryG.
106560 here
It took 323 secs to check 0 sequences from group NurseryA.
Output File Names:
wholetrees4.good.denovo.vsearch.pick.count_table
wholetrees4.good.unique.denovo.vsearch.chimeras
wholetrees4.good.unique.denovo.vsearch.accnos

mothur > remove.seqs(fasta=wholetrees4.good.unique.fasta,
    accnos=wholetrees4.good.unique.denovo.vsearch.accnos, count=
    wholetrees4.good.count_table)
Removed 1369 sequences from your fasta file.
Removed 1558 sequences from your count file.
Output File Names:
wholetrees4.good.unique.pick.fasta
wholetrees4.good.pick.count_table

mothur > summary.seqs(fasta=wholetrees4.good.unique.pick.fasta,
     count=wholetrees4.good.pick.count_table, processors=24)
                Start  End     NBases  Ambigs  Polymer  NumSeqs
Minimum:        1      180     180     0       3        1
2.5%-tile:      1      204     204     0       4        9047
25%-tile:       1      280     280     0       5        90464
Median:         1      294     294     0       6        180927
75%-tile:       1      301     301     0       6        271390
97.5%-tile:     1      301     301     0       10       352807
Maximum:        1      301     301     0       68       361853
Mean:           1      284.254 284.254 0       5.88943
# of unique seqs: 292402
total # of seqs: 361853
Output File Names: wholetrees4.good.unique.pick.summary
It took 3 secs to summarize 361853 sequences.
```

```
mothur > classify.seqs(fasta=wholetrees4.good.unique.pick.fasta
    , count=wholetrees4.good.pick.count_table, reference=
    UNITEv6_sh_dynamic_s.fasta, taxonomy=UNITEv6_sh_dynamic_s.
    tax, cutoff=60)
It took 2663 secs to classify 292402 sequences.
It took 14 secs to create the summary file for 292402 sequences
Output File Names:
wholetrees4.good.unique.pick.UNITEv6_sh_dynamic_s.wang.taxonomy
wholetrees4.good.unique.pick.UNITEv6_sh_dynamic_s.wang.tax.
    summary
wholetrees4.good.unique.pick.UNITEv6_sh_dynamic_s.wang.flip.
    accnos

#get.lineage was used for the production of the heatmap only.
mothur > get.lineage(fasta=wholetrees4.good.unique.pick.fasta,
    count=wholetrees4.good.pick.count_table, taxonomy=
    wholetrees4.good.unique.pick.UNITEv6_sh_dynamic_s.wang.
    taxonomy, taxon=Amanitaceae-Inocybaceae-Thelephoraceae-
    Thelephorales-Pezizales-Tuberaceae-Pyronemataceae-Gloniaceae
    )
Output File Names:
wholetrees4.good.unique.pick.UNITEv6_sh_dynamic_s.wang.pick.
    taxonomy
wholetrees4.good.unique.pick.pick.fasta
wholetrees4.good.pick.pick.count_table

mothur > remove.lineage(fasta=wholetrees3.good.unique.pick.
    fasta, count=wholetrees3.good.pick.count_table, taxonomy=
    wholetrees3.good.unique.pick.UNITEv6_sh_dynamic_s.wang.
    taxonomy, taxon=unknown-Protista)
Output File Names:
wholetrees4.good.unique.pick.UNITEv6_sh_dynamic_s.wang.pick.
    taxonomy
```

```
wholetrees4.good.unique.pick.pick.fasta
wholetrees4.good.pick.pick.count_table

mothur > summary.tax(taxonomy=wholetrees4.good.unique.pick.
    UNITEv6_sh_dynamic_s.wang.pick.taxonomy, count=wholetrees4.
    good.pick.pick.count_table)
It took 2 secs to create the summary file for 94484 sequences.
Output File Names: wholetrees4.good.unique.pick.
    UNITEv6_sh_dynamic_s.wang.pick.tax.summary

mothur > count.groups(count=wholetrees4.good.pick.pick.
    count_table)
NurseryA contains 77049.
NurseryD contains 18113.
NurseryG contains 23898.
Total seqs: 119060.
Output File Names: wholetrees4.good.pick.pick.count.summary

mothur > sub.sample(fasta=wholetrees4.good.unique.pick.pick.
    fasta, count=wholetrees4.good.pick.pick.count_table, size
    =18105, persample=t)
Sampling 18105 from each group.
Output File Names:
wholetrees4.good.pick.pick.subsample.count_table
wholetrees4.good.unique.pick.pick.subsample.fasta

mothur > count.groups(count=wholetrees4.good.pick.pick.
    subsample.count_table)
NurseryA contains 18105.
NurseryD contains 18105.
NurseryG contains 18105.
Total seqs: 54315.
Output File Names: wholetrees4.good.pick.pick.subsample.count.
```

summary

```
mothur > summary.seqs(fasta=wholetrees4.good.unique.pick.pick.
    subsample.fasta, processors=24)
                Start   End     NBases  Ambigs  Polymer NumSeqs
Minimum:        1       191     191     0       4       1
2.5%-tile:      1       264     264     0       5       1119
25%-tile:       1       286     286     0       5       11184
Median:         1       295     295     0       5       22367
75%-tile:       1       301     301     0       5       33550
97.5%-tile:     1       301     301     0       10      43615
Maximum:        1       301     301     0       22      44733
Mean:           1       291.77  291.77  0       5.64588
# of Seqs: 44733
Output File Names: wholetrees4.good.unique.pick.pick.subsample.
    summary
It took 2 secs to summarize 44733 sequences.

mothur > classify.seqs(fasta=wholetrees4.good.unique.pick.pick.
    subsample.fasta, count=wholetrees4.good.pick.pick.subsample.
    count_table, template=UNITEv6_sh_dynamic_s.fasta, taxonomy=
    UNITEv6_sh_dynamic_s.tax, cutoff=80)
It took 391 secs to classify 44733 sequences.
It took 3 secs to create the summary file for 44733 sequences.
Output File Names:
wholetrees4.good.unique.pick.pick.subsample.
    UNITEv6_sh_dynamic_s.wang.taxonomy
wholetrees4.good.unique.pick.pick.subsample.
    UNITEv6_sh_dynamic_s.wang.tax.summary
wholetrees4.good.unique.pick.pick.subsample.
    UNITEv6_sh_dynamic_s.wang.flip.accnos

mothur > cluster(fasta=wholetrees4.good.unique.pick.pick.
```

```
subsample.fasta, count=wholetrees4.good.pick.pick.subsample.
    count_table, method=agc, cutoff=0.05)
Output File Names: wholetrees4.good.unique.pick.pick.subsample.
    agc.unique_list.list

mothur > summary.seqs(fasta=wholetrees4.good.unique.pick.pick.
    subsample.fasta, processors=24)
                Start   End     NBases  Ambigs  Polymer NumSeqs7
Minimum:        1       191     191     0       4       1
2.5%-tile:      1       264     264     0       5       1119
25%-tile:       1       286     286     0       5       11184
Median:         1       295     295     0       5       22367
75%-tile:       1       301     301     0       5       33550
97.5%-tile:     1       301     301     0       10      43615
Maximum:        1       301     301     0       22      44733
Mean:           1       291.77  291.77  0       5.64588
# of Seqs: 44733
Output File Names: wholetrees4.good.unique.pick.pick.subsample.
    summary
It took 3 secs to summarize 44733 sequences.

mothur > make.shared(list=wholetrees4.good.unique.pick.pick.
    subsample.agc.unique_list.list, count=wholetrees4.good.pick.
    pick.subsample.count_table)
0.05
Output File Names:
wholetrees4.good.unique.pick.pick.subsample.agc.unique_list.
    shared

mothur > classify.otu(list=wholetrees4.good.unique.pick.pick.
    subsample.agc.unique_list.list, count=wholetrees4.good.pick.
    pick.subsample.count_table, taxonomy=wholetrees4.good.unique
    .pick.pick.subsample.UNITEv6_sh_dynamic_s.wang.taxonomy)
```

```
0.05 1143
Output File Names:
wholetrees4.good.unique.pick.pick.subsample.agc.unique_list
    .0.05.cons.tax.summary

mothur > get.oturep(fasta=wholetrees4.good.unique.pick.pick.
    subsample.fasta, count=wholetrees4.good.pick.pick.subsample.
    count_table, list=wholetrees4.good.unique.pick.pick.
    subsample.agc.unique_list.list, method=abundance)
0.05 1143
Output File Names:
wholetrees4.good.unique.pick.pick.subsample.agc.unique_list
    .0.05.rep.count_table
wholetrees4.good.unique.pick.pick.subsample.agc.unique_list
    .0.05.rep.fasta

mothur > count.groups(shared=wholetrees4.good.unique.pick.pick.
    subsample.agc.unique_list.shared)
NurseryA contains 18105.
NurseryD contains 18105.
NurseryG contains 18105.
Total seqs: 54315.
Output File Names:
wholetrees4.good.unique.pick.pick.subsample.agc.unique_list.
    count.aummary

mothur > count.seqs(shared=wholetrees4.good.unique.pick.pick.
    subsample.agc.unique_list.shared)
0.05
Output File Names:
wholetrees4.good.unique.pick.pick.subsample.agc.unique_list
    .0.05.count_table
```

# Appendix E

Cloning isolate (1.2.2 - C11) digested with *Eco*R1 aligned with *Thelephora terrestris* isolate FFP330 accession number: JQ711980.1

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1098 bits(571) | 0.0 | 571/571(100%) | 0/571(0%) | Plus/Minus |

```
Query  30   CTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGATCGAACGT  89
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  704  CTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGATCGAACGT  645

Query  90   TAAAAAAGCTGTCCTCGCTGAGGAGAGACATCTGTGAGCTCCAGCAAACCTTTGTGACCA  149
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  644  TAAAAAAGCTGTCCTCGCTGAGGAGAGACATCTGTGAGCTCCAGCAAACCTTTGTGACCA  585

Query  150  AAGGTTACCTGGCAGACAACAGCGAGCGTAGATATTTATCACACCCGTGATGCCACCAAA  209
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  584  AAGGTTACCTGGCAGACAACAGCGAGCGTAGATATTTATCACACCCGTGATGCCACCAAA  525

Query  210  CACTGGGAGGCTGATTAATTTGAGAGGAGCCGACCACAGGCCAGCAAAACCCCCAGAGTC  269
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  524  CACTGGGAGGCTGATTAATTTGAGAGGAGCCGACCACAGGCCAGCAAAACCCCCAGAGTC  465

Query  270  CAACTCATCATGGCAAACCATGAGAGTTGAGGTGTTCATGATACTCAAACAGGCATGCCC  329
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  464  CAACTCATCATGGCAAACCATGAGAGTTGAGGTGTTCATGATACTCAAACAGGCATGCCC  405

Query  330  CTCGGAATAGCCAAGGGGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGC  389
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404  CTCGGAATAGCCAAGGGGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGC  345

Query  390  AATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCC  449
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  344  AATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCC  285

Query  450  GTTGCTGAAAGTTGTATTGTATTGCGTTAGACGCGATGTACATTCCATAAAACTTTATTA  509
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  284  GTTGCTGAAAGTTGTATTGTATTGCGTTAGACGCGATGTACATTCCATAAAACTTTATTA  225

Query  510  CAGTGTGTGTGTAAAGACGTAGAACCACAGAAGGAAGACAGGGTCCCCCAGACCATAGAA  569
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224  CAGTGTGTGTGTAAAGACGTAGAACCACAGAAGGAAGACAGGGTCCCCCAGACCATAGAA  165

Query  570  CTACAGAGGGTGCACAGGTGTGAGTGGATGT  600
            ||||||||||||||||||||||||||||||
Sbjct  164  CTACAGAGGGTGCACAGGTGTGAGTGGATGT  134
```