ISOLATION, PROPAGATION AND RAPID MOLECULAR DETECTION OF THE KALAHARI TRUFFLE, A MYCORRHIZAL FUNGUS OCCURRING IN SOUTH AFRICA.

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ABSTRACT

Terfezia pfeilii is an edible mycorrhizal fungus that thrives in the Kalahari Desert of southern Africa. It is best known by desert dwellers for its flavour and as a source of nutrition. Although the genus *Terfezia* is generally regarded as being an ectomycorrhizal mycobiont, the exact mycorrhizal type formed by *T. pfeilli* and its' associated host plants remains uncertain. Discovery of the host plants for *T. pfeilii* would first be required in order to further investigate the life cycle and cultivation of this truffle. This study focussed on the isolation of mycelia from the ascocarp, optimising the growth conditions of the mycelial cultures, rapid molecular identification of *T. pfeilii*, investigation of potential helper bacteria and mycorrhizal synthesis experiments.

T. pfeilii ascocarps were harvested from the Spitskop Nature Reserve in Upington, South Africa. Ascocarps were successfully identified using both morphological and molecular methods. Despite the delayed growth mostly caused by contaminating microorganisms, the isolation of *T. pfeilii* mycelia culture was successful. Molecular techniques were used to confirm the identity of the pure culture. Further studies were conducted on ways to improve the growth conditions of the mycelial culture on Fontana medium. An optimum temperature of 32°C, the addition of Bovine Serum Albumin as a nitrogen source and a pH of 7.5 significantly improved the growth of *T. pfeilii in vitro*.

A rapid PCR-based molecular method was developed to speed up the identification of *T. pfeilii*. Specific primers that can exclusively amplify the ITS region of *T. pfeilii* were designed and used to identify both the ascocarps and the mycelial culture. The specificity of these primers was confirmed by their inability to amplify DNA from the isolates of contamining fungi obtained during the isolation process. Molecular comparison was made to confirm the reclassification of South African samples of *T. pfeilii* as *Kalaharituber pfeilii* as proposed by Ferdman *et al.*, (2005). However, in this study, the name *T. pfeilii* has been retained.

A total of 17 bacterial isolates were obtained from the fruiting bodies of *T. pfeilii* and these were tested for stimulation of mycelial growth *in vitro*, indole production and phosphate solubilising capabilities. Bacterial isolates that showed potential to be Mycorrhization Helper Bacteria (MHB) were identified as *Paenibacillus* sp., *Bacillus* sp. and *Rhizobium tropici*.

Selected plant seedlings were inoculated with *T. pfeilii* cultures or ascocarp slurry in order to re-establish the mycorrhizal association. After 8 months, light microscopy observations revealed an endomycorrhizal type association between *Cynodon dactylon* and *T. pfeilii*. This was confirmed with molecular analysis using specific *T. pfeilii* ITS primers. After 15 months, molecular methods confirmed *Acacia erioloba* as another host plant.

These results have provided essential information paving the way for further investigation into the life cycle and biology of the Kalahari truffle.

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List of Abbreviations

°E	Degree East
°S	Degree South
<	Less than
μ	Micron (10^{-6})
A	Adenine
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
Bp	Base pairs
BSA	Bovine Serum albumin
C	Cytosine
cm	Centimeters
CTAB	Cetytrimethylammonium bromide/sodium chloride
DNA	Deoxyribonucleic acid
ECM	Ectomycorrhizal fungi
EMBL	European molecular biology laboratory
FTN	Fontana medium
g	gram
G	Guanine
IAA	Indole acetic acid
i.e	that is
IGS	Intergenic spacers
ITS	Internal transcribed spacer
Kg	Kilogram
L/l	liter
LSD	Least significant difference
M	Molar
m	meter
mg	milligram
MHB	Mycorrhization helper bacteria
min	minute
mm	millimeter
MMN	Modified Melin-Norkrans
MP	Maximum parsimony
NA	Nutrient agar
NB	Nutrient broth
NCBI	National center for biotechnology information

NJ	Neighbour joining
P	Phosphorus
PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PFTN	Poor Fontana medium
PGPR	Plant growth promoting bacteria
pH	Potential hydrogen
Pi	Inorganic phosphorus
Po	Organic phosphorus
ppm	Part per million
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	ribonuclease
rpm	rotation per min
rRNA	Ribosomal ribonucleic acid
SAWS	South African weather service
SDS	sodium dodecyl sulphate
SEM	Scanning electron microscope
sp.	Species (singular)
spp.	Species (plural)
TBE	tris boric acid EDTA buffer
TE	Tris EDTA
TEM	Transmission electron microscope
Tm	Melting temperature
WA	Water agar

Dedication

Dedicated to the memory of my mother, who taught me how to read and write at home as well as my first year in school.

Acknowledgements

I thank the almighty God for his mercies and protection during the realisation of this thesis work.

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CHAPTER ONE

1 Literature Review

1.1 Introduction - Mycorrhizal association as a symbiotic association

Mycorrhiza, the Greek word for "root fungus", was first used by a German scientist, Frank in 1885 to describe the symbiotic association between a fungus and lateral roots of plants. In terms of structure, he described mycorrhizas as a combination of organs-mycelium of the fungus and the radicles of the host tree. Previously, the term "symbiosis" was used in 1879 by a botanist, Anton De Bary, to define a beneficial association between green and non-green-plants (lichens) and his description of the symbiotic process encompassed all other forms of interactions between two or more organisms living closely together regardless of the effects of one over the other. Interactions which were saprotrophic, parasitic and mutualistic were all thought to be symbiotic (Isaac, 1992). The meaning of "symbiosis" has evolved over the years and associations such as commensalism, parasitism, predation, herbivory, fungivory and amensalism have all been differentiated from symbiosis as interactions where one or both partners are not benefiting (Johnson *et al.*, 1997). Therefore, symbiosis can now be used interchangeably with mutualism or referred to as mutualistic symbiosis involving "give and take" relationships (Isaac, 1992; Smith and Read, 1997; Johnson *et al.*, 1997). In line with this, mycorrhizal associations were described and accepted as symbiotic (Smith and Read, 1997).

The mycorrhizal association is a mutual symbiosis that stimulates plant growth. The fungus member of this association absorbs and translocates nutrients from the soil into the plants (Cooke, 1977, Kendrick, 2001). Increase in the nutrients supply to the plant subsequently improves the physical and biochemical activities in the plant including the rate of photosynthesis which in turn enhances the production of carbon (Smith and Read, 1997). In return for this function, plants transfer carbon obtained during photosynthesis to the fungus (Kendrick, 2001). The rate of photosynthesis in mycorrhizal plants has been established to be higher than the rate in non-mycorrhizal plants (Eissenstat *et al.*, 1993). Studies have shown that mycorrhizal fungi, and not the roots, are mainly responsible for nutrient uptake (Johnson *et al.*, 1997; Smith and Read, 1997; Brundrett *et al.*, 1996). In rare cases, this situation can turn parasitic; a situation which is an exception rather than the rule (Johnson *et al.*, 1997). Dosskey *et al.*, (1990) proposed that if nutritional and water uptake effects remain constant, then the rate of photosynthesis will be directly proportional to the rate at which carbon is being transported into the mycorrhizal fungus. This model ensures sustainable energy, not only for the individual members (plant or fungus) of the symbiosis, but also for their association (Smith and Read, 1997).

Meanwhile, the success of any mycorrhizal association depends mostly on events that precede the coming together of both partners. Efficient communication between the symbiotic partners is vital at this stage. Koske and Gemma (1992) stated that substances such as water soluble and volatile exudates of plant roots provide enough nutrients for the mycorrhizal fungal propagules to germinate. After germination, a vegetative structure called an "appresorium" is produced. This structure avoids the suberised region and penetrates the host root.

Three main components are involved in all mycorrhizal associations namely; the host plant (phytobiont), the mycorrhizal fungus (mycobiont) and the soil which acts as the medium through which materials are exchanged (Fig. 1.1A) (Brundrett *et al.*, 1996). Under natural conditions, the interaction of these three components is influenced by various biotic and abiotic factors (Johnson *et al.*, 1997).

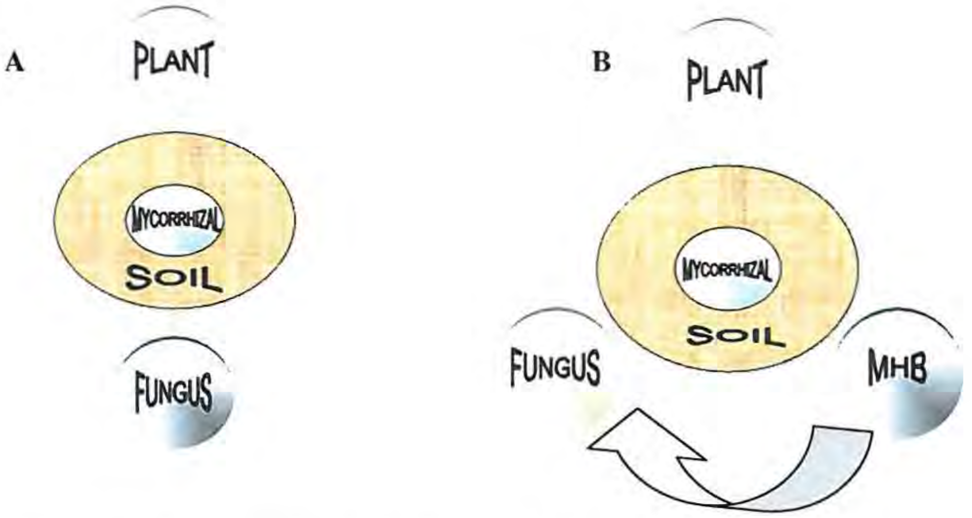

Figure 1.1: (A) The three components involved in mycorrhizal associations. (B) Effects of Mycorrhizal Helper Bacteria (MHB) on mycorrhizal formation. MHB have been reported to be involved in the formation of mycorrhizas.

1.2 Factors affecting mycorrhization

Many factors do affect the process of mycorrhizal colonisation of plants. These factors are difficult to treat individually because of their interdependency (Koske and Gemma, 1992). These factors can be abiotic or biotic. For instance, the rate of phosphorus absorption by the mycorrhizal fungus was found to relate to the ability of the extraradical hyphae to proliferate and cover a large surface area of the soil (Bolan, 1991), however, this same rate has been found to be dependent on some other factors such as light intensity (Koide, 1985). Most of the influences from the biotic factors are attributed to the complex nature of rhizosphere which supports many organisms (Garbaye, 1994). One of the most common biotic factors is the role of bacteria which have been identified to help in the process of mycorrhization (Fig. 1.1B).

1.2.1 Abiotic factors

Abiotic factors are non-living factors that affect the formation of mycorrhizal associations and these include light, tillage and soil nutrient levels.

Light availability is essential to photosynthesis and is directly proportional to the carbon level of the plant. Reduction in light intensity being received by mycorrhizal plants invariably affects production of carbon. This results in both the phytobionts and the mycobionts competing for carbon, thereby causing a growth depression (Buwalda and Goh, 1982; Johnson *et al.*, 1997).

Tillage is another abiotic factor that affects the establishment of mycorrhizal formation. It is a common agricultural practice that results in soil disturbance with the aim of enhancing decomposition of crop residues (Kabir, 2005). Soil distubances are also a result of urban development, road construction, grazing, erosion and mining operations. Studies conducted to determine the effects of soil disturbance on mycorrhizal associations have shown that the rate of phosphorus absorption was significantly reduced (Evans and Miller, 1990; Mcgonigle *et al.*, 1990). The main adverse effect of tillage is the disruption of the hyphal network, disconnecting the mycorrhizal fungus from the host plant (Kabir, 2005).

In addition, it has been established that soil phosphorus levels have a direct effect on the establishment of mycorrhizal fungi (Harley *et al.*, 1953; Jones *et al.*, 1991). Swift, (2004), indicated that the benefits of mycorrhizas are optimal when the phosphorus concentration is at or below 50mg/kg. Extremes in concentration, either too high or too low, will disturb the symbiosis resulting in decreased infective density and/or eliminating need for mycorrhizal association (Koide, 1991). Furthermore, the application of fertilizers and fungicides has been listed to affect the uptake of phosphorus. In a study by Alexander and Hadley, (1984), it was shown that the application of thiabendazole, a fungicide, to some mycorrhizal *Goodyera repens* inoculated with *Rhizoctonia goodyera-repentis*, significantly decreased the growth and phosphorus uptake by the mycorrhizal fungus. It has also been reported that the efficiency of mycorrhizal fungi is greatly reduced by the addition of a high concentration of labile inorganic phosphate fertiliser because this changes the composition of root exudates, therefore reducing the root colonisation by the fungus (Bolan, 1991; Smith and Read, 1997). Soil pH has also been reported to be directly proportional to the uptake of some nutrients in the soil, especially the nitrogen compounds (Smith and Read, 1997).

1.2.2 Biotic factors

In the past, mycorrhizal plants have been grouped according to their level of association with mycorrhizal fungi, dividing them into mycorrhizal dependent and non-mycorrhizal dependent plants. Mycorrhizal dependent plants were found to have special root characteristics that enhance their association with and reliance on mycorrhizal fungi (St. John, 1980). Manjunath and Habte, (1991) reported that density, mass, surface area, and length of the root are all directly proportional to the level of mycorrhizal dependency of the plants. Higher percentage of root hair incidence, higher shoot to root ratio and smaller diameter were also listed to indicate mycorrhizal dependency of plants. Roots characteristics such as the extensiveness, activeness and permeability of root systems have been reported to encourage nutrient uptake through mycorrhizas (Brundrett, 2002).

Explaining the entire roles of biotic communities that interact in an ecosystem is a complex task that cannot be totally elucidated. There are other soil microorganisms such as bacteria and other fungi that have direct and indirect impact on mycorrhizal fungi and their functions. For example, the feeding of herbivores on green plants affects these plants by reducing their photosynthetic capability. Subsequently, carbon production by the plants would be reduced and this may alter the function and potential of mycorrhizal fungi that depend on this carbon (Johnson *et al.*, 1997). High densities of insect herbivores therefore may inhibit the formation of mycorrhizal association. In a study by Gehring *et al.*, 1997, scale insects were reported to have significantly reduced ectomycorrhizal colonisation of some scale susceptible plants. Additionally, fungivores such as microarthropods that graze on the mycelial mass of the mycorrhizal fungus greatly reduce the extraradical growth, resulting in limited soil nutrient uptake by the mycorrhizal fungus (Johnson *et al.*, 1997; Klironomos and Ursic, 1998). Moore *et al.*, (1985) showed how four different microarthropods-*Folsomia candida, Onychiurus encarpatus, Onychiurus folsomi* and *Proisotoma minuta* grazed on mycelia of arbuscular mycorrhizal fungi and subsequently reduced the mycelial growth.

Most biotic factors that influence mycorrhizal formation are found in the zone of soil where the root exerts a great influence through its exudates. This competitive region is termed the rhizosphere and has been defined as the immediate zone of influence of the root that supports and provides energy for the growth of many microorganisms (Anderson, 1992). Around the rhizosphere is the mycorrhizosphere, the region that surrounds the mycorrhizal roots and extramatrical hyphae (Linderman, 1998). In the mycorrhizosphere, there are more direct influences of biotic factors on mycorrhization. The mycorrhizosphere is a nutrient rich zone (Johansson, *et al.*, 2004) as a result of high concentration of root exudates, remnants of root cells/tissue fragments, secretions which have sloughed off into the soil and cell lysates (Azcon-Agular and Barea, 1992). This area attracts and supports many microorganisms. Among them are the pathogenic, saprotrophic and symbiotic microorganisms (Fitter and Garbaye, 1994).

Mycorrhizal helper bacteria

Apart from the mycorrhizal fungi, many other beneficial microorganisms such as bacteria inhabit the mycorrhizosphere. Such bacteria include phosphate solubilizing microorganisms, nitrogen-fixers, plant-growth promoting rhizobacteria (PGPR), siderophore-producing microorganisms and Mycorrhization Helper Bacteria (MHB) (Azcon-Agular and Barea, 1992). Generally, mycorrhizal associated bacteria can have positive, negative or neutral effects on mycorrhizal development. Those bacteria that stimulate the mycorrhizal development have been named MHB (Fig. 1.1B) (Garbaye, 1994). These bacteria are found more frequently in the mycorrhizal environment where they have been shown to play an essential role in mycorrhizal formation (Johansson, *et al.*, 2004). Therefore, it was suggested that the mycorrhizal association is tripartite rather than bipartite (Fig. 1.1A and Fig. 1.1B).

The population and survival of the mycorrhizospheric bacteria are affected by the amount and quality of root exudates, competition for nutrients and the effects of secondary metabolites produced by either their symbiotic partners or competitors (Cairney and Mehag, 2002). Bacteria such as *Bacillus* species, *Paenibacillus* sp., *Serratia* sp., *Pseudomonas* sp., *Burkhoderia* sp. and *Streptomyces* sp. have been isolated and confirmed to be MHB (Aspray *et al.*, 2006a, Aspray *et al.*, 2006b; Bending *et al.*, 2002).

The mode of action of MHB is complex and not well understood (Smith and Read, 1997). Garbaye, (1994), proposed various hypotheses to support the mode of action of these bacteria in aiding mycorrhizal formation. These mostly focussed on direct and indirect effects of MHB on the plant root, the soil and mycorrhizal fungi. His suggestions included the effect of the bacteria in stimulating fungal growth; the production of substances by the bacteria that facilitate fungal penetration and/or recognition of the root by the fungus; production of metabolites that increases the short root formation in plants and the capability of these bacteria to influence the physico-chemical properties of the soil to favour the establishment of mycorrhizal association.

All the aforementioned factors are not only important for mycorrhizal formation but also affect the role of mycorrhizal fungi in the ecosystem. It is therefore necessary to ensure that factors enhancing mycorrhizal establishment are maintained in order for mycorrhizal fungi to perform its most important role in the ecosystem; nutrient absorption (Smith and Read, 1997).

1.3 Nutrient uptake by mycorrhizal fungi

Due to their lack of chlorophyll, fungi are heterotrophs deriving their organic compounds from other processes rather than photosynthesis (Isaac, 1992; Carlile *et al.*, 2001). Previous studies using radioactive isotopes have confirmed the bidirectional flow of compounds from plants to the mycorrhizal fungus and *vice versa* (Fig. 1.2). In such process, photosynthetates, most especially carbon compounds are transferred from plant to the fungus, while soil macro-nutrients such as phosphorus, nitrogen and micro-nutrients such as copper and zinc are transferred from the fungus to the plant. This process enhances the survival of mycorrhizal plants (Levin *et al.*, 1987; Isaac, 1992; Smith and Read, 1997; Aikio and Ruotsalainen, 2002). Absorption of nutrients by the mycorrhizal fungi involves the uptake of nutrients by the fungal hyphae, translocation and transfer of the nutrients to the host plants (Fig. 1.2).

The capablity of mycorrhizal fungi to assimilate nutrients efficiently is credited to their possession of an extensive external mycelial network that penetrates into the rhizosphere and competes effectively with other rhizospeheric microorganisms for these nutrients. These hyphae release enzymes such as proteases and phosphatases that enable the mobilisation of nutrients such as organic nitrogen and phosphorus in the soil (Smith and Read, 1997). Therefore, the enormous hyphal network that allows for contact with larger soil volumes and the growth of mycelia into nutrients pockets add to the attributes that make mycorrhizas a successful association (Bolan, 1991; Smith and Read, 1997). Mycorrhizal fungi have been found to increase the rate of nutrients absorption in plants up to a thousand fold (Smith and Read, 1997; Amaranthus, 1999).

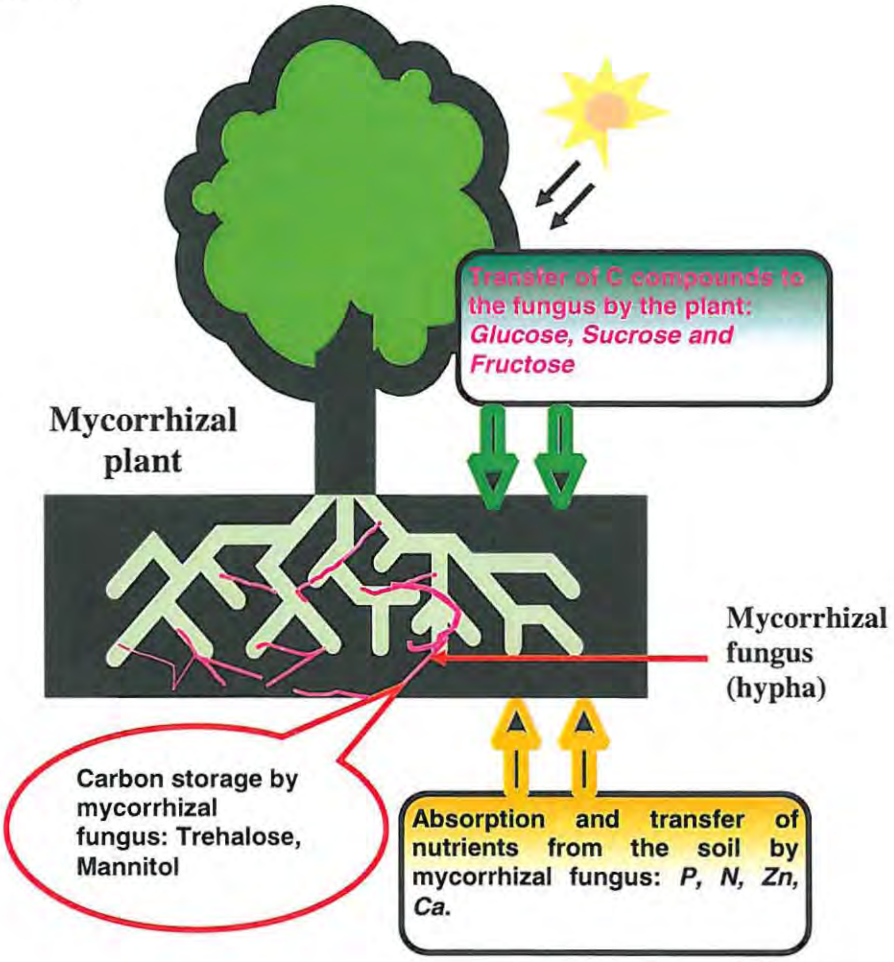

Figure 1.2: Nutrients movement in a mycorrhizal association. Carbon is transferred by the plant to the mycorrhizal fungus after photosynthesis in return for the nutrients absorbed (such as P, N) to the plants by the mycorrhizal fungus. Carbon compounds are stored in mycorrhizal fungus in the form of trehalose, mannitol and arabitol.

Studies have confirmed that mycorrhizal fungi absorb phosphorus more than any other nutrient (Bolan, 1991). Consequently, phosphorus (P) nutrition has been extensively studied and used in various experiments to evaluate the efficiency of mycorrhizal fungi (Smith and Read, 1997). Generally, P in the soil has a very low diffusion coefficient, therefore access to phosphate ions by plants is limited (Koide, 1991). Phosphorus, being a macro-nutrient required by plants, deficiencies will lead to growth limitation. Mycorrhizal fungi therefore ensure that plant P requirement is satisfied.

Less than 1% of the total phosphorus in the soil is available to plants (Bolan, 1991). Root hairs are helpful in accessing the P in the soil but their capability is restricted by their relatively short radius and absorbing capacity (Clarkson, 1985). This condition has necessitated a more appropriate system that will ensure a constant supply of P to the plant. Most plants therefore rely on mycorrhizal symbiosis to meet their phosphorus demand (Fig. 1.2) (Koide, 1991, Smith and Read, 1997). Various studies have attributed the increased P uptake of plants to the presence of mycorrhizal fungi (Koide, 1991; Bolan, 1991, Smith and Read, 1997).

Phosphorus in soil occurs in both inorganic (Pi) and organic (Po) forms. Pi mostly occurr as insoluble forms chemically bonded to Ca, Fe, Na and Al or to the surface of clay minerals. These forms of P are not readily available to the plant. On the other hand, Pi can be present in labile forms which are readily available to the plants (Smith and Read, 1997). Po are mostly derived from organic matter from soil organisms. Common types of Po in soil are inositolphosphates, phospholipids and nucleic acids which are not likely to be directly available to the plants (Smith and Read, 1997). Mycorrhizal fungi are able to utilise these phosphorus forms by solubilisation of insoluble Pi and/or hydrolysis of Po (Koide, 1991; Pant and Warman, 2000). This was highlighted in the study conducted by Jayakumar and Tan, (2005), where *Pisololithus tinctorius* was able to solubilise Pi both in pure culture and in association with *Acacia mangium*. It has also been reported that enzymes such as phosphatases can help in the hydrolysis of Po to Pi which can be readily absorbed by plants (Pants and Warman, 2000). In a study by Koide and Kabir, (2000), extraradical hyphae of *Glomus intraradices* were able to hydrolyse two different Po sources (5-bromo-4-chloro-3-indolyl phosphate and phenolphthalein diphosphate) to Pi, which can be transported and utilised by plants. Other studies have also reported the importance of ectomycorrhizal (ECM) associated enzymes such as acid phosphatases in the conversion of Po to Pi for the utilisation of plants (Antibus *et al.*, 1992; Bending and Read, 1995; Chalot *et al.*, 2002).

The decline in the population of some ECM fungal sporocarps recorded in Europe was once attributed to the deposition of the nitrogen (Arnolds, 1991). Nitrogen (N) is probably the second most important nutrient, after P that is absorbed by mycorrhizal fungi. Nitrates (NO_3^-) and ammonium (NH_4^+) ions are the most common forms of inorganic nitrogen. Though nitrates are more mobile than ammonia, plants utilise ammonia more readily than nitrates for their growths (Smith and Read, 1997). Marschner and Dell, (1994), investigated how these two nitrogen sources can be absorbed by different groups of mycorrhizal fungi, their results indicated that NH_4^+ was absorbed by arbuscular mycorrhizal (AM), ectomycorrhizal (ECM) and ectendomycorrhizal fungi while NO_3^- was absorbed by only ECM. They concluded that up to 25% of the entire nitrogen content of plants is derived from mycorrhizal fungi. In addition, mycorrhizal fungi are also capable of absorbing and transferring organic nitrogen such as proteins (Bovine Albumin Serum-BSA) and amino acids (glutamate and glycine) to plants, a condition which increases their capability to compete effectively with soil decomposers (Abuzinadah *et al.*, 1986; Lipson *et al.*, 1999; Hawkins *et al.*, 2000). Extrapolation of such results may only be possible in a natural condition if mycorrhizal colonised plants are supplied with substrates they will encounter on the field in the presence of other soil microorganisms (Smith and Read, 1997). Such was reported by Entry *et al.*, (1991) where mycorrhizal fungus *Hysterangium setchelli* participated in the decomposition of organic matter which led to the decline in nitrogen concentration in the litter of *Pseudotsuga menziesii*. The ability of ECM and ectendomycorrhizal fungi to absorb various forms of organic nitrogen has been attributed to their production of ectoenzymes such as acid proteinases (Marschner and Dell, 1994). This was discussed in a study by Leake and Read, (1990), where *Hymenoscyphus ericae* showed a high proteinase activity in the presence of BSA as compared to lower values recorded for ammonium, peptide (di-, tri, tetra- and hexa-alanine) and alanine.

1.4 Other mycorrhizal benefits

Other ecological functions of mycorrhizal fungi include the significant role of mycorrhizal fungi in water uptake and storage, as well as strengthening of plants against the effects of biotic and abiotic stresses. In a study conducted by Aliasgharzad *et al.*, (2006), it was discovered that the relative water content (RWC) and the leaf water content (LWC) of mycorrhizal soybean plants were greater than that of non-mycorrhizal soybean plants in a water-stressed condition. Consequently, these effects improved the entire fitness of the soybean as higher seeds number and shoot dry weights were obtained in mycorrhizal soybean. Another role of mycorrhizal fungi is to increase plant tolerance to heavy metals in the soil. This was shown in a study conducted by Jones and Hutchinson, (1986) where *Scleroderma flavidium* increased nickel tolerance of *Betula papyrifera*. Protection of mycorrhizal plants from heavy metals was also reported by Colpaert and Assche, (1993) where nine ECM fungi which included *Thelephora terrestris, Laccaria laccata, Scleroderma citrinum,*; *Paxillus involutus*, two strains of *Suillus luteus*, and three isolates of *Suillus* sp. were tested for their capability to protect and shield *Pinus sylvestris* from the toxic effects of cadmium. The examination of the plant seedlings after the experiment revealed a significant low cadmium level in mycorrhizal seedlings as compared to non-mycorrhizal seedlings.

Heavy metals absorbed by mycorrhizal fungi can be sequestered by polyphosphates in the fungus thereby making it impossible for the majority of the heavy metals to reach the plant tissues. This was confirmed in a study by Turnau *et al.*, (1993), where electron energy loss spectroscopy was used to evaluate activities in the mycorrhizal root of *Pteridium aquilinum*. A disadvantage of this process is the possible damage to the mycorrhizal fungi by the accumulation of these heavy metals which will consequently affect absorption of essential nutrients such as phosphorus and zinc (Smith and Read, 1997).

Additonally, mycorrhizal fungi have also been reported in the protection of plants against diseases. Several ECM fungi exhibit biological control activity through the formation of a sheath around the plant root, limiting the access of pathogenic microorganisms. Mycorrhizal fungi were reported to induce the biocontrol activity through improved resistance/growth of plants and changes in root morphology (Whipps, 2001). For example, a study investigated the effect of four ECM fungi (*Laccaria laccata, Hebeloma crustuliniforme, H. sinapizans* and *Paxillus involutus*) on chestnut (*Castanea sativa*) ink disease caused by *Phytophthora cambivora* and *P. cinnamomi* and it was shown how these mycorrhizal fungi prevented the pathogen from infecting the plant roots and at the same time improved the root and shoot development of the plant (Branzanti *et al.*, 1999).

1.5 Specificity of mycorrhizal fungi

Mycorrhizal fungi show varying degrees of specificity to host plants. This may be dictated by several factors such as the accumulation of phenolic compounds in the epidermal and cortical cells of the plants as suggested by Malajczuk *et al.*, (1982). Molina *et al.* (1992) described different reasons that are responsible for compatibility and specificity in mycorrhizal associations between both partners. These include the ability of the plants to form a mycorrhizal association, ability of the symbiotic partners of independent existence in any stage of their life cycles and the strictness of the plant in forming association (sometimes with just a group of mycorrhizal fungi). Other reasons highlighted included the host range of the mycorrhizal fungus, receptivity of the host plant to accommodate different mycobionts and influence of biotic and abiotic factors.

1.6 Characterisation of mycorrhizal fungi

Mycorrhizal associations have been classified into seven categories based on their structural characteristics formed during mycorrhizal development. These distinctions are based on the formation of hyphal septation, formation of mantle and Hartig net and plant/fungal taxa involved (Smith and Read, 1997). The major types of mycorrhiza are ectomycorrhizas, arbuscular mycorrhizas, ectendomycorrhiza, arbutoid, ericoid, monotropoid and orchid mycorrhizas (Smith and Read, 1997, Brundrett *et al.*, 1996). These groups are broadly differentiated by the nutrient exchange compartments they form within the root. Fungi that penetrate the root and show intracellular penetration of root cells are termed endomycorrhiza while those that stay outside of the root cells showing only intercellular penetration are termed ectomycorrhiza (Smith and Read, 1997; Friberg, 2001).

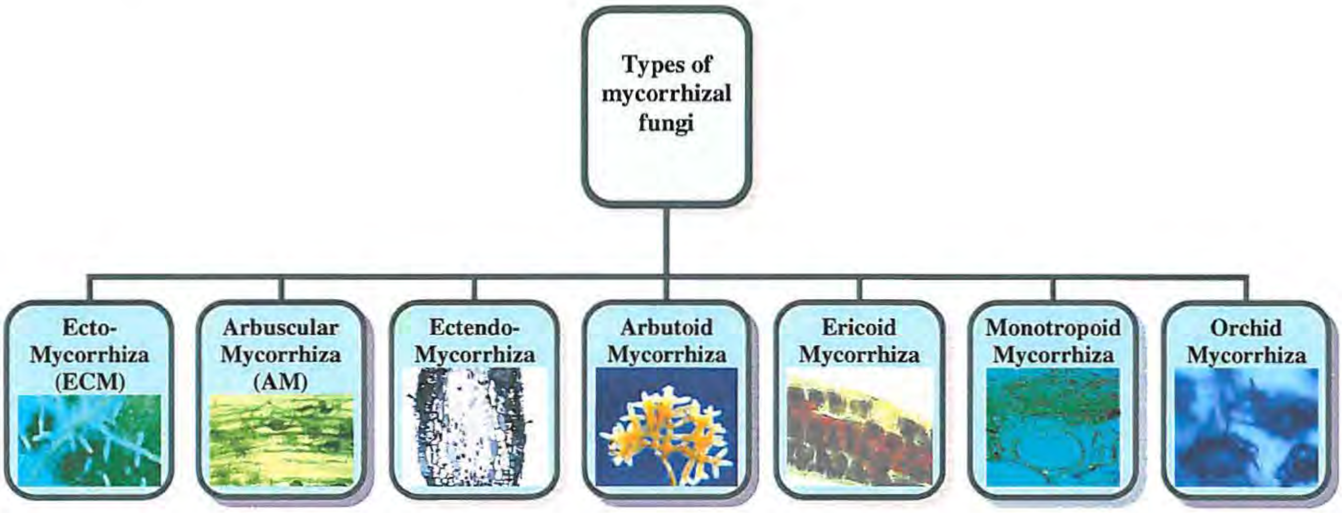

Figure 1.3: Classification of Mycorrhizal fungi (adapted from Smith and Read, 1997; Images from Kendrick, 2001, Yu *et al.*, 2001).

1.6.1 Ectomycorrhizal fungi

The term "Ecto" means outside. This group of mycorrhizal fungi form an association characterised by means of their structural mycelial formation that does not penetrate, but extends between the host root cells to form a Hartig net (Smith and Read, 1997) (Fig. 1.3). Between 7,000 to 10,000 species of ECM fungi have been reported worldwide (Taylor and Alexander, 2005). ECM associations are formed by higher Basidiomycotina for example *Agaricus*, *Amanita*, *Lactarius*, *Thelephora* and *Scleroderma* and a few Ascomycotina such as *Tuber*, *Terfezia* and Zygomycota (*Endogone*) (Isaac, 1992, Molina *et al.*, 1992, Lodge, 2000). Fossil evidence indicates that ECM fungi have been in existence for at least 50 million years, having evolved long after the AM fungi (450 - 500 million years ago) (Smith and Read, 1997). The continued existence of trees from the families of Pinaceae, Fagaceae, Betulaceae and Myrtaceae is partly due to their association with ECM fungi (Muchovej, 2004), which are important, particularly in environments where growth conditions are not ideal (Isaac, 1992).

ECM fungi are distinguishable from other mycorrhizal fungi by the presence of a sheath or fungal mantle (20 to 40μm thick) which is an assemblage of fungal tissue surrounding the lateral roots of the host plant. Hyphae penetrate between the epidermal and cortical root cells forming a Hartig net (Isaac, 1992; Smith & Read, 1997). ECM fungal colonisation imposes structural changes on the plant roots by thickening and enhancing root branching. The association also eliminates the development of root hairs. New roots are continuously colonised by hyphae from the main roots (Isaac, 1992).

ECM colonisation of a plant root is triggered by the release of root exudates such as water soluble and volatile exudates into the rhizosphere; this stimulates germination of the propagules and subsequent growth of the hyphae of the fungus, after which a mycelial mass aggregate is formed around the root outer surface (Bécard and Piché, 1989; Koske and Gemma, 1992). This phenomenon is followed by the hyphae exerting pressure on the cortical cell walls of the root, and sometimes, a localized enzymic degradation (through enzymes such as pectinases and cellulases) of the cell wall (Garcia-Garrido *et al.*, 1992; Bonfante and Perotto, 1995). The hyphae then grow between the cortical cells, pushing apart the cells to establish a network called the Hartig net. This formation rarely penetrates the cortical cells and will not extend beyond the endodermis (Isaac, 1992).

Sucrose, fructose, glucose, trehalose and mannitol have been identified in ECM infected plant tissues compared to non-ECM colonised roots which only contained sucrose, fructose, and glucose. Lewis and Harley, (1965a; 1965b), in their experiment on roots of beech, reported that carbon taken from plants by ECM fungi may not be utilised immediately but stored in a form that is unavailable to the plant. This creates a concentration gradient for the flow of carbon from the plant to the fungus. In a later study by Martin *et al.*, 1985; Martin *et al.*, (1988), a similar result was obtained using Nuclear Magnetic Resonance spectroscopy to monitor the metabolism of glucose by *Spaherosporella brunnea*. These studies most importantly revealed the significance of how carbon compounds are broken down through the mannitol cycle by ECM fungi.

Unlike the AM fungal counterparts, some ECM fungi have been successfully cultivated *in vitro*, but they generally grow slowly (Horton and Bruns, 2001, Finlay and Söderström, 1992). This has been attributed to their ability to utilise simple sugars (glucose, mannose and fructose) independent of their host plants and is a proof of the facultative nature of some ECM fungi. Studies have shown that some ECM fungi can also make use of dissacharides (sucrose and trehalose) and oligosacchirides (starch, glycogen and inulin) as a carbon source (Ohta, 1997). ECM fungi are unlikely to utilise complex carbohydrates such as lignin and cellulose except on rare occasions such as the case of *Cortinarius bruneus* which can degrade lignin and cellulose (Hutchinson, 1990a). It has also been shown that ECM fungi can participate in the degradation of complex carbohydrates as shown by Entry *et al.*, (1991). It was observed that the ECM fungus, *Hysterangium setchellii* was able to provide an improved microenvironment for easy decomposition of ligning and cellulose. The cultivation of ECM fungi on complex carbohydrates is a direct method that can be used to investigate the saprotrophic stage of their life cycle which can be used to cultivate them without the host plants (Hutchinson, 1990a). Ohta, (1997), studied and manipulated the nutrient requirements of two ECM fungi (*Hebeloma radicosum* and *Hebeloma* sp.) *in vitro* to grow a large quantity of mycelia which later lead to eventual production of the sporocarp without the host plant. Considering the intense competition among micororganisms for nutrients in the natural environments, it may be difficult for any ECM fungi to acquire the necessary quantity of nutrients needed for the production of fruiting bodies (Taylor and Alexander, 2005).

Growth of ECM fungi in the laboratory is essential for various research purposes such as production of inoculums, maintenance of the biodiversity of the ECM fungi and studies of their physiological growth parameters, as well as their fungus-host relationship (Brundrett *et al.*, 1996). However, it has been reported that repeated subculture of ECM fungi on agar media, over a long period of time affects their natural ability to colonize host plants (Thomson *et al.*, 1993). This problem can be overcome by inoculation onto, and re-isolation from a compatible host (Thomson *et al.*, 1993). Marx and Daniel, (1976), showed that viability could be retained for a period of 1 to 3 years by storing ECM mycelia in sterile water at 5°C.

Generally, ECM fungi have broad host ranges within restricted plant families and their hosts may be receptive to several ECM fungi (Molina *et al.*, 1992). This means a plant species can play host to a variety of ECM fungi and an ECM fungus can also colonise different suitable host plants (Isaac, 1992, Molina *et al.*, 1992). The advantage of this is the increased survival rate of new seedling species (dispersal of the seedlings) in new environments as they can easily associate with a variety of ECM fungi. Examples of ECM fungi with little host restriction include *Amanita aspera*, *Boletus calopus*, *Tuber borchii*, *Tuber brumale*, *Tuber melanosporum*, *Choiromyces venosus* and *Pisolithus tinctorius* (Molina *et al.*, 1992). This situation boosts the plants access to more nutrients because of the ability of the individual ECM fungus involved in the association to source nutrients in the soil on behalf of the plant (Bruns *et al.*, 2002). In contrast, some ECM fungi are host specific. These include those that can associate with one genus of host plant such as *Amanita diemii* (*Nothofagus* as host), *Suillus cavipes* (*Larix* as host) and *Tricholoma robustum* (*Pinus* as host) Specificity can restrict the geographic scope of the ECM fungus; those that are too specific will be restricted to areas where the host is present.

The investigation of the actual roles of ECM fungi in a natural environment is always problematic due to the associated mycorrhizal and saprophytic fungi. Apart from their roles in nutrient uptake, ECM fungi also fulfil other ecological roles such as contributing to the total soil microbial biomass and the production of dissolved organic carbon (in conjunction with the root of their host plant) contributing up to one-third of the entire soil microbial biomass (Wallander *et al.*, 2001, Hogberg and Hogberg, 2002). Cairney and Meharg, (2002) also reported in their review of the ability of ECM fungi, in conjuction with other mycorrhizospheric microorganisms, to degrade aromatic pollutants (such as petroleum). An example of this is the study conducted by Sarand *et al.*, (1999), where a mycorrhizal fungus, *Suillus bovinus* individually and in conjunction with Scots pine and *Pseudomonas fluorescens*, helped in the biodegradation of *m*-toluate which is an aromatic compound.

1.6.2 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi is named after the finely branched structures they produce intracellularly which are referred to as "arbuscules" (Fig. 1.3). This structure is present in most members of this group (Smith and Read, 1997, Muchovej, 2004). They are sometimes referred to as vesicular arbuscular mycorrhizal (VAM) but only in situations where species produces vesicles (Brundrett, 2002). AM fungi are believed to be much older than land plants because of their various primitive characteristics such as simple spores, absence of sexual reproduction and their relationships with a wide variety of plants (Brundrett, 2002, Morton, 1990) evolving over 500 million years ago (Smith and Read, 1997). They have been found associating with primitive plants such as mosses and ferns as well as a wide range of angiosperms and gymnosperms. AM fungi are obligate symbionts having no special enzymes to degrade simple or complex carbohydrates (Finlay and Söderström, 1992; Brundrett, 2002).

AM fungi have been classified under the phylum Glomeromycota (Walker and Schüßler, 2004). Members of the AM fungi family are genetically similar which makes it difficult to identify individual species (Hosny *et al.*, 1999, Pringle *et al.*, 2000). They have either aseptate or rarely septate hypha with inter- and intracellular colonization of the cortical cells of the host plants and absence of Hartig net or mantle (Smith and Read, 1997).

1.6.3 Ectendomycorrhizal fungi

In ectendomycorrhizal associations, there is a special scenario in which the fungal hyphae form a Hartig net and a primitive sheath around the roots. The hyphae penetrate the cortical root cells and there is a formation of reduced mantle thereby possessing both the characteristics of ectomycrrhizal and endomycorrhizal fungi (Molina *et al.*, 1992, Smith and Read, 1997) (Fig. 1.3). Some truffle species such as *Terfezia* spp. have been reported to form ectendomycorrhizal association (Bratek *et al.*, 1996, Kagan-zur *et al.*, 1998).

1.6.4 Other forms of mycorrhizal fungi

Other endomycorrhizal groups include the arbutoid mycorrhizas such as a *Leccinum* sp. which form a mycorrhizal association with two genera of Ericaceae (*Arbutus* and *Arctostaphylos*) (Molina *et al.*, 1992). The fungi colonise the plant root both intercellularly and intracellularly, but colonization is constrained to the epidermis and cortical cells (Molina *et al.*, 1992) (Fig. 1.3). Mycorrhizal fungi responsible for this association can form ECM on other plants (Molina and Trappe, 1982). Futhermore, there is another group known as the Monotropoid. These are mycorrhizal fungi associated with the plant family Monotropoideae, and form a thick fungal sheath. Little is known about the mycorrhizal fungi responsible for this association because of the inability to germinate the seeds of their host plant which are mostly parasitic plants (Molina *et al.*, 1992; Smith and Read, 1997).

Ericoid mycorrhizal fungi (such as *Hymenoscyphus ericae*) are associated with host plants in the family Ericales. Their hyphae are septate and grow intercellularly, their growth is however restricted to the epidermal cells (Molina *et al.*, 1992). Lastly, there is Orchid mycorrhiza in which the mycobionts responsible for this association belong to the group of Basidiomycotina. This group is characterised by an intracellular colonization but an absence of Hartig net, fungal sheath and vesicles (Fig. 1.3). An example of the group is *Rhizoctonia repens* (Smith and Read, 1997).

1.7 *Truffles- The Prize*

Truffles are fungi with underground (hypogeous) fruit bodies. Most truffles are edible mycorrhizal mushrooms that have been greatly admired due to their dual roles as a source of food (Hall and Wang, 1998) and their ability to improve the health and growth of their host plants (Giovanneti *et al.*, 1994). The study of truffles is referred to as Hydnology (Hydnon being Greek for truffle). Scarcity, sweetness, flavour, distinctive texture and good nutritional values are characteristics that have placed truffles in a well respected economic position (Pegler, 2002, Levin *et al.*, 1987, Taylor *et al.*, 1995, Mshigeni *et al.*, 2005). For years, the growth, ecology and biology of truffles remained a mystery as they were not plants, but grew on or beneath the soil, emerging and vanishing unexpectedly (Levin *et al.*, 1987, Pegler, 2002).

Recognition of truffles as an edible delicacy dates back to the bible as a source of food for the Israelites during their forty years stay in the wilderness. There, it was referred to as "Manna" (Exodus vxi) (Pegler, 2002). Historically, the existence of truffles has been subjected to many theories such as the claim in the first recognized literature on truffle by Theophraste, who stated that they appear as a result of thunderstoms. Dioscoridae claimed that truffles were roots. However, in 1711, Tounefort came close to describing the biology of truffles by depicting truffles as a gathering together of "grains" initially released from "vesicules". The adopted and current description of mycorrhizal associations which included most truffles was made by Frank in 1885. At this time, Frank was in the process of investigating the erratic distribution of truffles and is regarded as the pioneer of mycorrhizal research. (Delmas, 1978).

There are two major types of truffles: forest truffles and desert truffles (Pegler, 2002). Forest truffles are mostly "European" and are of more economic and culinary importance than desert truffles (Giovanneti *et al.*, 1994). Desert truffles aptly describe their preferred habitats and distinguish them from the more respected European truffles (Pegler, 2002). *Terfezia pfeilii* is a hypogeous ectomycorrhizal fungus (never appearing above ground) that thrives well in southern Africa (van der Westhuizen and Eicker, 1994). "Fagaa", "n'abba", and "omatumbula" are some of the names given to this truffle by the inhabitants of the areas where it is found (Peglar, 2002; Levin *et al.*, 1987; Mshigeni *et al.*, 2005).

1.8 *Kalahari Truffle*

1.8.1 Classification/Phylogenetics

The identification and classification of truffles have been significantly restricted by the complex nature of their cultivation in the laboratory as well as the difficulties in differentiating them at the vegetative states (Horton and Bruns, 2001). *Terfezia* species are taxonomically placed in the order Pezizales, under which the family of Terfeziaceae is placed (Fig. 1.4). The two most common genera of the Terfeziaceae family are the *Terfezia* and *Tirminia* (Kagan-Zur, 1998 and Fig. 1.4).

Recent molecular and phylogenetic scrutiny of the classification of *Terfezia pfeilii* by Ferdman *et al.*, 2005, has suggested the change of name and reclassification of *Terfezia pfeilii* to *Kalaharituber pfeilii.* This is due to phylogenetic confirmation that *T. pfeilii* does not belong to the *Terfezia* clade. This study did not include any South African isolates, so the name *Terfezia pfeilii* will be used in this study as the phylogenetic position of South African samples will be investigated (Objective 6).

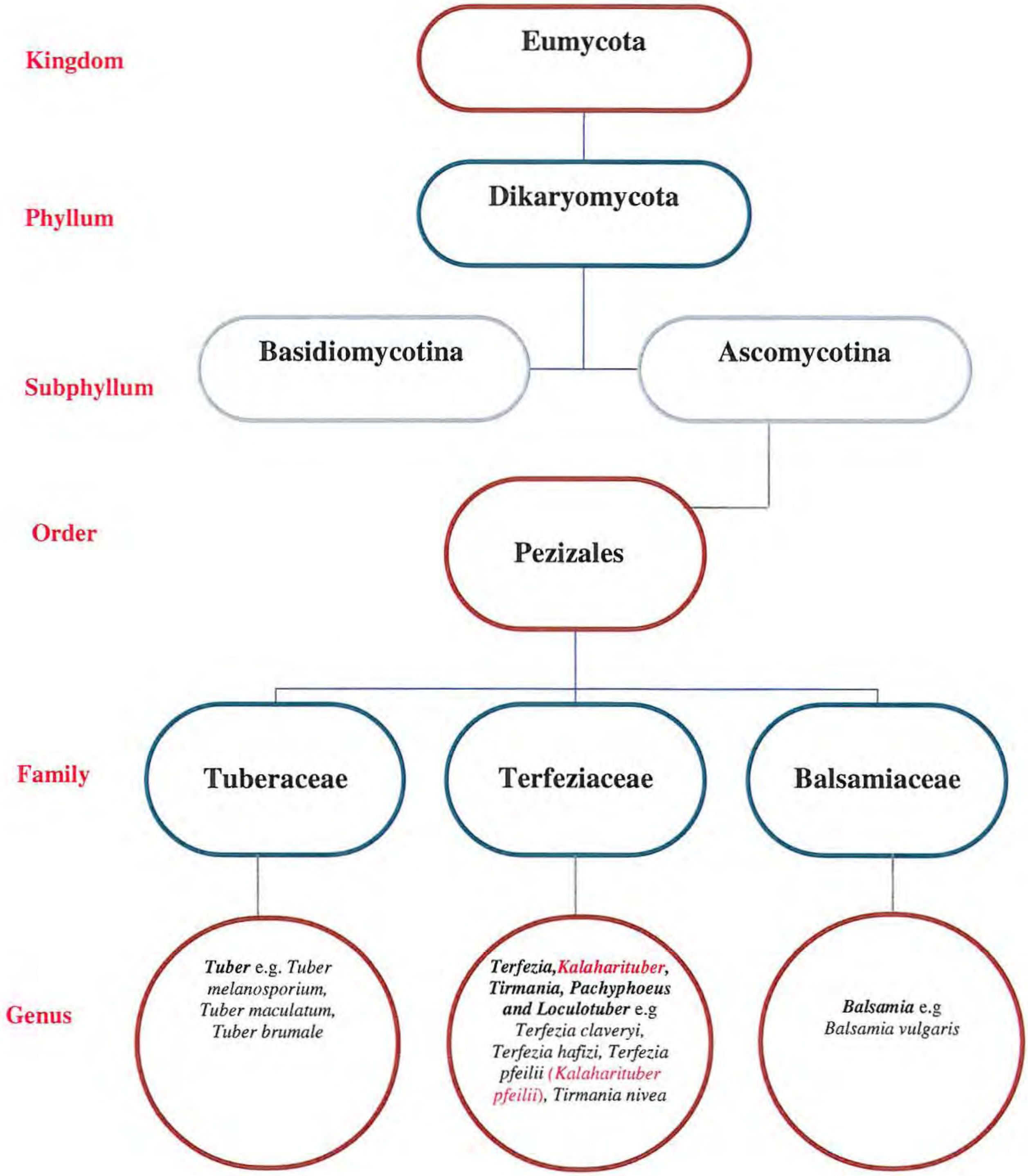

Figure 1.4: Classification of Truffles and the proposed reclassification of *T. pfeilii* as *Kalaharituber pfeilii* (adapted from Kendrick, 2001, Percudani *et al.*, 1999, Norman and Egger, 1999; Ferdman *et al.*, 2005).

1.8.2 Morphology / Life Cycle

The shape and size of the Kalahari truffle is comparable to that of a potato with an average size of about 20mm to 50mm in diameter (Fig. 1.5) (van der Westhuizen and Eicker, 1994). When sliced in half, the truffle has a cream white colour, which gradually turns yellow when exposed to air. Ascospores of *T. pfeilii* are ornamented, with densely distributed spines (Fig. 1.7-1) (Ferdman *et al.*, 2005). The hypogeous ascocarps of the Kalahari truffle are found 5 to 40cm below ground and their presence is indicated by cracks on the soil surface (Levin *et al.*, 1987, Taylor, *et al.*, 1995) (Fig. 1.5). Their hypogeous nature (van der Westhuizen and Eicker, 1994) has ruled out the option of wind dispersal of the ascospores contained in it. However, during the rainy period of the year, some areas of Kalahari Desert become water-logged and this causes part of the soil covering the truffle to be washed away, leaving a slight grass cover, thereby exposing the fruiting body (Taylor *et al.*, 1995). Local people say that the truffles became sand again, which probably means that they disintegrate and old spores are released into the soil.This may also be washed away by rain runoff (Botha, personal communication).

Figure 1.5: Ascocarp of *T. pfeilii* found under some grasses. Soil layer was removed from the truffle to expose the the fruiting body

Figure 1.6: Cracking surface of the sandy soil of the Kalahari Desert indicating the presence of a truffle

Truffles form an essential dietary component of some animals. These fungus-feeding animals are referred to as mycophagous and play an important role in dispersing the spores of the truffles through their fecal matter (North *et al.*, 1997; Claridge, 2002). Truffle spores remain viable after passing through the alimentary canal of mycophagous animals, a process confirmed to improve the germination of some truffle spores and other ECM fungi such as *Mesophellia* species (Lamont *et al.*, 1985; Zabel and Anthony, 2003). This is probably an indication that acid or heat pretreatment may improve germination of spores in the laboratory (Lamb and Richards, 1974; Izzo *et al.*., 2006). An animal feeding on *T. pfeilii* occurring in the Northern Cape region which could be aiding dispersal is *Suricata suricatta* (meerkat), however this has not been confirmed. Other mammals such as the rat kangaroos and rodents have been investigated and confirmed to help in the dispersal of other hypogeous fungi (Claridge *et al.*, 2002; Luoma, *et al.*, 2003). The faeces of a marsupial, *Benttogia penicillata,* was found to be an efficient ectomycorrhizal inoculant (Fig. 1.7) of *Eucalyptus calophylla* and *Gastrolobium bilobum* in the study conducted by Lamb and Richards, (1974). This can be compared to the post-fire recolonisation by some ECM fungi where resistant propagules need heat treatment to germinate as shown in the study by Izzo *et al.*, (2006). In their study, there was a significant increase in the frequency of occurrence of *Rhizopogon olivaceotinctus* after heat treatment at 75°C. It can therefore be depicted that such treatment may be necessary for faster germination of the spores of some ECM fungi.

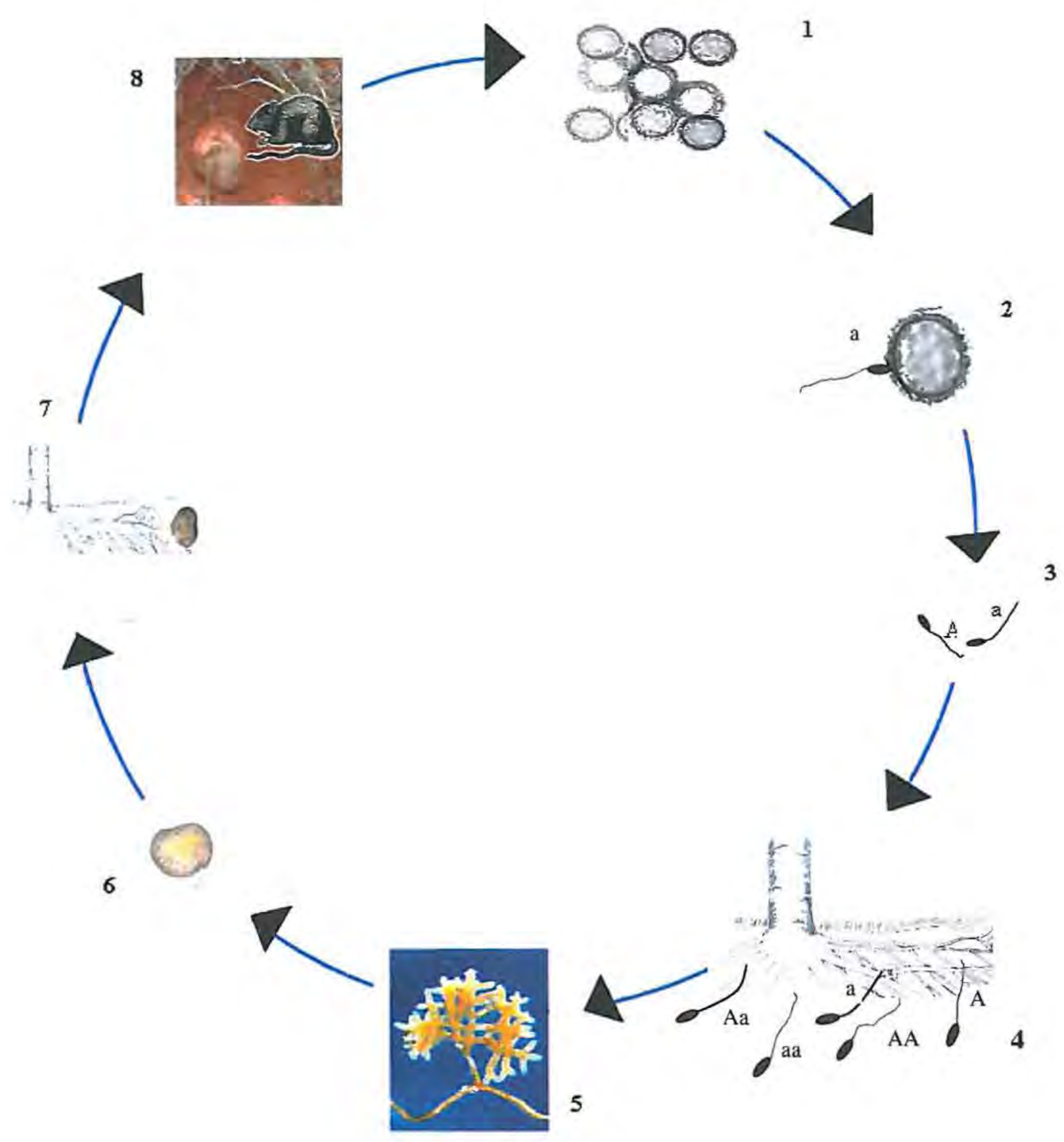

Figure 1.7: Proposed life cycle of *T. pfeilii*

Period of spore dormancy (Demlmas, 1978) though not known from *T. pfeilii*, has been reported to be reduced by mycophagy (**1**) Spores germinate producing single germination tube (**2**) Hyphae formed from the spores are monokaryons that can exist separately (A, a) or form a dikaryote with another hypha of its type, AA or aa (homokaryote) or with another hypha of another type, Aa (heterokaryote) after plasmogamy (**3,4**) (Roth-Benjerano *et al.*, 2004). All or any of these mycelial structures (AA, Aa, A, aa), monokaryon or dikaryon, have the capability to approach and colonise the root of the host plant. In close proximity to the root and like other ectomycorrhizal fungi, they probably develop appresoria which eventually lead to the penetration of the host root (**4**) (Roth-Benjerano *et al.*, 2004; Giovanetti *et al.*, 1994, Smith and Read, 1997). After the establishment of the symbiotic relationship with the host plant (**5**), mycelia aggregate together during which plasmogamy occurs, and this gradually leads to the formation and maturation of the fruit body, ascocarp (**6, 7**). After maturation of the ascocarp, spores are dispersed by either animals (Luoma *et al.*, 2003) that feed on the ascocarps or through the release of the mature spores into the soil (Taylor *et al.*, 1995) (**8**).

The information about truffles in general is limited by the lack of scientific knowledge at the various stages in the life cycle such as the exact mechanisms necessary for the formation of the fruiting bodies, germination of the spores and sexual/asexual reproduction (Mello *et al.*, 2006). Molecular methods are gradually being adopted in the resolution of the complexity involved in the understanding of truffles' life cycle. Recently, Paolocci *et al.* (2006) used molecular methods (polymorphic microsatellites) to proposed that the life cycle of *Tuber magnatum* is predominantly haploid as found in many ascomycetes.

A few studies on *T. pfeilii* have attempted to fill in the gaps of the knowledge. A proposed life cycle of *T. pfeilii* has been provided in Fig. 1.7. Putting together all the available pieces of information about *T. pfeilii*, the proposed life cycle could involve a period of spore dormancy, which precedes germination of the spores into mycelium (Fig. 1.7) (Delmas, 1978). The germination can be hastened by the dispersal of the spores by some animals such as *Suricata suricatta* (meerkat). Passage of the spores through the alimentary canal of such animals increases the chance of the spores germinating faster (Claridge, 2000; Luoma *et al.*, 2003). The germinating hypha then proceeds as a monokaryon to colonise the root of a potential host plant, or undergoes plasmogamy with another hyphae to produce a secondary mycelium that colonises the root (Fig. 1.7). The secondary mycelium could either be a homokaryon or a heterokaryon (Roth-Bejerano *et al.*, 2004). This is contrary to what has been observed in some *Tuber* species where only a dikaryotic hypha can colonise the root of the host plant (Fig. 1.7) (Poma *et al.*, 2006; Paolocci *et al.*, 2006). Though little information is available on the development of the hyphal network in the fruiting body, the presence of paired nuclei that have been recorded in the double ITS-RFLP profile of the ascocarps of *T. pfeilii* is an indication that the ascocarp is formed by development of heterokaryotic hyphae (Mello *et al.*, 2006), (Fig. 1.7).

Previously, Kagan-Zur *et al.*, (1999), investigated part of the life cycle of *T. pfeilii*. In their study, molecular analyses of ascocarps of *T. pfeilii* harvested from the same vicinity as some suspected host plants such as *Citrullus vulgaris*, revealed a double profile of two ITS-RFLP patterns after digestion with *Hin*f 1. This double profile was confirmed through the germinated spores from the ascocarps. The mycelial cultures that resulted from the germination exhibited either of the two profiles of the fruiting body but never both. A single ITS-RFLP pattern was obtained from the roots of *Citrullus vulgaris* harvested from the same area as the ascocarps. This is indeed an indication that the plant root could have been colonised by either hyphae that did not originate from the fruiting body or by a monokaryotic hyphae that underwent plasmogamy with another ITS-type hyphae just before the formation of the fruiting body (Fig. 1.7). Roth-Bejerano *et al.*, (2004), explained these events further by investigating the existence of homokaryotic and heterokayotic hyphae in *T. pfeilii* at some stages during development. Their results revealed the presence of multinucleate cells in both sterile mycelial cultures derived from the fresh ascocarp and ascospores. Paired nuclei were found in multinucleate cell from the ascocarp derived mycelial cultures indicating different origins while this pairing was not observed in cultures derived from ascospores indicating same origin.

Furthermore, there is a possibility that meiosis preceedes the ascospore formation, a procees that occurs inside the ascocarp primordia (Fig. 1.7) (Roth-Bejerano *et al.*, 2004). At maturity, ascospores are held inside the sacs called hymenium. *Tuber* ascospores have been reported to be multinucleate, similar to other ascomycetes (Mello *et al.*, 2005). In the truffle life cycle model by Paolocci *et al.*, (2006), it was suggested that though multinucleate, these nuclei are from the same meiotic stage and do not represent different meiotic products because the mycorrhizal mycelia that germinated from these ascospores and recovered from the root tips were all monomorphic. In addition, absence of heterozygosis in *Tuber* has eliminated the possibility of colonisation of the host root by heterozygotic mycelia. This situation is unlikely in *T. pfeilii* as heterokaryotic mycelia that are capable of colonising the root cells have been reported (Roth-Bejerano *et al.*, 2004).

Ascocarps are always found between April and June (van der Westhuizen and Eicker, 1994) after heavy rainfall combined with high levels of temperatures (Table 1.1). Similar climatic conditions were also suggested by Hall *et al.*, (1998) to trigger the fruitification of *Tuber magnatum*.

1.8.3 Nutritional values

Figure 1.8: Collection of harvested truffle (ready for cooking) in a plate with a sliced sample that turns yellow after exposure to air.

Desert truffles have been widely utilised as a direct source of food (Fig. 1.8), an ingredient in some dishes as well as a food condiment (Sawaya *et al.*, 1985). Nutritional studies have shown that *T. pfeilii* has more than 20% dry matter containing approximately 23% human digestible protein, 3-7.5% fat, 7-13% crude fibre, 60% carbohydrates and some amount of ascorbic acid (Ackerman *et al.*, 1975; Al-Shabibi *et al.*, 1982; Sawaya *et al.*,1985). The major fatty acid of the Kalahari truffle is linoleic acid (67.2% of the total fatty acid). Eight essential amino acids (histidine, lysine, phenylalanine, methionine, threonine, leucine, isoleucine and valine), four semi-essential amino acids (arginine, tyrosine, cystine and glycine) and six non-essential amino acids (serine, glutamic acid, aspartic acid, alanine, praline and glucosamine) as well as four different sugars (fructose, glucose, sucrose and sorbitol) are contained in the fruiting body of *T. pfeilii* (Ackerman *et al.*, 1975), making this truffle highly nutritional.

1.8.4 Distribution

Desert truffles thrive mostly in Africa (Libya, Tunisia, Morocco, Algeria, Egypt and South Africa), the Middle East (Iraq, Syria, Iran, Qatar, Kuwait and Saudi Arabia) (Pegler, 2002; Sawaya *et al.*, 1985) and Europe (Spain) (Gutierrez et al, 2003). In southern Africa, the distribution covers most of the Kalahari Desert region, an area of over 2.5 million km^2 between 24°S, 20°E and 29°S, 23°E (van der Westhuizen and Eicker, 1994, van Rooyen, 2001), starting from the Northern Cape Province in South Africa extending towards some parts of Botswana and Namibia (Fig. 1.9). The Kalahari is a plateau with an altitude that ranges between 900- 1,100m (van Rooyen, 2001). It is a sparsely populated region and consists of races such as Qung, Marsawa, Overherero, Heikum, Haina, Bakalahadi, Masarwa, Madenassena, Batete, San Bushmen, Bogo, Tannekhoe, Hottentot, Namaqua and Goriquas (Schwarz, 1928). Upington, Kimberly and Prieska are specific districts of South Africa where Kalahari truffles (Fig. 1.9) have been found (Levin *et al.*, 1987, van der Westhuizen and Eicker, 1994, Taylor *et al.*, 1995), whereas Oshamaka, Ongwedina and Omupupa are places in Namibia where *T. pfeilii* has been reported (Mshigeni *et al.*, 2005). In Botswana, harvesting has been reported in Lekalane, Kang, Ghanzi, Dutlwe, Khakhea, Werda, Takatakwane and Motokwe districts (Taylor *et al.*, 1995).

Three different types of truffles were identified in South Africa and reported by Marasas and Trappe, (1973) in their preliminary investigation of *Terfezia* spp. Characters such as asci and spores were used to categorise these truffles into *Choiromyces echinulatus*, *Terfezia pfeilii* and *Terfezia austroafricana*. *C. echinulatus* was reported to have uniseriate spores that are prominently echinulate with cylindrical asci, while *T. pfeilii* spores were reported to be mainly echinulate with spines and spores of *T. austroafricana* had prominent spinose-reticulate form with spines. So far, molecular analyses have been used to confirm the existence of only *Choiromyces echinulatus* and *Terfezia pfeilii*, several nucleotide sequences of these two species have been deposited in the GenBank (Wheeler *et al.*., 2006). *T. austroafricana* seems to be rarely encountered (van Schalk Wyk, personal communication) and has not been studied in any detail particularly at a molecular level. No nucleotide sequence of *T. austroafricana* has been found in the GenBank of both the National Center for

Biotechnology Information (NCBI) and the European Molecular Biology Laboratory (EMBL) (Wheeler *et al.*, 2006; Tamara *et al.*, 2006).

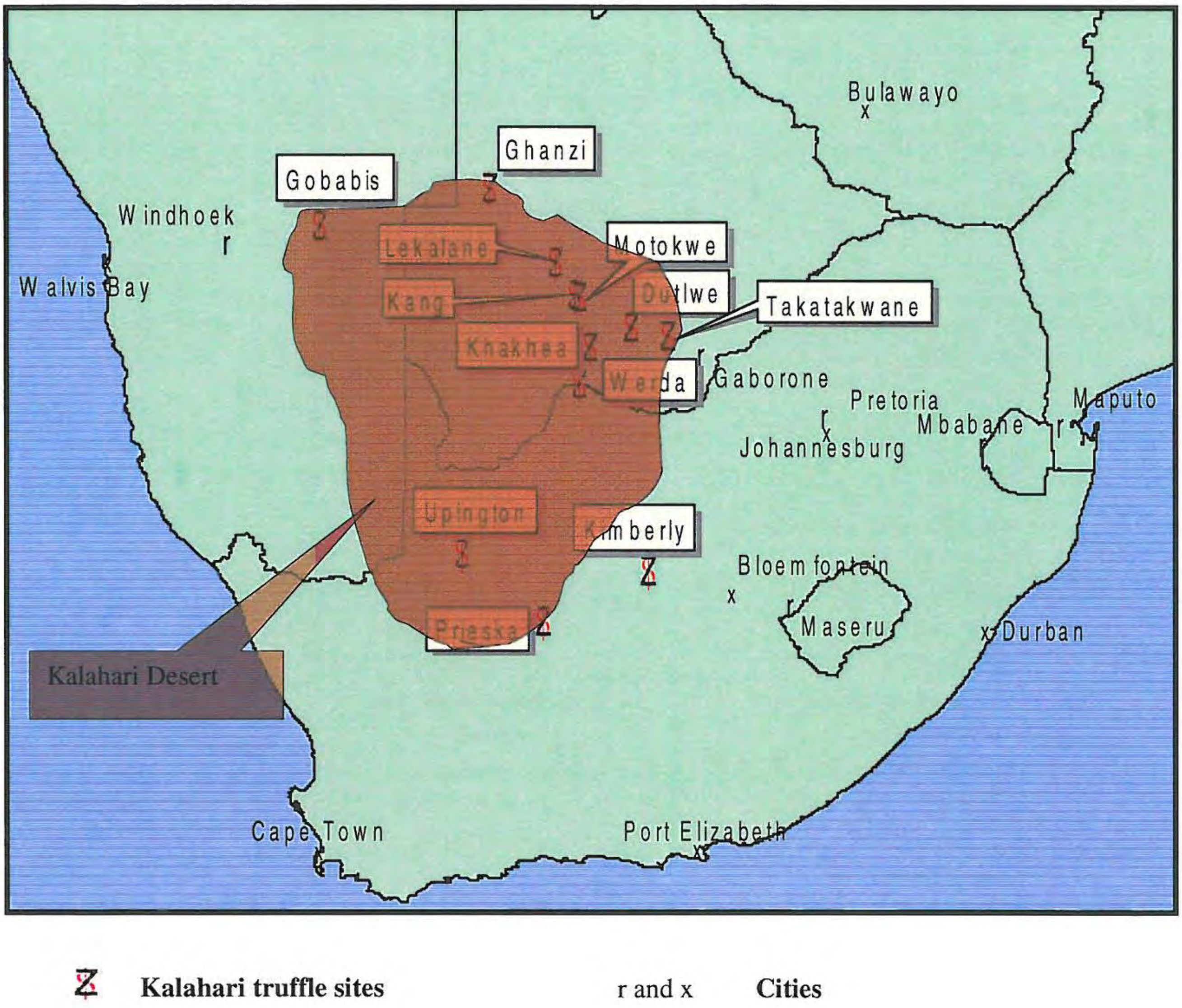

Figure 1.9: Map of southern Africa showing the location of the Kalahari desert and the distribution of *T. pfeilii* (Map drawn with Arc view GIS 3.2 edition).

Table 1.1: Climatological information based on monthly averages for the 30-year period 1961 – 1990. This indicates the average minimum and maximum temperature and average rainfall distribution in Upington (SAWS, 2006).

Month	**Temperature (° C)**		**Precipitation**
	Average Daily Minimum	**Average Daily Maximum**	**Average Monthly (mm)**
January	20	36	24
February	20	34	35
March	18	32	37
April	13	28	26
May	8	24	10
June	5	21	4
July	4	21	2
August	6	23	4
September	9	27	4
October	13	30	9
November	16	33	17
December	19	35	17
Average annual	**13**	**29**	**189**

Table 1.2: Characteristics of soil collected from Upington. Soil samples were analysed by Eco-Analytica laboratory, North West University, South Africa. (Data presented are means of 4 samples)

Nutrients	**Ca**	**Mg**	**Na**	**NO_3**	**NO_2**	**Total P**	**NH_4**	**C**	**Available P**	**pH**
	Millimole per liter							**(%)**	**(ppm)**	
Average values	2.100	4.440	1.495	1.890	0.034	0.406	0.595	0.148	11.44	5.968

1.8.5 Host plants and mycorrhizal status

The Kalahari does not entirely meet the definition of a desert and its classification as such has been queried by experts (van Rooyen, 2001). It has a dense population of shrubs, grasses and trees. In terms of the species/area ratio, the Kalahari Desert is poorly vegetated compared to other regions of southern Africa. The flora in the Kalahari consists of thorny Kalahari Dune Bushveld and shrubby Kalahari Dune Bushveld (van Rooyen, 2001). The search for hosts of *T. pfeilii* has been focused on plants that thrive well in the Kalahari Desert (van der Westhuizen and Eicker, 1994). However, finding the conclusive evidence of the phytobionts (plant host) of *T. pfeilii* seems to be the most challenging and unaccomplished part of Kalahari truffle research (Story, 1958; Taylor *et al.*, 1995; Kagan-Zur *et al.*, 1999).

Terfezia species generally form different types of mycorrhizal associations which can be ectomycorrhizal, endomycorrhizal or ectendomycorrhizal under different conditions (Fortas and Chevalier 1992; Kagan-Zur, 1998). In a study by Gutierrez *et al.*, (2003), *Terfezia claveryi* formed an endomycorrhizal association in a natural field condition, ectomycorrhizal / ectendomycorrhizal (with no sheath and Hartig net) in pot cultures and an ectomycorrhizal (with sheath and Hartig net) association under *in vitro* conditions with *Helianthemum almeriense.* Furthermore, Fortas and Chevalier, (1992) also reported that *T. claveryi* formed an ECM association in a phosphate rich soil (1400ppm of total soil weight), but this same species formed an endomycorrhizal association in phosphate-poor media (60ppm). In a light microscopic investigation of *T. terfezioides*, the mycorrhizal association was established to be an ectendomycorrhizal or endomycorrhizal type as both the "Hartig" net and the mantle were absent, but the hyphae of the fungus colonised the cortical cells with no indication of parasitism (Bratek *et al.*, 1996).

The mycorrhizal association that exists between *Terfezia* species and the family Cistaceae especially *Helianthemum* species has been proven to be ECM and sometimes endomycorrhizal (Fortas and Chevalier 1992; Gutierrez *et al.*, 2003). However, members of this family are exotic in South Africa or Southern Africa (Taylor *et al.*, 1995; Kagan-Zur *et al.*, 1999). Although *T. pfeilii* has been reported to be associated with plants such as *Acacia haematoxylon* (grey camel thorn), *Acacia erioloba* (camel thorn), *Stipagrostis* species (bushman grass) and *Cynodon datylon* (kweek/couch grass), the mycorrhizal association is yet to be proven (van der Westhuizen and Eicker, 1994; Taylor *et al.*, 1995; Kagan-Zur *et al.*, 1999). There is a need to focus attention on these plants and many others that inhabit the Kalahari Desert such as *Acacia luedetritzii* (false umbrella thorn), *Boscia lehmanniana* (Shepherd tree), *Eragostis lehmanniana* (Lehmann's love grass), *Centropodia glauca* (gha grass) and *Aristida meridionalis* (giant tree-awn) that inhabit the Kalahari Desert (van Rooyen, 2001). A study by Kagan-Zur *et al.*, (1999), reported that wild water melon, *Citrullus vulgaris* formed an endomycorrhizal type of association with *T. pfeilii.* Studies of the effects of factors such as climatic, topographic and geopedological conditions as suggested by Delmas, (1978), might also assist in the quest for *T. pfeilii* phytobionts.

1.8.6 Molecular characteristics

Molecular analysis is essential in truffle studies (Lafranco *et al.*, 1998). Molecular techniques have contributed immensely to the knowledge of truffles, especially in the areas of classification, identification and differentiation of morphologically related species (Mello *et al.*, 2002). Traditional morphological and analytical methods have been unable to accurately resolve the problems associated with the identification of mycorrhizas, especially in situations where species are morphologically similar, such as in some truffles (Urbanelli *et al.*, 1998, Horton and Bruns, 2001). With the advent of more precise and effective molecular methods especially Polymerase Chain Reaction (PCR) many of these problems have been resolved (Sejalon-Delmas *et al.*, 2000). The focus of the PCR method is the amplification of the ribosomal genes and spacers of which products can be further analysed using PCR based techniques such as Restriction Fragment Length Polymorphism (RFLP) or sequencing to provide a proper identification/classification of the fungus. With some restrictions, the use of Internal Transcribed Spacer (ITS)-RFLP method has become a very popular and effective molecular technique in mycology. This method has also been applied in an attempt to investigate the phytobionts of less understood mycorrhizas such as truffles (Kagan-Zur *et al.*, 1999).

A molecular approach was adopted in the search for the phytobionts for the Kalahari truffle by Kagan-Zur *et al.*, (1999), where they compared the PCR amplified ITS fragments from the fruit bodies to that obtained from the "suspected" host plants (*Citrullus vulgaris*, *Sorghum bicolor* and an unidentified weed) around where the ascocarp was harvested. Their efforts were not entirely successful, resulting in only one likely host being detected which was watermelon (*Citrullus vulgaris*). Surprisingly, morphological studies of the roots of *C. vulgaris* revealed an endomycorrhizal type of association (Kagan-Zur *et al.*, 1999). This highlights the importance of combining light microscopic techniques with those of molecular analyses in the identification of truffles and their associations.

The role of molecular techniques in truffle researches has continued to increase. For example, the use of molecular analysis in quality assurance of truffle products will be very useful especially with issues concerning food poisoning, as raised by Ackerman *et al.,* (1975) where the toxic *Choiromyces echinalatus*, was wrongly identified as *T. pfeilii* because of the morphological similarities between the two. Furthermore, molecular analyses are also needed to clarify the reported existence of the three different types of desert truffles (*C. echinulatus*, *T. pfeilii* and *T. austroaficana* (Marasas and Trappe, 1973) in South Africa.

1.9 Motivation for the study

Few studies have attempted the investigation of Kalahari truffle in South Africa in terms of its related species, host and its biology (Taylor *et al.*, 1995). Moreso, it is the only indigenous truffle in South Africa (Taylor *et al.*, 1995) with high prospects for commercial production like most European truffles. This potential cannot be achieved if the host plants and the mycorrhizal status remain unknown. Host plants are important for the formation of mycorrhiza without which mycorrhizal fungi cannot complete their life cycles and produce fruiting bodies (Taylor and Alexander, 2005).

1.10 Hypothesis and Objectives

1.10.1 Hypothesis

T. pfeilii forms an ectomycorrhizal relationship with selected host plants, similar to that of other ascomycotinous *Terfezia* genera.

1.10.2 Objectives

To prove this hypothesis, the following objectives were investigated:

1. To collect and identify ascocarps: Ascocarps were collected from Upington and subsequently identified by morphological and molecular methods.
2. To obtain *in vitro* cultures of *T. pfeilii*: The main aim of this objective was to obtain an *in vitro* pure culture of *T. pfeilii* that can be used for other investigations such as studies on physiological growth parameters, mycorrhizal synthesis experiment and mycorrhizal helper bacteria experiment.
3. To successfully identify in vitro pure cultures of *T. pfeilii* morphologically and molecularly: Morphological and molecular methods were used to accurately identify the pure cultures and distinguish truffle cultures from contaminants.
4. To determine the physiological growth parameters required for optimal *in vitro* culture: This particular study was aimed at reducing the time required to obtain the pure culture and at the same time increase the quantity of mycelial culture of *T. pfeilii* that can be grown *in vitro*. Eventual optimisation of the physiological growth parameters will also improve the understanding of these growth parameters as it relates to the maximum growth of *T. pfeilii* in the field.
5. To develop a method for rapid identification of *T. pfeilii* using molecular techniques: This objective was particularly aimed at reducing the time required for complete identification of important stages in the life cycle of *T. pfeilii*. This would allow rapid and easy confirmation of the identity of the ascocarps, pure culture and mycorrhiza of *T. pfeilii*. Proper and quick identification of various forms of *T. pfeilii* is pertinent to the success of the entire study.
6. To investigate the proposed reclassification of the *T. pfeilii* as *Kalaharituber*: Investigation into the proposed reclassification of *T. pfeilii* is necessary as no South African sample was used in the initial investigation conducted for the proposed name change (Ferdman *et al.*, 2005). Comparative analysis of ITS sequences from South African samples with those used by Ferdman *et al..*, 2005, will enable a contribution towards the general acceptance or rejection of the proposed name.

7. To re-establish the mycorrhizal association on selected host plants through mycorrhizal synthesis experiments: this objective will enable the determination of the potential host plants.
8. To assess the mycorrhizal status of *T. pfeilii* on various selected host plants. Roots of inoculated plants were subjected to staining and molecular analysis to determine if and what type of mycorrhizal were present.
9. To isolate and investigate the *in vitro/in vivo* potential mycorrhizal helper bacterial effects: This objective was to determine the helper effects of the bacteria associated with the ascocarp of *T. pfeilii.*

CHAPTER TWO

2 Isolation and Optimisation of Physiological Growth Parameters of *Terfezia pfeilii in vitro*

2.1 Introduction

Some ECM fungi are facultative biotrophs (Smith and Read, 1997) meaning that at a stage in their life cycles they are capable of independent existence. The presence of this saprotrophic stage has allowed scientists to grow them in pure cultures (Lacourt *et al.*, 2002). ECM fungi are difficult to cultivate in the laboratory because of their complex nutritional requirements, and there are still many that cannot be cultivated in the laboratory, hence believed to be obligate symbionts (Molina and Palmer, 1982; Isaac, 1992; Horton and Bruns, 2001). However, successes have been recorded in growing some ECM fungi *in vitro* and this has assisted in investigations into their biology and ecology (Molina and Palmer, 1982). Pure cultures of ECM fungi have been used for various research purposes such as inoculum production, studies on plant host-fungus interaction, nutrient requirement/uptake by the fungus, interactions of ECM fungi with other rhizospheric microorganisms, determination of physiological growth parameters and genetic diversities among ECM fungi (Wong and Fortin, 1989, Garbaye, 1994, Brundrett *et al.*, 1996, Iotti *et al.*, 2002). To obtain pure cultures of ECM fungi, isolations are generally made from fruit body tissues, ectomycorrhizal roots, sclerotia, rhizomorphs and sexual spores (Molina and Palmer, 1982).

The most commonly used method of isolation from truffles is from the ascocarp using the gleba that consists of vegetative mycelia and spores. In this case, a piece of the gleba is aseptically removed and inoculated onto a suitable medium (Brundrett *et al.*, 1996).

Correct identification is necessary to ensure and confirm the identity of the samples before and after isolation (Brundrett *et al.*, 1996). Identification begins in the field with selection of fruiting bodies at different stages of development. Young and fresh fruiting bodies are needed for the isolation of the pure culture, whereas matured ascocarps are essential for correct identification, all these require a good knowledge of the life history of the truffle (Molina and Palmer, 1982). The time of appearance of fruiting bodies for various truffles varies, but it is April-June for *Terfezia pfeilii* (van der Westhuizen and Eicker, 1994).

Identification based only on morphological characteristics is time consuming and may not be sufficient to distinguish species of the same genus (Lafranco *et al.*, 1998). Molecular analysis has provided a more suitable technique that is accurate and confirmatory, especially for the identification of closely related truffles (Sejalon-Delmas, *et al.*, 2000; Lacourt *et al.*, 2002; Iotti *et al.*, 2006).

2.1.2 Identification of pure culture

The process of isolating a pure culture of ECM fungi can be affected or delayed by contaminating micororganisms, most especially, fungal contaminants which sometimes have similar mycelial morphology in culture and nutritional requirements as the intended isolate (Muller and Loeffler, 1976; Smith and Read, 1997). Therefore, success of the isolation process lies in the ability to differentiate between the intended isolate and these contaminants (Brundrett *et al.*, 1996). A combination of a wide range of physiological, biochemical and morphological characteristics such as the utilisation of carbon and nitrogen sources, temperature requirements, fungal static and fungal toxic properties, septa formation and growth rate have been suggested by Hutchinson, (1990a and b) for the identification of ECM cultures. However, these methods are time consuming and not reliable especially in the identification of closely related species (Pinkas *et al.*, 2000). For example, it may be difficult to utilise such methods in situations where different strains of the same organisms have different characteristics such as one reported by Saltarelli *et al.*, (1999) where some strains of *Tuber borchii* were misidentified as contaminants because of differences in some of their features. The introduction and current use of molecular analyses to differentiate between the contaminants and the pure culture has assisted in verifying purity of cultures obtained from isolations. Mostly, PCR-based methods are routinely being used to confirm identification (Urbanelli *et al.*, 1998, Horton and Bruns, 2001, Sejalon-Delmas *et al.*, 2000).

2.1.3 Media for the Growth of ECM fungi

General purpose media such as Modified Melin Norkrans medium (MMN), Pachlewski medium (PACH), Potato Dextrose Agar (PDA), Yeast Extract Agar among others have all been successfully used to isolate ECM fungi (Brundrett *et al.*, 1996). These media generally combine various ingredients and include mineral nutrients, a carbohydrate source, vitamins and agar. Media formulations have included maltose, glucose, malt extract and dextrose as sources of carbohydrates with D-glucose being the most common choice (Brundrett *et al.*, 1996; Molina and Palmer, 1982). In the case of nitrogen utilisation, most ECM fungi have been confirmed to readily utilise NH_4^+ and NO_3^- and some organic nitrogen compounds *in vitro*. Additionally, the pH of the media is also very important, as it strongly affects the quantity of nitrogen that can be utilised in the media (Smith and Read, 1997). Apart from nitrogen and carbon, other macronutrients such as phosphorus, potassium, sulphur and magnesium are needed in high quantities for the media formulation. Meanwhile, copper, iron, manganese, molybdenum and zinc are required in trace amounts for the complete formulation of the media (Molina and Palmer, 1982). Media additives such as vitamins and antibiotics have been found to increase the chances of isolating a pure culture. These additives either have direct effects on the growth of the mycelium or act by inhibiting the growth of contaminant microorganisms (Brundrett *et al.*, 1996).

More selective media supporting the growth of specific ECM fungi have also been utilised. An example of this is Fontana medium that has been successfully used to isolate and grow some truffle species especially *T. pfeilii*. (Fontana, 1968; Kagan-Zur *et al.*, 1999). Caution must however be exercised when formulating media for ECM fungi because of the differences in the nutrient utilisation by different strains. For instance, four different strains of *Amanita caesarea* had different nitrogen utilisation capabilities when grown on MMN containing BSA, di-ammonium hydrogen phosphate and calcium nitrate as nitrogen sources (Daza *et al.*, 2006). In such situations, a number of different strains of the same organisms will need to be studied before the media can be formulated.

2.1.4 Physiological Growth parameters of *T. pfeilii* on Fontana medium

The capability of ectomycorrhizal fungi to form a symbiotic association with the host plant and grow under both *in vivo* and *in vitro* conditions is greatly influenced by physical characteristics and nutrient availability. Such factors include carbon, nitrogen, temperature and pH (France and Reid, 1984; Daza *et al.*, 2006).

The knowledge about the nutrient requirement of some ECM fungi has been used to formulate different media for the growth (Molina and Palmer, 1982). It has been shown that the efficiency of these media for the cultivation of ECM fungi can be improved by alterating growth parameters such as nutrient composition (France and Reid, 1984; Mischiatti and Fontana, 1993). Most studies have focussed on adjustment by substitution, addition and subtraction of various macronutrients especially carbon, nitrogen and phosphorus (Holligan and Jennings, 1972; France and Reid, 1984; Antibus *et al.*, 1992; Hutchinson, 1990a; Ceccarolli *et al.*, 2001).

Carbon is an important source of energy for the growth of all mycorrhizal fungi (Isaac, 1992; Carlile, 2001). To fully exploit the symbiotic association, an understanding of carbon nutrition by ECM fungi is necessary (Jakobsen, 1994). Carbon requirements of ECM fungi had been investigated *in vitro* on artificial culture media and there is evidence that the utilisation of carbon compounds among and within ECM fungal groups varies (Ceccaroli *et al.*, 2001; Daza *et al.*, 2006). Many studies have confirmed the presence of trehalose and mannitol as the major carbon compounds stored in the mycelia of ECM fungi (Martin *et al.*, 1988, Lewis and Harley, 1964b, 1989, Holligan and Jennins, 1972). One such study was conducted by Rangel-Castro *et al.*, (2002), using ^{13}C- Nuclear Magentic Resonance analyses of *Cantharellus cibarus*. Their results confirmed that the storage forms of carbohydrates in this fungus were mostly trehalose and mannitol.

Another well investigated macronutrient is nitrogen. ECM associations have the capacity to provide a greater surface area for the increased uptake and assimilation of nitrogen from the soil (France and Reid, 1984). Although most ECM fungi can utilise both organic and inorganic nitrogen sources, ammonium and nitrate are the two most common forms of nitrogen that are readily utilisable by the ECM fungi (Smith and Read, 1997). Pure culture experiments have been used by various workers to determine the nitrogen requirements of ECM fungi (Molina and Palmer, 1982; France and Reid, 1984; Daza *et al.*, 2006). Meanwhile, the capability of ECM fungi to utilise organic forms of nitrogen under the influence of proteases (Leake and Read, 1990) have been widely studied using pure culture experiments and the results indicated that some species or sometimes strains showed preferences for different organic forms of nitrogen such as various proteins, amino acids and chitin (France and Reid, 1984; Abuzidanah and Read, 1986 I; Leake and Read, 1990; Smith and Read, 1997; Rangel-Castro *et al.*, 2002; Rangel-Castro *et al.*, 2002; Daza *et al.*, 2006). For example, Abuzidanah and Read, (1986) reported the high preference of some strains of *Amanita muscaria* and *Paxillus involutus* for BSA as the nitrogen source at pH of 6 and above.

Phosphorus is the most widely studied macronutrient of ECM fungi (Smith and Read, 1997). ECM fungi are generally known for their ability to mobilise and continuously transfer phosphorus from soil to the phytobionts (Marschner and Dell, 1994). It has been reported that ECM fungi can utilise phosphorus in various forms, both organic and inorganic (Smith and Read, 1997). Most of the phosphorus absorbed by ECM fungi is not utilised by the fungus but transferred to the host plant (Smith and Read, 1997). This is probably the most significant symbiotic role of ECM fungi. However, Po is not readily utilisable by ECM fungi but is converted (solubilisation) to Pi with the aid of phosphatases (Jayakumar and Tan, 2005). Meanwhile, production of phosphomonoesterases by some ECM fungi has been reported to enhance the utilisation of Po. In relation to this, a greater phosphomonoesterases activity was reported around the mycorrhizal roots of spruce and rhizomorphs of *Thelephora terrestris* when compared to the non-mycorrhizal spruce by Dinkelaker and Marschener, (1992), an indication of the role of this enzyme in the utilisation of Po.

Physical factors also have significant roles to play in the growth of ECM fungi (Hung and Trappe, 1983; Daza *et al.*, 2006). Hung and Trappe, (1983), categorised ECM fungi based on their pH requirement into pH optimum and pH tolerant, and went further to investigate the effect of pH among and within species of some ECM fungi. Their results indicated that there are differences in the pH requirements of ECM fungi in the field and those growing on culture media. They also discovered that intraspecific variations do exist in the pH tolerance of some ECM fungi. On the other hand, optimum temperature for the growth of ECM fungi has been suggested to reflect the growth conditions obtainable in the field for ECM fungi (Daza *et al.*, 2006).

This study reports on the isolation of *in vitro* cultures of *T. pfeilii* and investigates into the physiological growth parameters thereof. The isolation of the *in vitro* culture will provide mycelial culture of *T. pfeilii* that can be used for other mycorrhizal purposes most especially the investigation of the host plant through mycorrhizal synthesis.

2.2 Materials and methods

2.2.1 Collection and isolation of *T. pfeilii*

Harvesting of the ascocarp was conducted during the fruiting period of the *T. pfeilii*, from April to June, 2006. These were harvested from the Spitskop Nature Reserve in Upington, Northern Cape province, South Africa (S28° 22.780', E21° 09.420'). Cracks at the surface of the soil as shown in Fig. 1.6 indicated the presence of the hypogeous fruiting body. Both young and old ascocarps were collected for the purpose of inoculation and morphological identification, respectively. Sand particles attached to the ascocarp were gently brushed off and the ascocarp surface-sterilised briefly with 50% ethanol. Ascocarps with no physical trace of either microbial or insect infection were selected for the isolation experiments (Molina and Palmer, 1982).

After collection, glebal tissues from the young and fresh ascocarps were immediately inoculated onto MMN (Marx, 1969) (Appendix I) and PDA (Biolab; Cat.# HG00C100.500) in the field under aseptic techniques. The remaining ascocarps were not sterilized in the field but transported to the laboratory inside dry paper bags in a cooler box.

Laboratory isolation of the ascocarps

The use of glebal tissue as a method of isolation was one of the 3 options available. The second method which involves isolation of the spores using old fruiting bodies could have afforded the mycelia to probably grow faster (Roth-Bejerano *et al.*, 2004) but because of the risk of contamination, this method was not considered. The third method was not feasible because *T. pfeilii* mycorrhiza was not collected.

Ascocarps collected in the field were stored at 8°C in a cool room and processed as soon as possible. After surface sterilisation, ascocarps were gently split open and sections from inside the ascocarps (explants) of between 10mg and 15mg were taken and inoculated onto the Fontana (FTN) (Fonatna, 1968) and MMN (Marx, 1969) media (Appendix I). Plates inoculated in the field that were showing signs of contamination were also sub-cultured onto these media and incubated at 25°C.

Bacterial contaminants were easily differentiated because of their different appearance on the medium. The bacteria were re-inoculated onto new nutrient agar for further investigation (Chapter 4). Fungal contaminants were observed microscopically by preparing a tape mount and staining with trypan-blue (Appendix IV) (Harris, 2000). The tape mounts were made by pressing a small piece of scotch tape (sticky side) on a fungal colony growing on the medium, the tape was then placed on microscope slide with the sticky side of the tape facing upwards, a few drops of trypan blue were then applied to the sticky area and a cover slip was placed on top of the mounts (Harris, 2000). This was then examined under the compound microscope (Nikon YS100).

Fungi that produced asexual conidia were eliminated at this stage because they were identified microscopically as being contaminants. Non-sporulating, septate fungal cultures from Fontana medium were transferred to fresh Fontana media using a cock borer of 4mm diameter and the new cultures maintained for further molecular identification.

Preparation of the pure culture

Mycelia from the isolates were scraped off the surface of Fontana agar with a flamed wire loop and suspended in autoclaved 1.5ml microcentrifuge tubes containing autoclaved triple distilled water. The suspension was vortexed to separate the clustered mycelia. A 50µl aliquot of each suspension was spread onto new plates of Fontana medium with the aid of an autoclaved glass spreader. After five days, distinct colonies of the fungi were sub-inoculated onto new plates to obtain pure culture of the fungi.

2.2.2 Identification of the ascocarps

Morphological identification

Morphological identification of the ascocarp was conducted using an identification manual (Trappe, 1979), and the description given by van der Westhuizen and Eicker, (1994). Characteristics such as subglobose brownish ascocarps, solid gleba and irregular diameters comparable to a large potato (with an average diameter of 20mm to 50mm) were used in the morphological identification of the ascocarps.

Molecular Identification of ascocarp

DNA Extraction

The Cetylmethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) was optimised and used for extraction of DNA from the ascocarp. A small quatity of fresh weight gleba (100mg) was placed inside a mortar and 750µl of 2% CTAB was added. This was then crushed with a pestle. The homogenate was transferred into clean 1.5ml microcentrifuge tubes and vortexed for 2 min. A 20µl volume of proteinase K - 20mg/ml (Cat.# V302B) was added and gently mixed to degrade the protein content of the sample that may interfere with the PCR reaction. The homogenate was incubated at 37°C for one hour to allow the enzyme to react and later heated at 65°C for 15 min to dissolve the CTAB and increase the lysing efficiency. A 200µl volume of phenol/chloroform (1:1) was added, vortexed for 2 min and centrifuged at 14,000 rpm at room temperature for 5 min. The supernatant (400µl) was transferred into another clean 1.5ml microcentrifuge tube. A 2X volume (800μl) of 100% ice cold isopropanol was added to precipitate the DNA. The mixture was kept on ice for 20 min and later centrifuged at 14,000 rpm for 10 min. The supernatant was carefully discarded. The pellet was rinsed with 500μl of ice cold 70% ethanol by centrifuging at 14,000 rpm for 4 min. The supernatant was discarded and pellet air-dried inside a lamina flow bench. The pellet was resuspended in 100μl of TE buffer which contains Tris HCl, NaEDTA and water (Appendix I) and stored at -20°C.

Polymerase Chain Reaction

A Qiagen PCR master mix (Cat. # 201445) in a premix format was used in the PCR. The master mix contained *Taq* DNA Polymerase, PCR Buffer, magnesium chloride ($MgCl_2$) - 1.5mM, and 200μM of each of the dNTPs. A final PCR volume of 50μl was used in each reaction, which contained the following: Master mix (11.5μl), autoclaved milliQ water (29.5μl), primer 1; ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Tm - 49.7°C (0.4μM) (Gardes and Bruns, 1993), primer 2; ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; Tm- 52.1°C (0.4μM) (White *et al.*, 1990) , BSA-3μl of 3mg/ml solution and genomic DNA (2μl). The PCR was performed in a Hybaid PCR Sprint .The cycling conditions included an initial denaturing cycle of 3 min at 94°C followed by 30 cycles of 1 min at 94°C for DNA denaturing, annealing temperature of 50°C at 30s and 2 min DNA synthesis at 72°C. There was a final elongation period of 72°C that lasted for 8 min. The PCR product was separated electrophoretically on a 1% agarose gel and visualised on ethidium bromide-UV fluorescence to determine the size of the amplified bands.

Sequencing

The PCR product obtained was cleaned using the PROMEGA Wizard SV Gel and PCR purification kit (Cat.# A9280) and resuspended in 30μl of nuclease-free double distilled water. Cleaned PCR product was sent to the Rhodes University Sequencing Facility. The product was sequenced using ABI prism BigDye Terminator v3.1 Ready Reaction Cycle sequencing kit (Cat.# 4336917) according to manufacturer's instructions with the primers that were used in the PCR: (ITS1F and ITS4). The sequences were electrophoresed on an AB3100 Genetic Analyser (AppliedBiosysematics). Forward and reverse sequences obtained were aligned using

BioEdit software prior to BLASTing. Sequences were thereafter BLASTed on the NCBI website (Hall, 1999) to confirm the nearest identical organism. Genera obtained from the GenBank with high percentage identity (>95%) were assumed to be very significant.

2.2.3 Growth of *T. pfeilii* on different growth media

T. pfeilii inoculated on MMN, PDA and FTN media were investigated to determine if any of these media supported the growth better than FTN media at room temperature (25°C). *T. pfeilii* was inoculated on three replica plates of each medium. Growths were measured daily.

2.2.4 Cultural and hyphal characteristics of *T. pfeilii* isolates

The morphology of the colony of the *T. pfeilii* isolate growing on FTN medium was observed with the naked eye and hyphal structures were observed under the microscope using the tape mount method (Section 2.2.1) to check for the presence or absence of hyphal septation. Other characteristics recorded were colour, flat growth and hyphal penetration of the nutrient medium.

Identification of the Isolates

Molecular Identification

The same protocol used for the DNA extraction from the ascocarp (Section 2.2.2) was applied here except for the use of mycelial material instead of the ascocarpic material. The mycelium growing on the FTN medium plate was scraped off the plate surface and suspended in 750μl volume of CTAB inside 1.5ml microcentrifuge tubes. The mycelial material was crushed with a sterile micropestle. PCR and sequencing were conducted as described in Section 2.2.2 .

2.2.5 Physiological growth parameters

FTN medium (Appendix I) was used for all the investigated parameters and mycelial isolates of *T. pfeilii* were transferred from FTN medium. A molecular confirmatory test (Section 2.2.4) was carried out on the mycelial cultures to confirm the identity of *T. pfeilii* and to ensure contaminants were absent as a control measure. Agar plugs of 4mm diameter were cut from the actively growing edge of cultures and inoculated at the centre of the plates, this was used as the starting inoculum for the experiments. All inoculated plates were incubated in the dark at 32°C except for the temperature experiment, where other temperature ranges were investigated (section 2.2.6). Petri-dishes were sealed with parafilm and placed in cellophane bags that were initially sterilised by wiping with 70% ethanol, to prevent the cultures from drying up and contamination. Colony growth diameter and/or mycelial dry weight were measured to determine the effect of the parameter being tested on growth. Colony diameter was measured daily starting from the third, fourth or fifth day when there was visible hyphal proliferation. The colony diameter was measured daily along two transects and the average of the two readings recorded in all the treatments. The mycelial dry weight was measured after the last measurement of the colony diameters on plate experiments. This was done by gently scraping the culture medium and the mycelial mats off the Petri-dishes and placing these into a 100ml screw cap bottles for autoclaving at 121°C for 15 min. After autoclaving, mycelia mats were then filtered through a pre-weighed Whatman filter paper of 90mm diameter placed inside a Buchner funnel. The culture medium adhering to the mycelia was washed off with hot water during filtration. Both the harvested mycelial mats and the filter paper were oven dried at 80°C for 24 hours and reweighed to determine the biomass of the mycelial mats. This method can lead to the loss of water soluble compounds (Baar *et al.*, 1997). All the experiments ran for a maximum of 15 days.

2.2.6 Temperature and pH

An average temperature range which was a reflection of the average daily temperature of the habitat (Kalahari Desert) where the ascocarps were collected was used in this study. For the temperature experiment, inoculated plates were incubated at 15°C, 20°C, 28°C, 32°C and 37°C temperatures that persist in the Kalahari Desert. Three replicates of each treatment were used for this experiment. The growth at 15°C, 20°C and 37°C were disregarded for the statistical analyses as no growth was recorded at these temperature ranges. The pH values of 5, 6, 7, 7.5 and 8 were investigated.

Hydrochloric acid (1M HCl) and sodium hydroxide (1M NaOH) (rather than buffers) were used to adjust the pH of the medium before autoclaving and verification was done after autoclaving. Different buffering solutions that were initially used for the pH experiments supported no growth of the fungus. This can be related to the change in basic constitution of the Fontana media by the buffering solutions thereby making it difficult for the fungus to grow. Buffers can introduce many extraneous variables into growth media (Hung and Trappe, 1983). Four replicates plates were inoculated for each pH value.

2.2.7 Carbon

ECM fungal utilisable carbon compounds already reported in the literature (Ceccaroli *et al*.., 2001; Daza *et al*.., 2006), were chosen for this study. Carbon sources investigated were sucrose, fructose, mannitol and mannose. A quantity of 6.5g per litre of each of the carbon sources was substituted for the glucose contained in the basal FTN medium (Appendix I). Six replicates of each carbon source were prepared. The control for this experiment retained the original constitution of the FTN medium with glucose being the source of carbon.

2.2.8 Phosphorus

The two phosphate sources investigated were sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) and di-ammonium hydrogen orthophosphate $(NH_4)_2HPO_4$, these were substituted for the potassium dihydrogen orthophosphate, (KH_2PO_4) contained in the basal FTN medium (Appendix I). A quantity of 0.33g per litre of these phosphate sources were used in the experiment. The control retained the original constitution and quantity of the KH_2PO_4 in the FTN medium.

2.2.9 Nitrogen

Two nitrogen sources were investigated by substituting them for casein hydrolysate. These included a protein; BSA and an amino acid; L-arginine. A quantity of 0.33g per litre of these nitrogen sources were used in the experiment. The control retained the original constitution and quantity of the casein hydrolysate in the FTN medium (Appendix I). BSA was filter-sterilised through an autoclaved 0.45µm membrane filter and added to the medium after autoclaving.

2.2.10 Statistical Analysis

Data obtained from *in vitro* investigation into the physiological growth parameters were analysed using a repeated measure ANOVA. This type of ANOVA is used when samples of the same characteristics are measured under different conditions (Statsoft, 2004). In this case, the fungal colony diameter is the measurement and day is the condition that changes. Fischer LSD was then selected to evaluate the level of significance. Statistica 7 software was used for all analyses.

2.3 Results

2.3.1 Collection and isolation of *T. pfeilii*

The ascocarps collected were identified morphologically with the help of local experts and identification manuals (Fig. 2.1), and the confirmatory identification was carried out using molecular analyses. The purity of the five isolates was enhanced by the methods used for preparation of the isolates to establish growth from individual hyphae. This method is comparable to the single spore fungal isolation and the pure bacterial cultures using serial dilution and spread plate methods (Brudrett *et al.*, 1996, Madigan *et al.*, 1998; Giomaro *et al.*, 2005).

Figure 2.1: Ascocarps harvested at Upington, South Africa were morphologically identified as *Terfezia pfeilii*

Seventeen bacterial contaminants were subcultured and kept for further investigations (Chapter 4). Sporulating fungal contaminants were successfully eliminated microscopically as these produced reproductive structures and spores. Four non-sporulating fungal isolates (designated A, P, D and E) were obtained form Fontana medium within 4 to 11 weeks after inoculation onto Fontana medium. At the eleventh week, it was observed that isolate P started to grow (Fig. 2.2).

Isolate P demonstrated slower growth on FTN media, having only covered 50% of the plate in 11 weeks. Growths of *T. pfeilii* on three different media were later compared and it was revealed that the fungus cannot grow on MMN but grew on both FTN medium and PDA. The growth on FTN medium was better (Fig. 2.3) than that of PDA. This could be due to the ability of the fungus to utilise nutrients contained in FTN medium better than those in PDA.

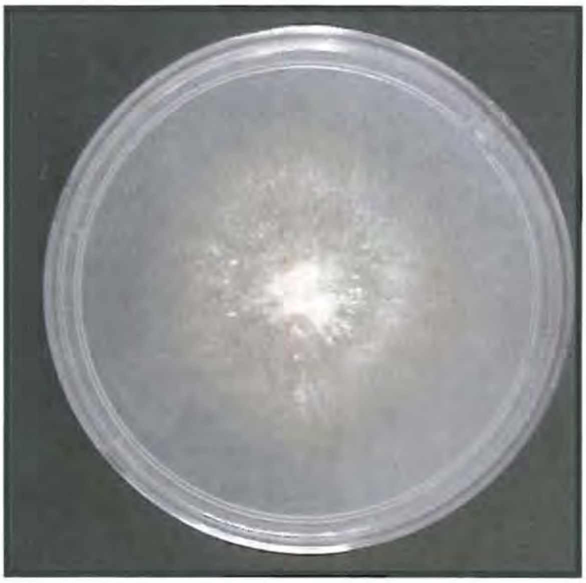

Figure 2.2: Isolate P on Fontana medium. This was isolated at the eleventh week after inoculation

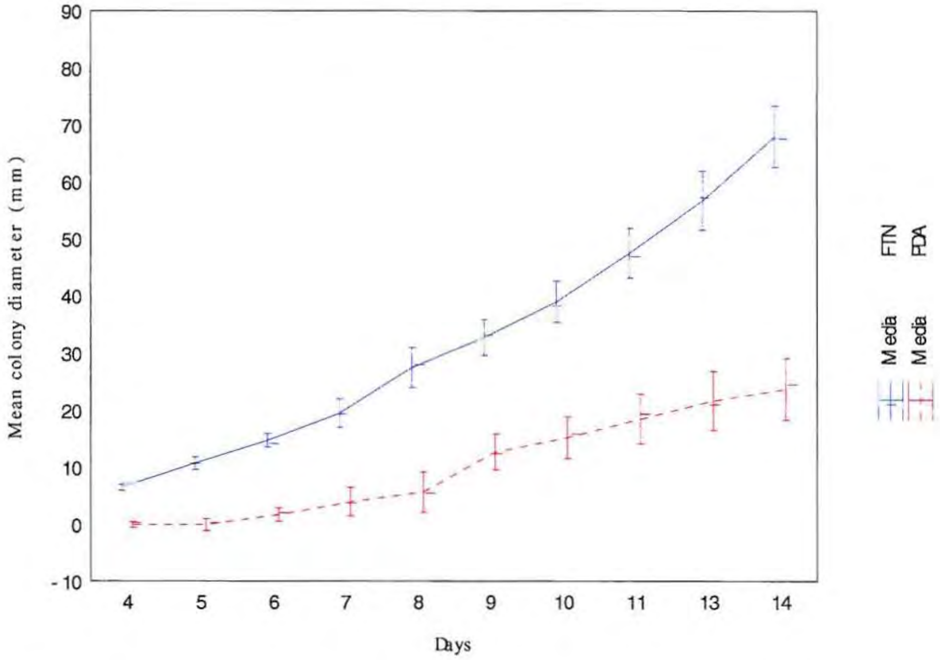

Figure 2.3: Comparison of growth of *T. pfeilii* on two different media; Fontana media (FTN) and Potato Dextrose Agar (PDA). $F_{(9,54)} = 67.110$, $p<0.05$, Vertical bars denote 0.95 confidence intervals.

2.3.2 Identification of ascocarps and fungal isolates

The identity of the 4 remaining fungal isolates and the ascocarps that were earlier identified by morphologically, were confirmed with molecular analyses (Table 2.1). Initially, the CTAB method did not give any amplification from the ascocarp material and one of the isolates (P). Optimisation of the PCR conditions and the addition of BSA enhanced amplification. After the separations of the PCR products electrophoretically, clean and clear bands of DNA of different lengths were obtained which indicated successful amplification (Fig. 2.4).

Figure 2.4: PCR amplified products using (ITS1F and ITS4) of all the fungal isolates obtained during the isolation process and the ascocarps. The PCR products were separated electrophoretically on ethidium bromide stained 1% agarose gel. Lane 1- Molecular marker (100 base pairs), Lanes 2, 3 - amplified DNA from ascocarp (S) at different dilutions of 10^{-2}, 10^{-3} respectively, Lanes 4, 5-amplified DNA from pure culture of Isolate P at different dilutions of 10^{-2}, 10^{-3} respectively, Lane 6 -amplified DNA from pure culture of Isolate E, Lanes 7, 8 -amplified DNA from pure culture of Isolates A and D respectively.

Both forward and reverse sequences were obtained by using the ITS1F and ITS4 primers. The alignment of forward and reverse sequences in BioEdit software programme (Hall, 1999) (Table 2.1) was used to confirm the sequencing result. BLAST analysis confirmed that the ascocarp and one of the fungal isolates (P) to be that of *T. pfeilii* (Table 2.1). Other fungal isolates identified were two *Fusarium* species and one *Phoma glomerata* (Table 2.1). Two closely related sequences were obtained for both the ascocarp and the mycelial culture of *T. pfeilii*.

Table 2.1: NCBI BLAST result of the fungal nucleotide sequences obatined from the isolation process. All sequences obtained after the BLAST were identified with >95% confidence.

Fungal Isolates identified	GenBank Accession number	Most significant alignment	% Identity	E value
Isolate A	AY183371	*Phoma glomerata*	98	0.0
Isolate P	AF301420	*Terfezia pfeilii*	99	0.0
Isolate D	DQ093699	*Phoma glomerata*	96	1e-124
Isolate E	AY986957	*Fusarium equiseti*	97	0.0
Ascocarp (S)	AF301422	*Terfezia pfeilii*	98	0.0

2.3.3 Physiological Growth parameters

Temperature and pH

The quickest and higest *in vivo* growth of *T. pfeilii* as measured by colony diameter was obtained at the temperature of 32°C (Fig. 2.5; Table 2.2). There was no growth at 15°C, 20°C and 37°C, so data at these temperatures were not included in the statistical analysis (the software programme does not compute the zero values). When analysed statistically, growth at the temperatures differed significantly from each other (Fig. 2.5). The results indicated that *T. pfeilii* can grow at an optimum temperature of 32°C. The optimum temperature obtained in this experiment was used as the incubating temperature for subsequent experiments.

Table 2.2: Growth (mm) of *T. pfeilii* after 12 days on Fontana medium at different temperatures. LSD – Least significant difference. Values are means of three replicates. Means followed by the same letter across the row are not significantly different at $P<0.05$.

Parameter	Temperature			
	32°C	**28°C**	**20°C**	**LSD**
Average colony diameter (mm)	76a	61b	13c	1.00

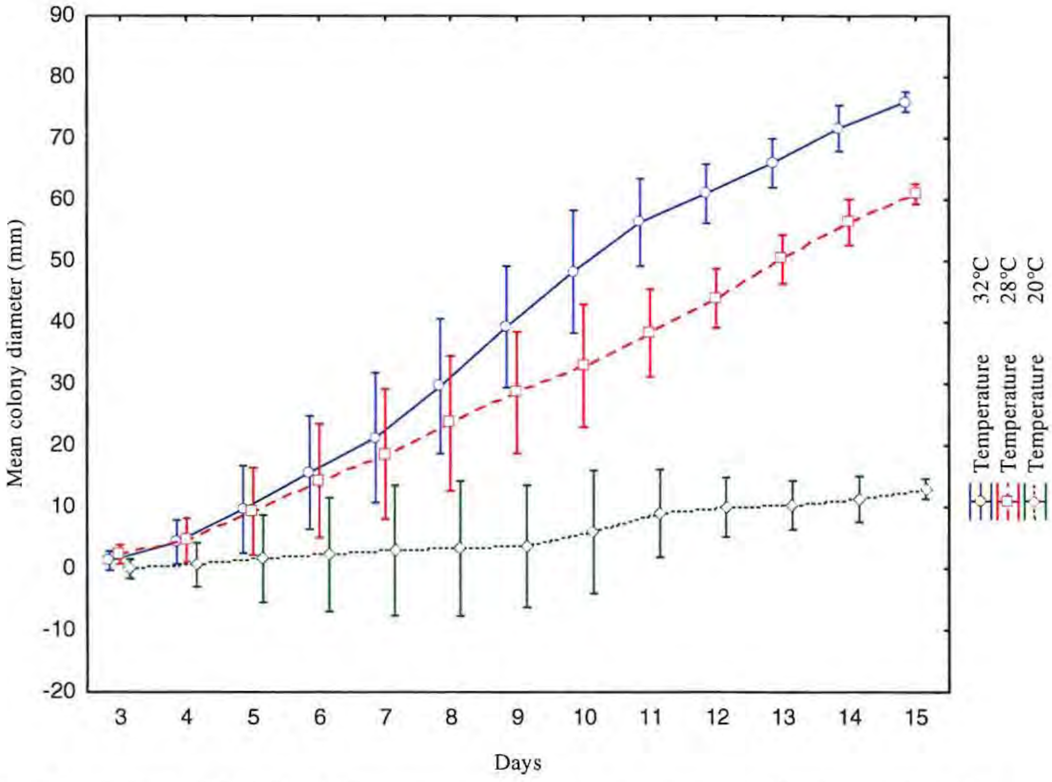

Figure 2.5: Growth of *T.pfeilii* at different temperature ranges as measured on Fontana medium for a period of 15 days. $F_{(24, 72)}=45.973$, $p<0.05$. Vertical bars denote 0.95 confidence intervals

Among the pH ranges investigated, pH 7.0 to 8.0 supported the highest growth of *T. pfeilii* on FTN medium as growths at this pH range were not significantly different from one another (Fig. 2.6). The growth at these pH values were significantly higher from the growth achieved at the lower pH of 5 and 6 (Table 2.2). A relatively low pH of 5.0 severely restricted the fungal growth (Table 2.3).

Table 2.3: Growth of *T. pfeilii* at different pH after 14 days on Fontana medium at different pH ranges. LSD – Least significant difference. Values are means of three replicates. Means followed by the same letter across rows are not significantly different at $P<0.05$.

Parameter	pH value					
	5.0	**6.0**	**7.0**	**7.5**	**8.0**	**LSD**
Average colony diameter (mm)	21.5a	69.7b	76.0c	76.0c	76.0c	1.000

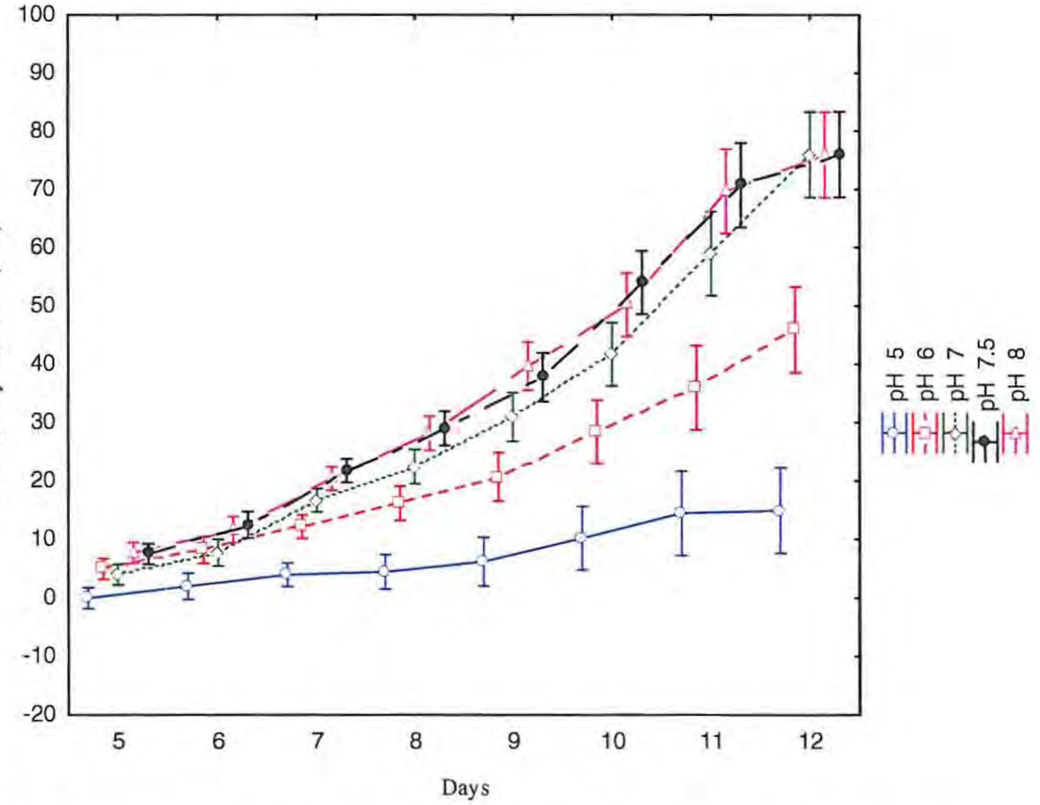

Figure 2.6: Growth of *T. pfeilii* at different pH values on Fontana medium over a period of 12 days. $F_{(28, 105)}=32.061$, $p<0.05$. Vertical bars denote 0.95 confidence intervals.

Carbon

When comparing growth on media containing different C sources, different results were obtained depending on whether growth was measured in terms of colony diameter or biomass. Growth with fructose as the C source produced more biomass than maltose whereas the opposite was recorded for colony diameter. The difference in growth as measured by colony diameter, between the glucose and sucrose C sources was not significant but significant differences were recorded in biomass (Fig. 2.8). Despite these variations, the glucose contained in the basal medium, and sucrose supported the growth of *T. pfeilii* better than any other source of carbon as shown by the statistical analysis (Fig. 2.7; Table 2.4). Fructose as a carbon source supported the least growth of *T. pfeilii* on Fontana medium.

Table 2.4: Growth of *T. pfeilii* after 12 days on Fontana medium with different carbon sources. LSD – Least significant difference. Values are means of six replicates.
Means followed by the same letter across rows are not significantly different at $P<0.05$.

Parameter	C Sources					
	Glucose	**Sucrose**	**Fructose**	**Maltose**	**Mannitol**	**LSD**
Mean colony diameter (mm)	50.0a	48.2a	33.2b	38.6ab	41.5ab	0.853
Mean dry weight (mg)	50.7c	28.7a	20.7ab	12.5b	21.7ab	0.869

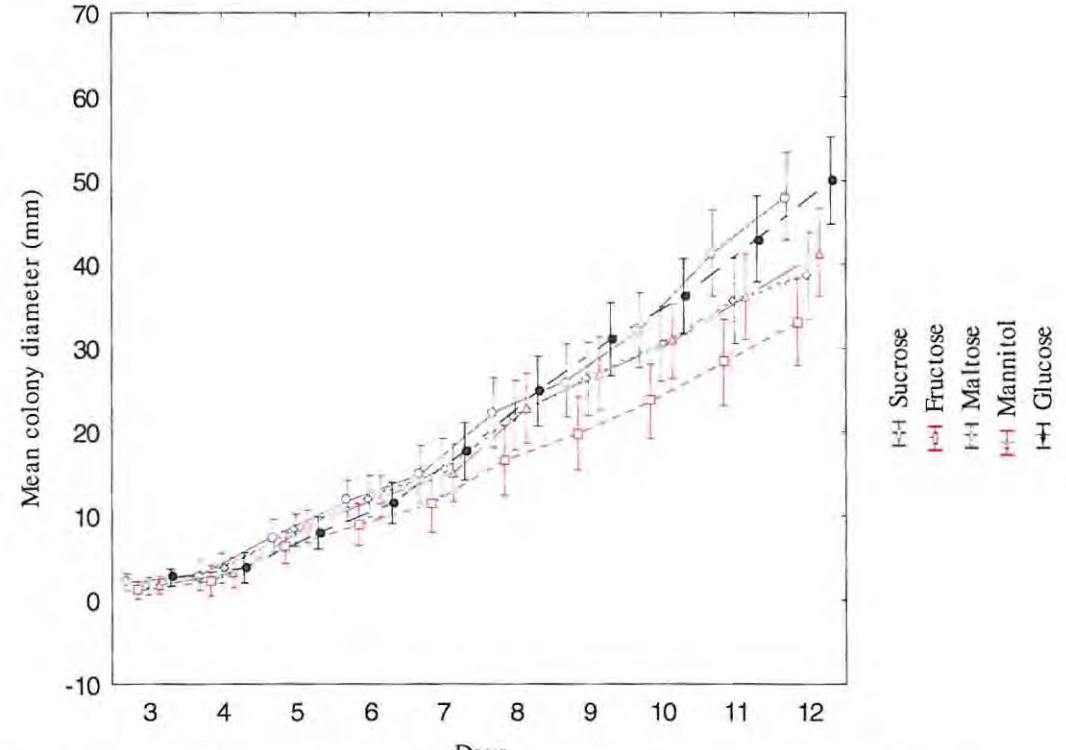

Figure 2.7: Growth of *T.pfeilii* as measured by colony diameter on Fontana medium with different carbohydrate sources for a period of 12 days. $F_{(36, 225)}=6.3548$, $p <0.05$. Vertical bars denote 0.95 confidence intervals.

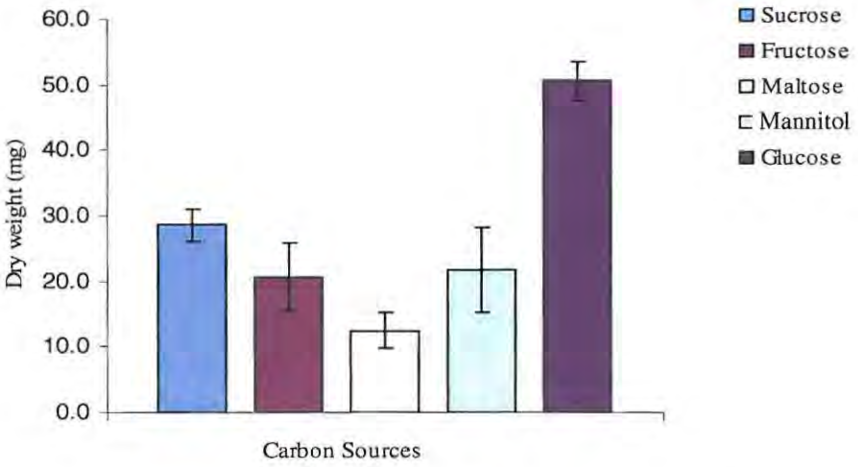

Figure 2.8: Biomass, as measured by dry weight, of *T.pfeilii* grown on Fontana medium with different carbohydrate sources. $F_{(4, 25)} = 11.986$, $p<0.05$, Values are means of 3 replicates. Vertical bars denote 0.95 confidence intervals.

Phosphorus

When analysing the growth of *T. pfeilii* on different phosphorus sources, contradictory results were noted when comparing growth as measured by colony diameter and biomass as measured by dry weight (Fig. 2.9; Fig. 2.10; Table 2.5). In terms of growth, the statistical analysis revealed no significant difference between the two phosphate groups tested and the control at the end of the experiment (Fig. 2.9; Table 2.5). However, dry weight measurements contradicted these results. The dry weight results indicated that greater biomass was generated with the control experiment than the two sources; sodium phosphate and ammonium phosphate (Fig. 2.9; Table 2.5)

Table 2.5: Growth of *Terfezia pfeilii* after 13 days on Fontana medium with different phosphate sources LSD – Least significant difference. Values are means of six replicates. Means followed by the same letter across rows are not significantly different at P<0.05.

Parameter	Phosphate source			
	Potassium phosphate	**Na phosphate**	**Ammonium phosphate**	**LSD**
Mean colony diameter (mm)	50.0a	53.8a	55.0a	0.836
Mean dry weight (mg)	50.7b	33.5a	34.5a	0.892

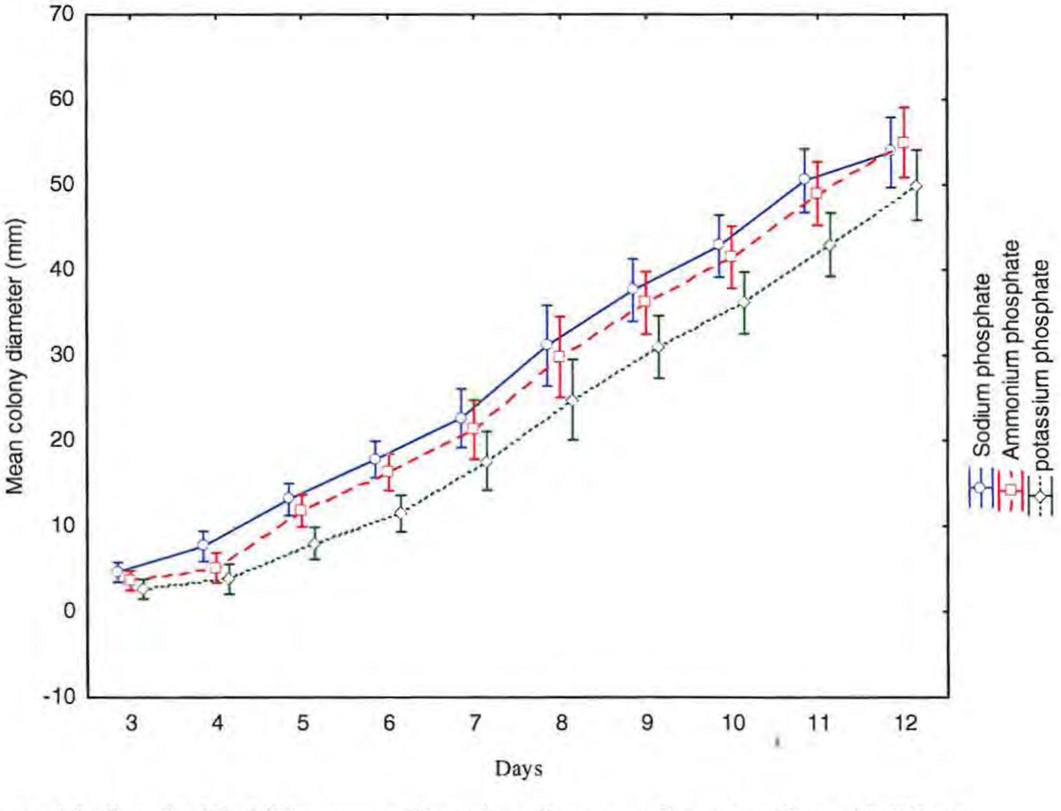

Figure 2.9: Growth of *T.pfeilii* as measured by colony diameter on Fontana medium with different phosphate sources for a period of 13 days. $F_{(18, 135)}=1.3306$, $p<0.05$. Vertical bars denote 0.95 confidence intervals.

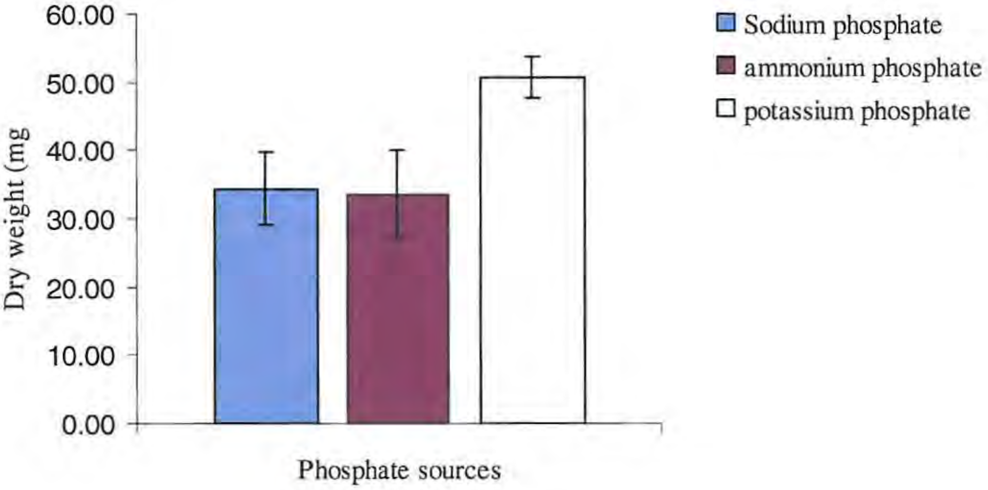

Figure 2.10: Biomass, as measured by dry weight of *T. pfeilii* growth on Fontana medium with different phosphate sources. $F_{(2,15)}=3.5252$, p<05. Values are mean of 3 replicates Vertical bars denote 0.95 confidence intervals.

Nitrogen

Statistical analysis of the data obtained from nitrogen experiment indicated that BSA supported the best growth of *T. pfeilii* on Fontana medium both in terms of biomass and colony diameter (Fig. 2.11; Fig. 2.12; Table 2.6). However, different results were obtained when comparing growth and biomass on media modified wih L-arginine. The growth on L-arginine as the source of nitrogen was significantly higher than that of the control and equivalent to that achieved on BSA but there was significant higher in biomass when compared to control (Fig. 2.11; Fig. 2.12; Table 2.6). BSA on the other hand gave a significantly higher biomass than both the control and L-arginine.

Table 2.6: Growth of *T. pfeilii* after 13 days on Fontana medium with different nitrogen sources. LSD – Least significant difference. Values are means of six replicates. Means followed by the same letter across rows are not significantly different at $P<0.05$.

Parameter	Nitrogen source			
	Casein hydrolysate	**BSA**	**L- Arginine**	**LSD**
Mean Colony diameter (mm)	56.5a	76.0b	76.0b	1.000
Mean dry weight (mg)	50.7a	62.2b	49.7a	0.815

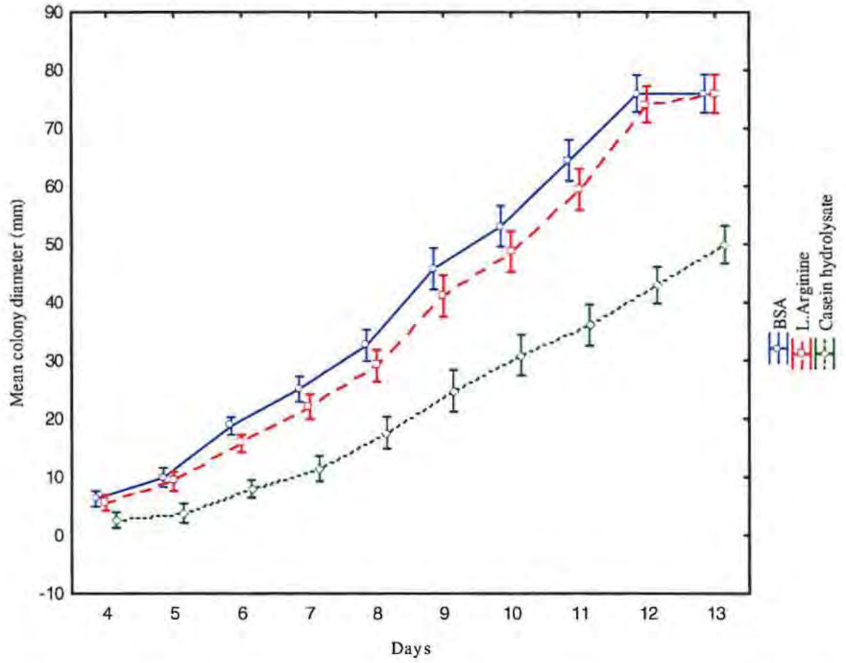

Figure 2.11: Growth of *T.pfeilii* as measured by colony diameter on Fontana medium with different nitrogen sources for a period of 13 days. $F_{(18,90)}=48.173$, $p<0.05$. Vertical bars denote 0.95 confidence intervals.

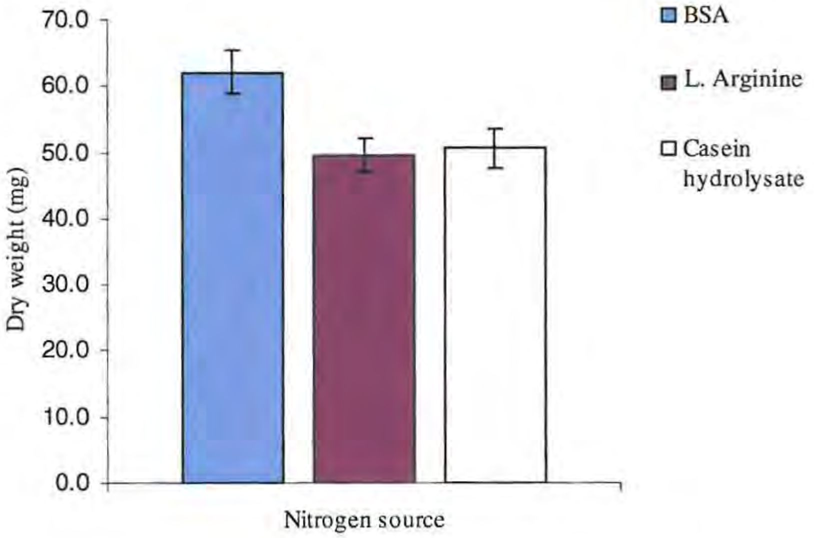

Figure 2.12: Biomass, as measured by dry weight of *T. pfeilii* grown on Fontana medium containing different nitrogen sources. $F_{(2,\ 15)}=5.4451$, $p<0.05$. Values are means of 3 replicates. Vertical bars denote 0.95 confidence intervals. L.ARG. = L-Arginine.

2.4 Discussion

Due to the level of similarity among different groups of truffles such as *Tuber magnatum*, *T. borchii* and *T. puberulum* at some stages in their life cycles, it is difficult to use morphological means for their identification (Urbanelli *et al.*, 1998). Prior to the advent of molecular analysis, the problems encountered in the use of morphological methods to accurately identify pure cultures isolated from fruiting bodies or ectomycorrhizas of truffles led to the use of some other methods such as the "reinfection" of the host plants (mycorrhizal synthesis experiments). The isolate could then be secondarily identified by identification of its resulting mycorrhiza to confirm the identity of the isolate (Mischiati and Fontana, 1993). Morphological methods alone are not always confirmatory and mycorrhizal synthesis experiments are slow and rely on a secodary method of identificaton to confirm the success of the synthesis (Mischiati and Fontana, 1993). Molecular methods have an advantage mostly because of the rapidity and ability to reliably identify from complex samples such as degrading living tissues and delicate vegetative stages of various ECM fungi (Smith and Read, 1997; Horton and Bruns, 2001).

An advantage when working with ECM fungi is that some of them can be grown artificially on media as opposed to AM fungi (Smith and Read, 1997). The task of isolating the pure culture of *T. pfeilii* was difficult due to contamination and the slow initial growth of the vegetative mycelia from the ascocarp on FTN medium. Younger fruit bodies (immature spores) were selected for the isolation because of their undifferentiated fresh tissues and the reduced contamination level (Molina and Palmer, 1982).

Media additives especially antibiotics such as chloramphenicol could have removed some of the bacterial contaminants (Pinkas *et al.*, 2000), but this was not done because the bacterial contaminants were further investigated in later studies (Chapter 4). The purity of the fungal isolates was essential to the eventual successes of the further studies of physiological growth parameters, *in vitro* mycorrhizal synthesis, MHB and mycorrhizal syntheses experiments (Wong and Fortin, 1989; Garbaye, 1994; Brundrett *et al.*, 1996).

This study confirmed that FTN medium is a good medium for the isolation of *T. pfeilii* (Roth-Bejerano *et al.*, 2004). Apart from the initial slow growth from the gleba, the use of the Fontana medium was successful in promoting the proliferation of the hyphae. Growth was obtained between one and two weeks, a situation which is unobtainable in most other truffles species such as *Tuber magnatum* which takes approximately 30 days to produce a colony diameter of 50mm on a good medium (MMN) (Mischiati and Fontana, 1993). The advantages of the isolation and the fast growth of the *T. pfeilii* on FTN medium are enormous, but the most important one is the availability of the pure culture of any quantity at any time of the year.

The molecular analysis (ITS) was used to confirm both the identities of the samples and isolates. Percentage identity of 98% and 99% obtained from the BLASTing of the nucleotide sequences confirms both the pure culture P and ascocarp S as *T. pfeilii* pure culture and ascocarp of *T. pfeilii*, respectively.

The quantity of available mycelial cultures that can be produced within a short period of time is crucial for physiological and biochemical studies of culturable mycorrhizal fungi (Mischiati and Fontana, 1993). Similar to other truffle species that produce ascocarps in certain period of the year (Hall and Wang, 1998), *T. pfeilii* ascocarps are are not available throughout the year, a situation which invariably necessitates a need for other forms of vegetative materials such as *T. pfeilii* mycelial cultures that can be utilised for research and commercial purposes throughout the year.

In their symbiotic forms, ECM fungi perform their roles in nutrient uptake/utilisation in relation to their ability to co-exist and compete with other rhizospheric organisms (Smith and Read, 1997). Therefore, it must be realized that the ability of ECM fungi to utilise various nutrients on general media cannot be totally extrapolated with the situations in the field.

Only the colony diameters were measured for the temperature and pH experiments. Optimum temperature of 32°C obtained was a reflection of the usual high environmental temperatures experienced in the Kalahari Desert as indicated in Table 1.1. The high average maximum temperature of 32°C to 36°C precedes the appearance of ascocarps between the months of January and March in this area. This result is in accordance with the findings of Daza *et al.* (2006) where the optimum temperature recorded for the *in vitro* cultivation of *Amanita caesarea* corresponded to the environmental temperature in the field. Though fruiting bodies cannot be produced *in vitro*, high temperature may be crucial to the development and formation of the fruiting bodies. In the pH experiment, the optimum pH was within the range of 7.0 to 8.0. This is higher than the average pH obtained from the soil analysis collected from the field (Table 1.2) which was between 5.48 and 6.41. This corresponds to soil analysis results in a study by Hung and Trappe, 1983, where there was no relationship between the pH at which ECM fungal cultures (such as *Amanita muscaria, Hebeloma crustuliniforme, Cenococcum geophilum*) grow *in vitro* and the pH of the habitat (USA and Finland) where the ECM fungi were collected. This result could also indicate that non buffered media were more suitable for the growth of *T. pfeilii* because as the fungus grows, pH of the media is likely to be changed due to the release of the metabolites that may have positive effects on growth.

The different results recorded in the carbon experiment (colony diameter versus dry weights) (Fig. 2.7; Fig. 2.8) indicated that carbon has a great influence on morphology of the fungal growth (Ceccaroli *et al.*, 2001; Daza *et al.*, 2006). This probably means that fructose allowed more growth of the fungus into the agar than encouraging horizontal proliferation. However, despite the disparity recorded between the growth and biomass (Fig. 2.7; Fig. 2.8), sucrose and glucose C sources proved to support better overall growth of *T. pfeilii* than other carbon sources tested. Though mannitol has been reported as the main soluble carbohydrate in ascomycetes, the poor growth attained by *T. pfeilii* on this carbon source could be an indication of the insufficient production of enzymes of the mannitol cycle such as mannitol phosphatase (Ceccaroli *et al.*, 2001).

There are differences with respect to radial growth versus biomass results recorded in the phosphate experiments (Fig. 2.9 and Fig. 2.10, Table 2.5). In some studies, ammonium compounds have been widely used as an inorganic form of nitrogen to investigate the nitrogen utilisation of ectomycorrhizal fungi (Keller, 1996; Baar, *et al.*, 1997; Yamanaka, 1999). However, in this study, ammonium phosphate was used as the source of phosphate rather than a source of nitrogen. This could have affected the outcome of this experiment because this compound could serve as both phosphate and nitrogen sources. This can lead to increased nitrogen concentrations of the Fontana medium that already had peptone and casein hydrolysate as sources of nitrogen. Nevertheless, the increased growth (colony diameter) recorded for sodium phosphate and ammonium phosphate (Fig. 2.9; 2.10) was an indication that these two phosphate sources encourage better hyphal proliferation which has a potential advantage during colonisation of the root by the hyphae (Garbaye, 1994). Comparing the phosphate content of FTN media to that of the habitat (Table 1.2), the higher P value of the latter is an indication that more phosphorus might be needed for the optimal growth of *T. pfeilii in vitro*. However, this kind of assumption should be carefully extrapolated because mycorrhizal fungi transfer most of the phosphorus absorbed from the soil to their host plant (Smith and Read, 1997). Since there is no host plant in the *in vitro* germination of the mycelia on petri dishes, addition of more phosphorus might as well deter the growth of the fungus.

In the nitrogen experiment, it is necessary to mention that casein hydrolysate was not the only nitrogen source in the FTN. Therefore, the effects produced by BSA, L-arginine and casein hydrolysate on the growth and biomass of *T. pfeilii* were in addition to that of nitrogen in the peptone (can also be a source of carbon), which was kept constant during the experiment. BSA increased the growth of *T. pfeilii* more than any other nitrogen sources tested. The growth was significantly higher from the basal nitrogen source both in terms of the growth and biomass (Fig. 2.11; Fig. 2.12; Table 2.6), while a significant higher growth was only observed in the mycelial dry weight when compared to the growth on L-arginine as a nitrogen source. This is in accordance with the study conducted by Daza *et al.* (2006) where BSA, in terms of hyphal proliferation, proved to be a good source of nitrogen for some strains of *Amanita caesarea.* Such enhancing results were also reported by Baar *et al.* (1997) where glycine supported the growth of *Coltricia perrennis* and *Laccaria bicolour.* The ability of *T. pfeilii* to grow well on BSA as a nitrogen source could be an indication that the fungus could compete efficiently with saprophytes under a natural condition (Abuzinadah *et al.,* 1986). The potential of *T. pfeilii* to grow well on BSA could also be related to the possible production of a specific protein enzyme. This theory is supported by findings of Leake and Read (1990) where *Hymenoscyphus ericae* released very high concentration of proteinase within 36 hours of inoculation onto a medium containing BSA as a nitrogen source. The production of this enzyme was attributed to the presence of the BSA as a substrate that regulates the production of this enzyme in the fungus. Such scenarios could improve the ability of the fungus to compete with other microorganims for polymeric nitrogen sources (Leake and Read, 1990).

The lower values of C, Ca, Mg and Na (absent) in FTN medium (Appendix I) compare to these values in the analysed Kalahari soil (Table 1.2) is an indication that FTN medium needs further optimisation. In addition, the low C content of the Kalahari soil may not be able to meet the entire C requirement of *T. pfeilii*, therefore, necessitates the reliability of the fungus on the host plants for C.

In summary, the optimum conditions founf in this study for the growth of *T. pfeilii in vitro* on FTN medium are a pH of 7.0, 7.5 and 8.0, incubation at a temperature of 32°C, glucose as carbon source, dihydrogen orthophosphate as the phosphate source and BSA as the source of nitrogen. Comparing the effects of all the different nutrients studied on the growth of *T. pfeilii*, BSA had the most significant effect (Fig. 2.11; Fig. 2.12).

The difficulty involved in sourcing casein hydrolysate for laboratory use due to governmental regulations regarding the importation of animal proteins, has indeed necessitated a need for an alternative source of nitrogen for FTN medium. This same restriction sometimes applies to BSA as well but not L-arginine. However, *T. pfeilii* may possibly utilise other forms of organic nitrogen in the field and this may be an indication of the capability of its saprophytic existence because this type of nitrogen source mostly comes from decomposing organic matter. The results obtained in this study will provide insights into the field requirements for the growth of *T. pfeilii* and also help in improving the *in vitro* mycorrhizal synthesis conditions with potential host plants. Glucose and sucrose have been previously reported as forms of carbon that can be transferred by the host plants to the mycobionts (France and Reid, 1988). Caution must however be taken in expecting this same result for all *Terfezia pfeilii* cultures because of the possible existence of different strains of this mycorrhizal fungus. Previous studies have confirmed that different strains of the same species of culturable mycorrhizal fungi such as *Amanita caesarea* and *Tuber borchii* exhibit differences in their requirements for temperature, pH, carbon and nutrients (Hung and Trappe, 1983; Saltarelli *et al.*, 1999; Ceccaroli *et al.*, 2001; Daza *et al.*, 2006). The existence of differences between different strains of *T. pfeilii* as suggested by Roth-Bejerano *et al.* (2004) has not been investigated in this study. A broader study on different strains of South African *T. pfeilii* is necessary to investigate the possible existence of individual physiological growth parameters. Another interesting experiment would be to investigate the effects of different concentrations of the nutrients on the growth of *T. pfeilii in vitro* (Baar *et al.*, 1997), which would help in the quantification of nutrients needed for various mycorrhizal experiments.

Finally, having obtained a mycelial culture of *T. pfeilii* and improved on the ways of producing this culture, it will be easier to obtain the pure culture within a shorter period of time for use in mycorrhizal synthesis experiments. Since identification of the culture is very important, the development of a reliable and rapid method for the identification of pure cultures is needed. This will reduce the total length of time and cost needed to achieve results in mycorrhizal synthesis experiments.

CHAPTER THREE

3 Development of a Rapid Identification of *Terfezia Pfeilii* Using Molecular Techniques and an Investigation into the Reclassification

3.1 Introduction

Several methods have been proposed and used for the identification of ECM fungi. The most commonly used methods are based on morphology and field survey of fruiting bodies of ECM fungi (Horton, 2002; Martin and Rygiewicz, 2005). These methods have provided broad knowledge on the diversity and ecology of ECM fungi, especially ECM root tips, which were often disregarded (Agerer, 1987-2002; Trappe, 1979; Ingleby *et al.*, 1990). However, the accuracy and reliability of morphological methods in the identification of ECM fungi have been of concern (Kagan-Zur *et al.*, 2001, Horton and Bruns, 2001). Morphological characteristics such as colour and shape have been used for rapid sorting of species because they are easily observable (Agerer, 1987-2002; Ingleby *et al.*, 1990). The complete identification of species with this method is only possible with a well characterised system that requires many details and extensive database observation, which are time consuming. The more detail that is required proportionally increases the length of time required for final identification, resulting in a reduced number of samples that can be examined. Subsequently, the more time it takes to identify a sample, the more prone the system is to errors (Horton and Bruns, 2001). This is the main bottle-neck of morphological identification methods of ECM fungi. Another problem is the possibility of root colonisation by more than one mycorrhizal fungus as indicated in some plants that can play hosts to multiple mycobionts (Molina *et al.*, 1992).

The advent of molecular technology (PCR) has re-established fresh interest in the study of ECM fungi because of the significant solutions it has provided for the thorough identification of various species of ECM fungi (Horton and Bruns, 2001). Different stages of mycorrhizal development that would otherwise be slow, complicated or impractical to identify by morphological or cultural based methods have been investigated and elucidated using molecular techniques. Studies on evolution, classification and taxonomy of ECM have been greatly improved by molecular methods (Bruns and Bidartondo, 2002).

In general microbial ecology, molecular analysis is mostly used for both identification and quantification. However, the quantification aspect does not always apply to most mycorrhizal fungi. This is due to the macroscopic aggregates they form during their life cycle which can be counted or weighed (Horton and Bruns, 2001). Meanwhile, advanced molecular techniques can still be used in some cases to quantify some mycorrhizal fungi for some particular purposes such as the investigation of spatial distribution of below ground ECM fungi (Guidot *et al.*, 2002).

The success of molecular technology in ECM fungal studies has been widely attributed to the ability to influence regions on and around some ribosomal RNA genes and spacers (Lafranco *et al.*, 1998). These regions are important because of the distinct characteristics they possess; most especially they can be reproduced exponentially using PCR (Mullis and Faloona, 1987; Horton and Bruns, 2001).

Prior to the amplification of the genomic DNA by PCR, it is necessary to obtain pure DNA in order to eliminate possible inhibitors of this process. Different biochemical and cellular composition of different samples do affect the quality of DNA extracted and this has necessitated the modification of routine extraction protocols for fungi (Lafranco *et al.*, 1998).

3.1.1 Polymerase Chain Reaction

PCR is the amplification of a specific region of DNA sequence of an organism which requires the reactions of a buffer (containing Tris, potassium chloride and magnesium chloride), the four different deoxyribonucleotides, forward and reverse primers and a thermostable DNA polymerase (Lafranco *et al.*, 1998, Mello *et al.*, 2002). PCR has the ability to amplify from a wide variety of samples including minute and degraded quantity of tissues (Bruns and Bidartondo, 2002, Sejalon-Delmas *et al.*, 2000).

Modifications to the PCR technique are used to obtain DNA needed for other molecular techniques (Lafranco et a, 1998). A type of modification of PCR is nested PCR. Nested PCR is a two-stage type of PCR which involves the initial amplification with the universal primers and a second round amplification with more specific primers (Martin and Rygiewicz, 2005). The specific primers have shorter products and their sequences located within the products (sequence) generated from the first PCR (Zeze *et al.*, 1998). Although nested PCR increases the sensitivity, it may give false-positive results due to contamination from the first round PCR (Diaz and Fell, 2005).

3.1.2 Internal Transcribed Spacer

A region called the Internal Transcribed Spacer (ITS) has been a good target for the PCR amplification of the DNA in mycorrhizal studies (Gardes *et al.*, 1991; Gardes and Bruns, 1993; Lafranco *et al.*, 1998). The ITS region is the most widely used nuclear region for the molecular analysis of mycorrhizal fungi exhibiting important characteristics such as high copy numbers and presence of conserved sequence. It is situated between the small subunit (18S) and the large subunit (28S-LSU) ribosomal RNA (rRNA) genes (Horton, 2002) (Fig. 3.1). One of the greatest advantages of this region in molecular analyses of ECM fungi is its high variability compared to coding genes. The variability is mainly due to the presence of the two non-coding regions which have between them the 5.8S rRNA subunit (Bruns and Bidartondo, 2002). The size of these three regions is in the range of 650 to 900 base pairs (bp) for most fungi (Horton and Bruns, 2001). This region has been exploited to design both universal primers (such as ITS1 and ITS4) and fungal specific primers (such as ITS1F and ITS4B) (White *et al.*, 1990; Gardes and Bruns, 1993).

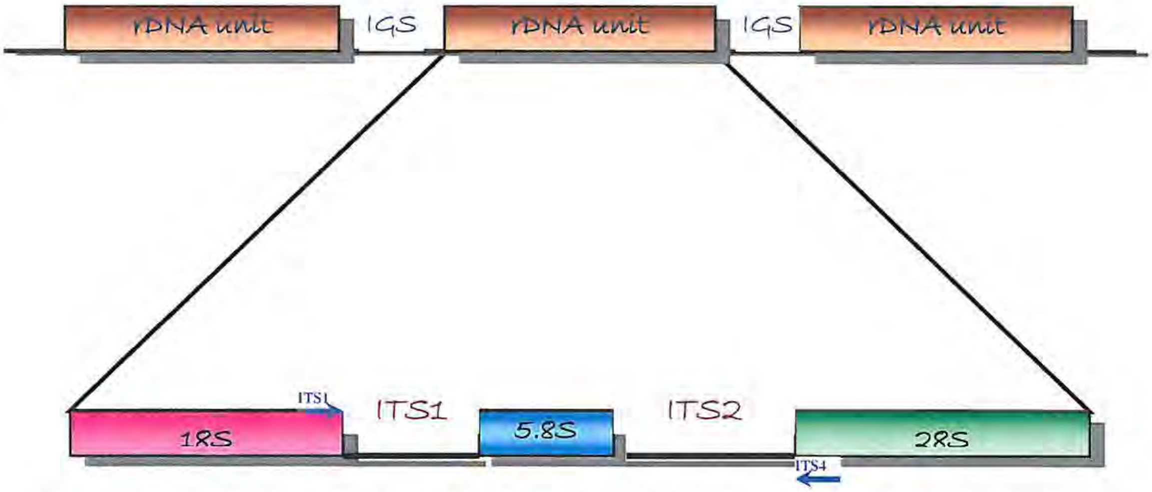

Figure 3.1: Schematic representation of the repeated units of rDNA showing the subunits that constitutes the rDNA (adapted from Lafranco *et al.*, 1998)

Primers (oligonucleotides) are short single-stranded DNA fragments that bind, on recognition, to specific complimentary strands on the genome. They are usually designed in opposite directions (forward and reverse) using the two flanking sequences of the target DNA template (Lafranco *et al.*, 1998) as illustrated in Fig. 3.1 where the two ITS regions were the target section of the DNA. Several specific primers have been designed to enhance the rapid identification of mycorrhizal fungi at various stages of their life cycles. Examples include those for *Tuber magnatum* and *Tuber borchii* (Horton and Bruns, 2001; Amicucci *et al.*, 1998; Mello *et al.*, 1999; Douet, *et al.*, 2004). Some factors have been highlighted as necessary for the design of good primers. According to van Tuinen *et al.*, 1998, such factors include the length of the primer, which should be between 18 to 28 nucleotides long, the even distribution of all four nucleotides, avoidance of polypurine/polypyrimidine stretches, avoidance of complementary 3′ ends of the primers and equal or similar melting temperatures (Tm) for the primer pairs. Computer software programmes such as Gene Runner and Primer3 Output have been developed for the design of primers to replace the usual manual method (Hastings Software, 1994; Rozen and Skaletsky, 2000).

3.1.3 Protocols in molecular analysis of Ectomycorrhizal fungi

The discovery of PCR has been associated with and followed by other molecular techniques that essentially rely on the PCR amplified products. Protocols such as nested PCR, the use of fragment length to identify interspecific variations, Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), microsatellites and sequencing have been developed as PCR based techniques (Lafranco *et al.*, 1998).

RAPD is a method is used in analysing polymorphic DNA differences between strains. Polymorphism can be as a result of insertions and deletions (Saunders and Owen, 1998; Bergero, 2000). However, reproducibility has been a major cause of concern for this method (Saunders and Owen, 1998).

Another PCR based molecular technique is RFLP. This is simply the side by side comparisons of the products of restriction endonuclease enzyme digests of the PCR products to assist in identifying samples to genus level or sometimes species level. The use of this method has been very popular because of its rapidity and cost effectiveness in analysing samples. However, the use of this method alone has its limitations in the area of intraspecific variation among species (Gardes *et al.*, 1991). This was highlighted in a study on a desert truffle which resulted in different band patterns recorded after the digestion of *Terfezia boudieri* with *HinfI* (Aviram *et al.*, 2004).

3.1.4 Truffles and molecular analyses

The introduction of molecular techniques has assisted in resolving the complexities involved in the biology and ecology of truffles (Mello *et al.*, 2002). The high commercial interests in truffles and the morphological similarities exhibited by some truffles of varying commercial values has spurred the search for confirmatory methods of identification that are more precise and reliable. For both commercial and research purposes, truffles need to be properly identified especially at the three significant stages of their life cycles: the mycelial stage, the mycorrhizal stage and the fruit body stage (Amicucci *et al.*, 1998). As a result of their hypogeous nature, it is difficult to actually characterise truffles morphologically during the developmental stages of their life cycles, for example fruit bodies are with immature spores and mycorrhizal tips are in early developments (Lacourt *et al.*, 2002).

Truffles species can appear identical in morphology, but these similarities do not apply to their commercial values. An example of this is the case of *Tuber melanosporum*, *Tuber brumale* and *Tuber indicum*, which all share similar morphological characteristics in most part of their life cycles, but *T. indicum* is inferior to both *T. melanosporum* and *T. brumale* while *T. brumale* is also inferior to *Tuber melanosporum* in terms of economic values (Douet *et al.*, 2004). Thorough identification methods are therefore needed to eliminate the mistakes, risks and fraud associated with the sales, eating and studies of the various forms of the truffles (inoculums, inoculated plants and ascocarps) (Amicucci *et al.*, 1998, Mello *et al.*, 1999).

The use of molecular techniques in the identification of truffles has not been problem-free. Issues of contaminating microorganisms and inhibitors of amplification as well as the high cost of molecular analysis have slowed down the pace of success in this area. PCR inhibitors such as high concentrations of phenolics, melanine and some polymers have been identified in truffles at various stages of development (Sejalon-Delmas *et al.*, 2000; Douet *et al.*, 2004). Most of these PCR inhibitors are associated with the root of the host plants. Another problem associated with the investigation of mycorrhizal association of truffles using PCR is the capability of the commonly used universal primer pair, ITS1F and ITS4 to amplify the ITS regions from other contaminating fungi colonising the roots as well as those of some plants species (Gardes and Bruns, 1993; Bruns and Bidartondo, 2002). Molecular analysis of truffles had undergone a series of modifications with cost and time now playing a major role in the selection and use of any particular molecular technique. Presently, one of the most widely used methods in the identification of truffles is the design and use of specific primers in PCR which has been adjudged fast, reliable and economical (Gardes and Bruns, 1993; Mello *et al.*, 1999; Amicucci *et al.*, 2001).

3.1.5 Molecular phylogeny/reclassification of *T. pfeilii*

Phylogeny has been described as the evolutionary record of an assemblage of entities (Harrison and Langdale, 2006). Recent advances in molecular biology have shifted the attention from the use of morphological characters to molecular characters for phylogenetic analyses (Hall, 2001). Submitted sequences in the GenBank database can now be used to examine phylogenetic relationships within and among species of different organisms (Hall, 2001). Diez *et al.*, (2002), investigated the phylogeny of desert truffles using molecular data. Their study provided insight into the evolution of desert truffles as they were able to confirm the earlier morphological grouping of *Terfezia* species and *Tirminia* species in the same clade using molecular phylogenetics. However, in their phylogenetic analyses, the omission of *Terfezia pfeilii* left a gap in their conclusion. This gap was later bridged by the study conducted by Ferdman *et al.*, (2005) which included a phylogenetic analysis of *Terfezia pfeilii* using the ITS and LSU DNA regions. Other sequences used in their analysis included closely related samples such as *Choiromyces echinulatus, C. venosus, C. alveolus, T. boudieri, T. claveryi,* and *Mattiromyces terfezoides*. After using the Neighbour Joining (NJ) and Maximum Parsimony (MP) methods of the phylogenetic reconstruction, *T. pfeilii* was found to fall outside the *Terfezia* clade. It was therefore proposed that since *T. pfeilii* does not belong to the same monophyletic group as other *Terfezia* species, it should be renamed as another genus and *Kalaharituber* was suggested. Samples of *T. pfeilii* used for the study by Ferdman *et al.,* (2005) were only from Botswana. No samples from South Africa were included.

This study focussed on the development of a rapid molecular technique that can be used to identify, monitor and evaluate various studies on *T. pfeilii* such as classification and mycorrhizal synthesis experiment (Chapter 5), of *T. pfeilii*. The molecular technique in question employs the advantages of PCR to design specific primers that can hasten and reduce the cost of identification of *T. pfeilii*. Also, the proposed reclassification of *T. pfeilii* as *Kalaharituber pfeilii* was investigated using South African samples by molecular phylogenetic approaches.

3.2 *Materials and Methods*

3.2.1 Initial identification of samples

All samples were first identified morphologically prior to molecular analyses (Chapter 2). Ascocarps and mycelial cultures were identified by molecular methods (Section 2.2.2). The CTAB method was used to extract from ascocarps and the mycelial culture as discussed in Section 2.2.2.

Prior to the PCR, the extracted genomic DNA fragments were separated electrophoretically in 1% agarose gel containing 0.5μg/ml ethidium bromide and visualised using Kodak DC 120 gel imaging system to check for the quality of DNA extracted. PCR and sequencing protocols were as described previously (Section 2.2.2).

3.2.2 Alignment of sequences

Alignment was used to compare and establish the relationships between the nucleotide sequences of the fruiting body and the pure culture of *T. pfeilii*. ClustalW internet software programme (Thompson *et al.*, 1994) was used for the alignment.

3.2.3 Design, specificity and sensitivity of the primers

Sequences obtained from the amplification of ITS1F and ITS4 primers were compared to deposits in the GenBank database. Having obtained 98% and 99% identity to the isolates of *T. pfeilii* with Accession numbers AF301420 and AF301422 in the GenBank (Section 2.3.2; Table 2.1), the two sequences were aligned and used for the design of the new primers.

The Gene Runner software programme (Hastings Software, 1994) was used for the design of new primers. Both forward and reverse primer pairs were designed considering factors such as the melting temperature, non-complementarity of the 3' ends, the G+C (between 40 - 60%) contents, non-occurrence of secondary structures, absence of cross hybridization and the length (18 -28 nucleotides) of the oligonucleotides (van Tuinen *et al.*, 1998). The primers were synthesised by Integrated DNA Technologies (IDT).

Primers were tested on both ascocarp and pure culture of *T. pfeilii* to confirm their efficiency. The PCR products were separated electrophoretically and visualised on an ethidium bromide stained 1% agarose gel to determine the sizes of the product. The primers were tested for specificity on a variety of samples that included the ascocarp and pure culture of *T. pfeilii* and the contaminating fungi that associated with the fruiting body; previously identified as *Fusarium equiseti* and *Phoma glomerata* (Section 2.3.2; Table 2.1)

3.2.4 PCR conditions

The PCR conditions were optimised in a final volume of 50μl that consisted of 1.25 units of Promega *Taq* DNA polymerase- (Cat.# M830B), 5μl of Promega 10x buffer, 0.875mM of Promega $MgCl_2$, 0.2mM of Promega dNTP mix, 32.25μl of autoclaved double distilled water, 0.4μM of both forward and reverse primers (newly designed), 2μl of genomic DNA and (BSA-3μl of 3mg/ml). The PCR was performed with a BIO-RAD MJ Mini Personal Thermal Cycler that allowed temperature optimisation of the PCR conditions; under cycling conditions that consisted: 1 cycle of initial denaturing at 94°C; 30 cycles of 1 min at 94°C for denaturing, 30s at 55°C-annealing, 2 min at 72°C and final elongation of 72°C for 5 min. The PCR products were separated electrophoretically and visualised on ethidium bromide-stained 1% agarose gel. PCR were also carried out at different concentrations (3μl of 3mg, 5mg, 7mg and 10mg per 10ml) of BSA to determine the BSA concentration that works best for the amplification.

3.2.5 Phylogenetic reconstruction

Sequencing and editing

The forward and reverse ITS sequences of *T. pfeilii* earlier obtained from the ascocarp (isolate S) and pure culture (isolate P) (Section 2.3.2) were assembled, checked and edited using Sequencher^{TM} version 4.2.2 (Gene Codes Corporation). Other ITS sequences including that of *T. pfeilii* used by Ferdman *et al.*, (2005) in their analyses, were obtained from the GenBank (Table 3.1) and aligned with *T. pfeilii* sequences generated in this study. All sequences were automatically aligned using ClustalW internet software (Thompson, *et al.*., 1994).

After alignment, sequences were exported into MacClade version 4.06 (Maddison and Maddison, 2000) for more alignment using manual method. The matrix was rechecked and modifications made manually to minimise the number of nucleotide differences among the sequences. The aligned matrix of the ITS sequence are as given in Appendix III.

Methods used by Ferdman *et al.*, (2005) were adopted for the phylogenetic analysis. Neighbour Joining (NJ) and Maximum Parsimony (MP) methods were used for phylogenetic reconstruction of desert truffles, focussing only on the ITS regions. Sequences of *T. pfeilii* earlier obtained from the mycelial culture and the ascocarp (Section 2.3.2) were included with those used by Ferdman *et al.*, 2005 to analyse the phylogenetic grouping of the South African *T. pfeilii* samples. A total of twenty five sequences including sequences obtained from the GenBank (Table 3.1) and that of South African isolates were included in the analysis; sequences of all other desert truffles were obtained from the GenBank database on the NCBI website. Alignments of all the samples were conducted with BCM search launcher using cluster W1.8 option. The programme PAUP (Swofford, 2002) was used for the analyses. To root the tree, *Tuber melanosporum* was selected as the outgroup. The reliability and topology of the tree was evaluated using a bootstrap value of 1000. Gaps were treated as missing data.

Table 3.1: List of sequences used by Ferdman *et al.*, (2005) from the GenBank. A total of 23 different ITS nucleotide sequences were used and this included the outgroup, *Tuber melanosporum*.

Species	Accession Number
Terfezia claveryi	AF301421
Terfezia claveryi-3	AF387648
Terfezia arenaria-1	AF276675
Terfezia arenaria-2	AF276674
Terfezia boudieri-1	AF092096
Terfezia boudieri-2	AF092097
Terfezia boudieri-3	AF092098
Terfezia boudieri-42a2	AF301418
Terfezia leptoderma-1	AF276678
Terfezia leptoderma-2	AF276679
Terfezia olbiensis-1	AF387657
Terfezia olbiensis-2	AF387656
Tirmania pinoyi	AF276669
Tirmania nivea	AF276668
Choiromyces echinulatus-12	AF435829
Choiromyces echinulatus-44894	AF435823 **changed to AF435825**
Mattirolomyces terfezoides-1	AF276681
Mattirolomyces terfezoides-2	AF276680
Terfezia pfeilii-a	AF301420
Terfezia pfeilii-b	AF301422
Tuber melanosporium	AF167097
Choiromyces venosus	AF003910
Choiromyces alveolatus	AF501258

3.3 *Results*

After confirming the identity of the pure culture (P) and the ascocarp (S) through sequencing as discussed in chapter 2, the ITS sequence obtained which was approximately 721bp in length, was used in the design of the new specific primers.

Table 3.2: List of primers designed using Gene Runner (Hastings Software, 1994)

Primer name	Tm(°C)	Primer sequence
TPF1	**54.6**	5^1 –TACCCTGTTGCTCCACTG-3^1
TPR1	**51.7**	5^1 – ACCTTCTTATCCAATGAGTCC-3^1

The designed set of primers; TPF1 (forward) and TPR1 (reverse) (Table 3.2) were designed with Gene Runner software. The pair was able to specifically amplify the ITS region from both the ascocarp and mycelial cultures with no trace of amplification from the isolated contaminant fungi (Fig. 3.4). As expected an amplicon of 410bp (Fig. 3.3; Fig. 3.4) were generated for the *T. pfeilii* samples.

```
P           TTTTCTTGGTTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC 60
S           -------------------------------------AAGGTTTCCGTAGGTGAACCTGC 23
                                                 ***********************

P           GGAAGGATCATTATTGAGTAAGCTTTATTGTAGCTTTCTCTCTTATCCCTTTGTTTACTT 120
S           GGAAGGATCATTATTGAGTAAGCTTTATTGTAGCTTTCTCTCTTATCCCTTTGTTTACTT 83
            ************************************************************

P           TACCCTGTTGCTTCCACTGGACAGTGTGAGCTTTGCTGGCAGTTGAAGAAGTTCAATTGT 180
S           TACCCTGTTGCTTCCACTGGACAGTGTGAGCTTTGCTGGCAGTTGAAGAAGTTCAATTGT 143
            ************************************************************

P           AGCAAGTGAGCCCTCTGGTTTTGGTGCACTCGGTACCATTGCTGGGGAGTTTGCCGGTGG 240
S           AGCAAGTGAGCCCTCTGGTTTTGGTGCACTCGGTGCCATTGCTGGGGAGTTTGCCGGTGG 203
            ********************************** *************************

P           GTAGCCCCTTTTATAATCAAAACCTGTGTAATAGAGAAACCTTTTTGTCTGATATTAAAT 300
S           GTAGCCCCTTTTATAATCAAAACCTGTGTAATAGAGAAACCTTTTTGTCTGATATTAAAT 263
            ************************************************************

P           GAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATG 360
S           GAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATG 323
            ************************************************************

P           AAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGTGAATCATCGAATC 420
S           AAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGTGAATCATCGAATC 383
            ************************************************************

P           TTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCAT 480
S           TTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCAT 443
            ************************************************************

P           CACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATTTGAGGACTCATTGGA 540
S           CACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATTTGAGGACTCATTGGA 503
            ************************************************************

P           TAAGAAGGTTTTACTCCTATGGGTGAATTCTTCTATCCAGAAAGTTATAGGCAGTACTGG 600
S           TAAGAAGGTTTTACTCCTATGGGTGAATCCTTCTATCCAGAAAGTTATAGGCAGTACTGG 563
            **************************** *******************************

P           TTAGTTCTTCTGTACTGGGCGTAATAATTTACTTTTATTCTCGTCTAGAAAAGGTGAATA 660
S           TTAGTTCTTCTGTACTGGGCGTAATAATTTACTTTTATTCTCGTCTAGAAAAGGTGAATA 623
            ************************************************************

P           GGTGCTTGCCTTGAACCCACAAGTTATGTTAACTGGGTGACCTCAGATCAGGTAGG---- 716
S           GGTGCTTGCCTTGAACCCACAAGTTATCTTAACTGGGTGACCTCAGATCAGGTAGGGATA 683
            *************************** ****************************

P           ----------------
S           CCCGCTGAACTTAAGC 699
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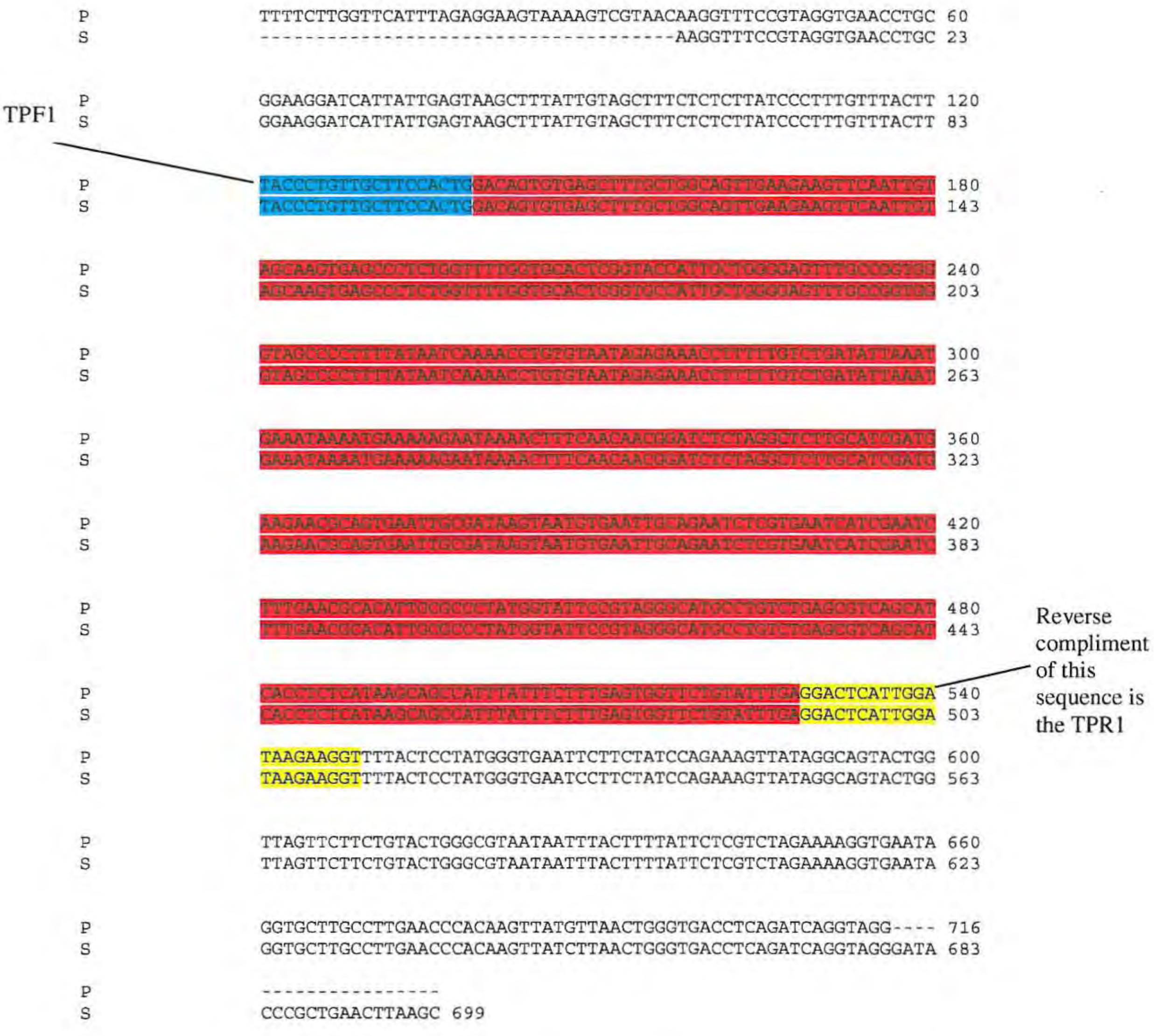
Figure 3.2: Nucleotide sequence alignments of the ascocarp and pure culture of *T. pfeilii* (derived from ascocarp). The alignment of the nucleotide sequences obtanined from the pure culture (P) and the ascocarp (S) indicated a very close similarity between the two. (CLUSTAL W (1.83) multiple sequence alignment).

Alignment of the sequences generated with the universal primer pair, ITS1F and ITS4 (Fig. 3.2) showed 3 mutational differences (substitution), one is the transition at positions 215 on isolate P (pure culture) and position 178 on isolate S (ascocarp) (A to

G). At positions 688 on P and 651 on S, there is transversion (G to C) and at positions 569 on P and 532 on S, there is transition (T to C).



```
P    TTTTCTTGGTTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC 60
S    ------------------------------------AAGGTTTCCGTAGGTGAACCTGC 23

P    GGAAGGATCATTATTGAGTAAGCTTTATTGTAGCTTTCTCTCTTATCCCTTTGTTTACTT 120
S    GGAAGGATCATTATTGAGTAAGCTTTATTGTAGCTTTCTCTCTTATCCCTTTGTTTACTT 83

P    TACCCTGTTGCTTCCACTGGACAGTGTGAGCTTTGCTGGCAGTTGAAGAAGTTCAATTGT 180
S    TACCCTGTTGCTTCCACTGGACAGTGTGAGCTTTGCTGGCAGTTGAAGAAGTTCAATTGT 143

P    AGCAAGTGAGCCCTCTGGTTTTGGTGCACTCGGTACCATTGATGGGGAGTTTGCCGGTGG 240
S    AGCAAGTGAGCCCTCTGGTTTTGGTGCACTCGGTGCCATTGATGGGGAGTTTGCCGGTGG 203

P    GTAGCCCCTTTTATAATCAAAACCTGTGTAATAGAGAAACCCTTTTGTCTGATATTAAAT 300
S    GTAGCCCCTTTTATAATCAAAACCTGTGTAATAGAGAAACCCTTTTGTCTGATATTAAAT 263

P    GAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATG 360
S    GAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATG 323

P    AAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGTGAATCATCGAATC 420
S    AAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGTGAATCATCGAATC 383

P    TTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCAT 480
S    TTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCAT 443

P    CACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATTTGAGGACTCATTGGA 540
S    CACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATTTGAGGACTCATTGGA 503

P    TAAGAAGGTTTTACTCCTATGGGTGAATTCTTCTATCCAGAAAGTTATAGGCAGTACTGG 600
S    TAAGAAGGTTTTACTCCTATGGGTGAATCCTTCTATCCAGAAAGTTATAGGCAGTACTGG 563

P    TTAGTTCTTCTGTACTGGGCGTAATAATTTACTTTTATTCTCGTCTAGAAAAGGTGAATA 660
S    TTAGTTCTTCTGTACTGGGCGTAATAATTTACTTTTATTCTCGTCTAGAAAAGGTGAATA 623

P    GGTGCTTGCCTTGAACCCACAAGTTATGTTAACTGGGTGACCTCAGATCAGGTAGG---- 716
S    GGTGCTTGCCTTGAACCCACAAGTTATCTTAACTGGGTGACCTCAGATCAGGTAGGGATA 683

P    ----------------
S    CCCGCTGAACTTAAGC 699
```

Figure 3.3: Isolates P and S represented above are the aligned ITS sequences of *T. pfeilii* earlier obtained from the pure culture and ascocarp respectively. The two sequences were used to design the new specific primers TPF3 and TPR1 that generated 410bp. Alignment was done with Clusta W (Thompson *et al.*, 1994)

Forward primer (TPF1) -5′– *TACCCTGTTGCTTCCACTG* -3′ 🟦
Reverse primer (TPR1) – 5′ -*ACCTTCTTATCCAATGAGTCC*–3′ 🟨
Expected sequence region to be amplified . 🟥

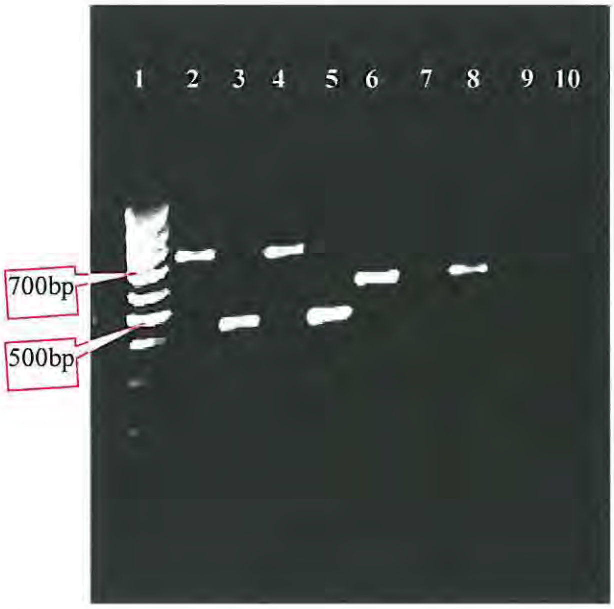


Figure 3.4: Ethidium bromide stained 1% agarose gel showing the selective amplification of *T. pfeilii* by the primer pair TPF1 and TPR1. Lane 1 -100bp ladder, amplification by the universal primers; ITS1F and ITS4 from the ascocarp of *T. pfeilii* –Lane 2, pure culture of *T.pfeilii* - Lane *4, Fusarium equiseti* (isolated contaminant fungus) - Lane 6 and *Phoma glomerata* (isolated contaminant fungus) - Lane 8. However, the newly designed specific primers TPF3 and TPR2 selectively amplified DNA only from the ascocarp- Lane 3 and pure culture - Lane 5 of *T. pfeilii* and could not amplify DNA from *Fusarium equiseti* -7 and *Phoma glomerata* - Lanes 9, 10 is the control with water substituted for genomic DNA.

In the ITS nucleotide alignment conducted for the phylogenetic analysis, the final aligned matrix was 759bp in length (Appendix III). Four hundred and forty two (58.23%) characters were variable and 384 characters (50.59%) were parsimony informative. Data on Retention Index (RI), Consistency Index (CI) and tree length values are as listed in Table 3.3.

Table 3.3: Summary statistics of data sets analysed using MP and the resulting tree statistics.

DNA Marker	No of trees	RI	CI	Tree length
ITS	3	0.824	0.706	926

A single tree was generated using NJ analysis (Fig. 3.6). Three trees were generated using MP analysis, out of which a strict consensus maximum parsimony tree was constructed (Fig. 3.5). The ITS phylogenetic analyses supported three major lineages in both NJ and MP analyses (Fig. 3.5 and Fig. 3.6). Group C lineage is comprised of the outgroup, *Tuber melanosporium* and *Choiromyces venosus* / *Choiromyces alveolatus.*

The other three lineages recovered (Groups A, A' and B) are detailed below. Group A' consists of *Terfezia claveryi, Terfezia arenaria, Terfezia bouldieri, Terfezia leptoderma, Terfezia olbiensis, Tirmania pinoyi, Tirmania nivea* and *Choiromyces echinulatus* (Fig. 3.5; Fig. 3.6). Group A' has three subgroups, containing other *Terfezia* species (excluding those of Group A) (Fig. 3.5; Fig. 3.6) that is well supported. This subgroup has a bootstrap value of 89% and 100% generated by NJ and MP analyses respectively. Also recovered in Group A' were a subgroup of *Tirmania* spp and *C. echinulatus* that have bootstrap values of 100%.

In the analyses conducted for this study (Fig. 3.5 and Fig. 3.6) Group A in both analyses has the four sequences/samples of *T. pfeilii*, two from South Africa (P and S) and two obtained from Genbank, a and b, from Botswana (Table 3.1; Ferdman *et al.*, 2005). These four nucleotide sequences of *T. pfeilii* clearly form a monophyleptic group that is well supported by a bootstrap value of 100% in both analyses.

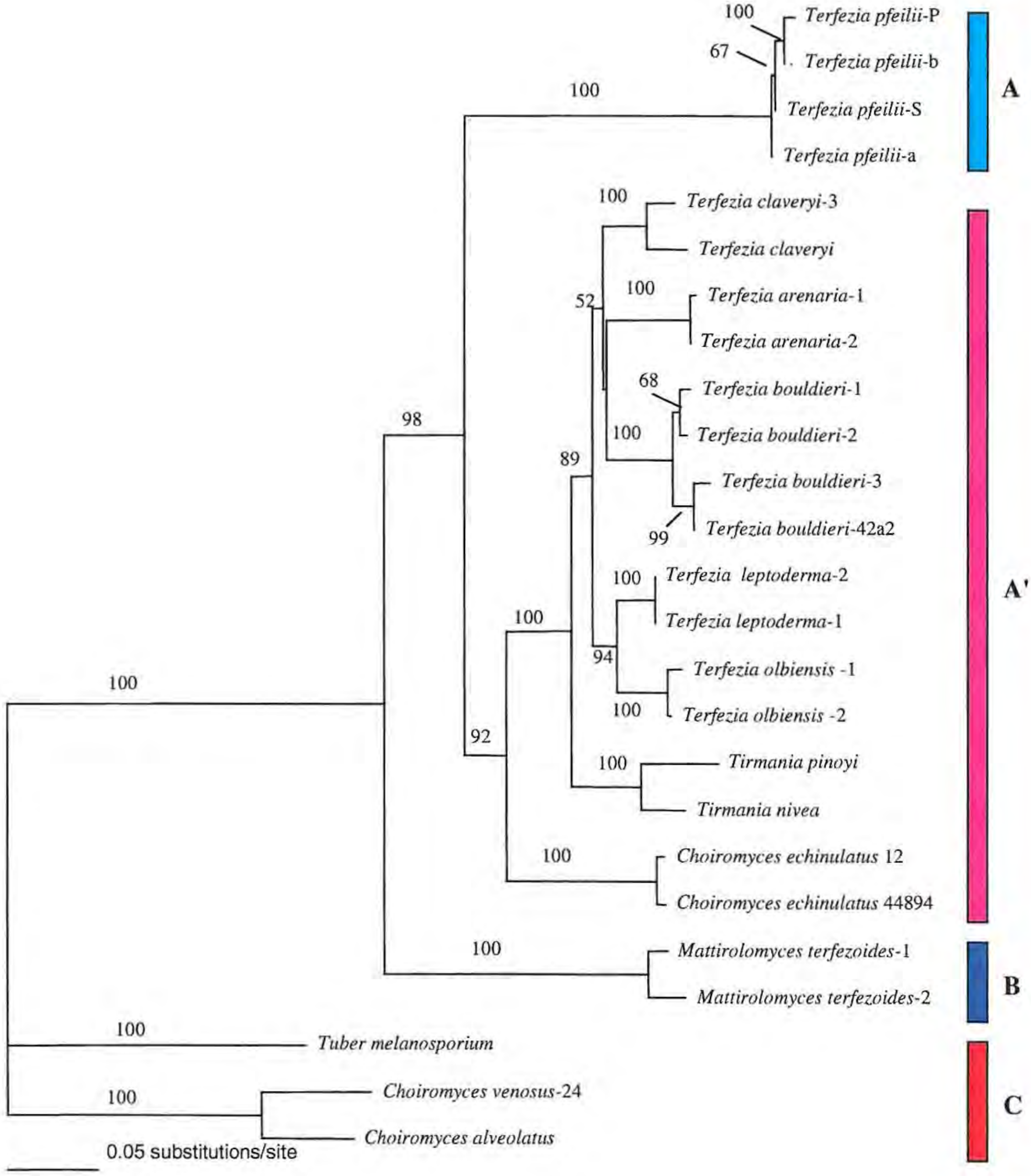


Figure 3.5: Neighbour joining tree constructed from ITS sequences of *Terfezia pfeilii* (P and S) harvested from Upington, South Africa and other related family members using the Jukes-Cantour evolutionary model. Major groups denoted by bars to the right are discussed in the text. Bootstrap values (1000 replicates) of >50% are indicated above the relevant nodes.

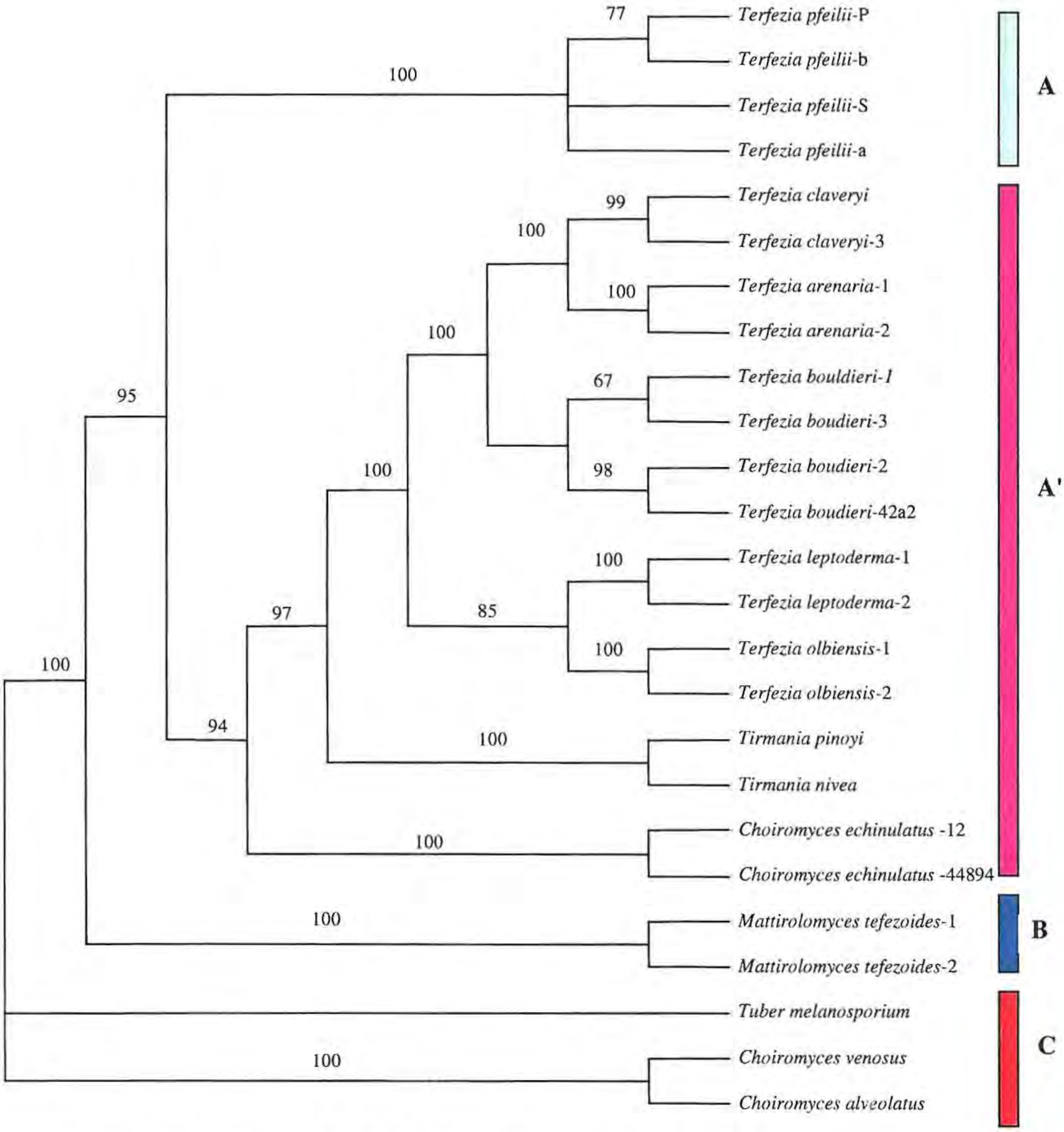


Figure 3.6: Strict consensus tree of three equally parsimonious trees based on ITS sequences obtained from the Maximum Parsimony analysis of *Terfezia pfeilii* (P and S) harvested from Upington, South Africa and some other related family members. Major groups denoted by bars to the right are discussed in the text. Bootstrap values (1000 replicates) of >50% are indicated above the relevant nodes.

Group B consists of two *Mattiromyces* samples, *Mattirolomyces tefezoides* and *Mattirolomyces tefezoides,* that is separated from the *Terfezia/Tirmania/Choiromyces* clade (A and A') which is well supported by a bootstrap value of 100% in both analyses.

It is important to mention that these trees were constructed based on the sequences generated by Ferdman *et al.* (2005) and the results obtained by these authors are shown in Fig. 3.7. The only differences in the current analysis are the inclusion of South African samples for the phylogenetic reconstruction and the use of *Choiromyces echinulatus* with accession number AF435825 instead of AF435823. Accession AF435823 is not an ITS sequence and might have been a typographical error used by Ferdman *et al.* (2005). The phylogenetic trees obtained from both studies showed similar topology.

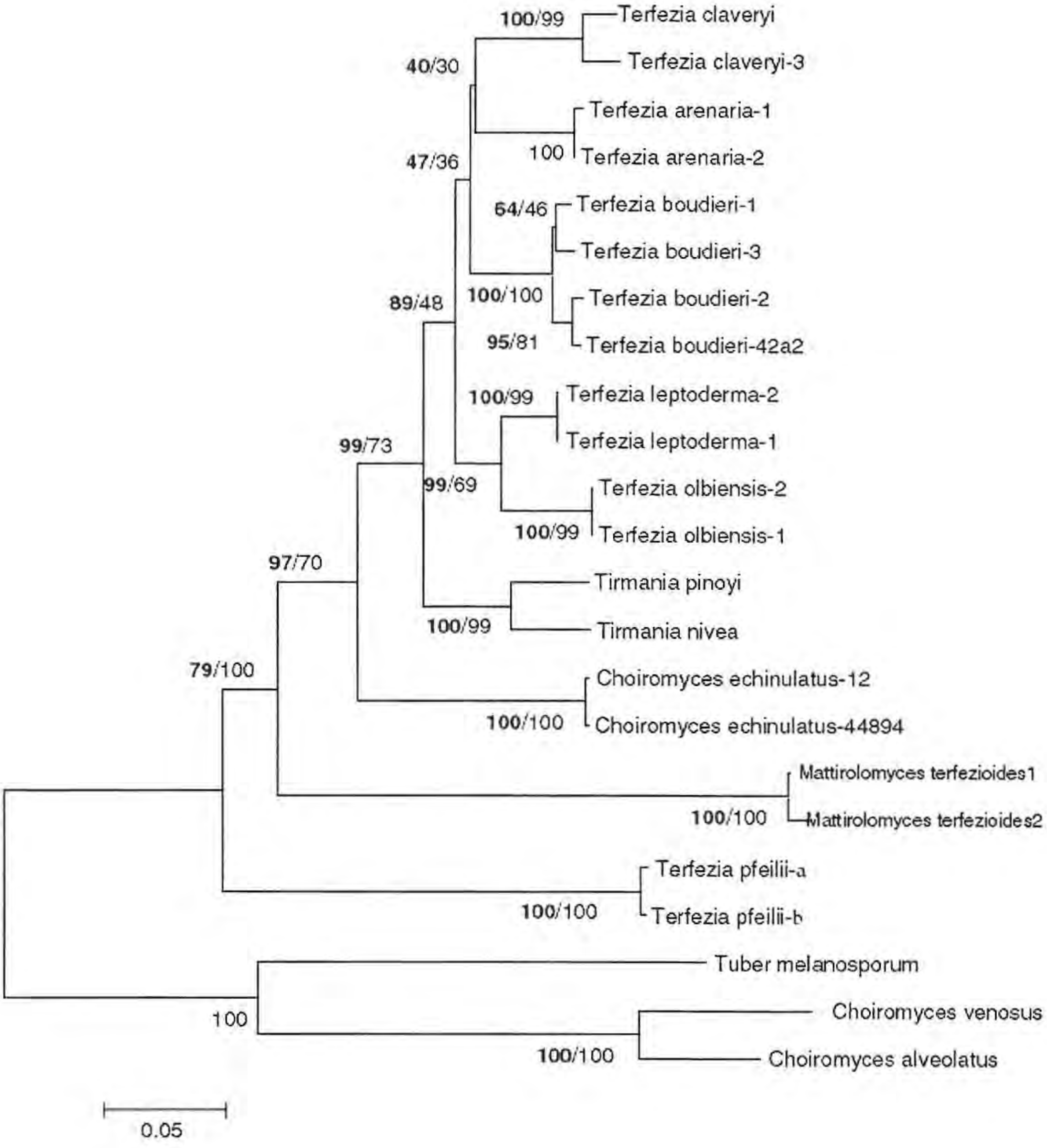


Figure 3.7: Neighbour joining tree from ITS sequences as constructed by Ferdman *et al.*, 2005. This analysis does not include any South African sample.

3.4 Discussion

The use of morphological methods in the identification of mycorrhizal fungi is very important. Though it may not be confirmatory (Horton and Bruns, 2001), its application is essential in the rapid sorting of species especially in the field. As suggested by Horton and Bruns, (2001), a combination of both morphological and molecular identification is needed for the quick and complete identification of mycorrhizal fungi. In this study, morphological identification was mostly used in the identification of ascocarps in the field. It was also useful in the screening of the pure cultures during the *in vitro* isolation of *T. pfeilii.* The morphological identification provided a good starting point for the rapid molecular identification of *T. pfeilii* by reducing the total number of samples needed to be identified with molecular methods. The physical identification of the ascocarp material was not difficult because this species is the only known truffle in the area. This knowledge made the identification of the ascocarp on the field less laborious.

Inclusion of BSA in the PCR helped in binding the PCR inhibitors (Iotti and Zambonelli, 2006) that were preventing the amplification using the universal primer pair, ITS1F and ITS4 (Section 2.3.2). The use of BSA was therefore continued with the new primers. Although an optimum concentration of BSA relies on the biochemical composition of the mycorrhizal fungi (Iotti and Zambonelli, 2006), efforts to optimise the BSA concentration did not make any difference, all the concentrations tested were equally effective. The universal nature of ITS1F and ITS4 made it difficult to differentiate among fungal samples amplified with these primers. The capacity of the ITS primers to amplify from all fungi was recorded in this study as they amplified from all the fungal samples including the contaminant fungi- *Fusarium equiseti* and *Phoma glomerata* (Lafranco *et al.*, 1998). Therefore, the design of specific primers was essential for species identification without need for sequencing.

The PCR products obtained from all the samples could be processed in several ways for the final identification. Sequencing could have been a reliable option as it was successfully used to identify the PCR products of the ascocarps and the pure culture of *T. pfeilii* (chapter 2), but it is expensive and relatively time consuming. The use of

RFLP is another option. This method is faster and cheaper than sequencing but the ITS fragment from RFLP does not always result in a single RFLP pattern for each species (Gardes *et al.*, 1991, Aviram *et al.*, 2004). Such intraspecific ITS variations can lead to false RFLP results, therefore, this method was not used. The design of specific primers that selectively amplify from *T. pfeilii* samples was therefore necessary because it is relatively fast, cheap and could exclude intraspecific variability problem and once the primer specificity is proven, sequencing would not be necessary.

The primers were designed to amplify a section of the ITS region (Fig. 3.3). The region has been widely used for this purpose in other fungi such as *Tuber borchii* and *Tuber magnatum* (Amicucci *et al.*, 1998; Menotta *et al.*, 2004, Douet *et al.*, 2004) The ITS region (Fig. 3.3) provided good priming sites for the design of specific primers that will only amplify from *T. pfeilii.* Gene Runner software was used for the design of the primers. The protocol developed for the identification of the ascocarps and the pure culture was simple. It is a single PCR step of identification using the newly designed primers.

The designed primer pair TPF1 / TPR1 was able to amplify selectively from the mycelial culture and ascocarp of *T. pfeilii* when tested on all the fungal samples obtained during isolation process showing no trace of amplification from the *Fusarium equiseti* and *Phoma glomerata* (Fig. 3.2). On the other hand, the universal primer pair ITS1F and ITS4 amplified from all the fungal samples. The same length of base pairs was recorded in the amplification of both *Fusarium equiseti* and *Phoma glomerata.* This made it difficult to differentiate these two fungi based on the amplicons generated by the universal primers. This result will definitely be useful in identifying pure cultures of *T. pfeilii* and the process will in future, allow a quick way of separating pure cultures from contaminant fungi that disturb and delay the process of isolation.

It is apparent that the earlier predictions (Gardes *et al.*, 1991) that taxon-selective amplification of the ITS region will become popular in the identification of fungi has materialised. This type of method will be useful in characterisation of the fruit bodies, confirmation of the identity of the mycelial cultures, investigation of host plants and mycorrhizal status of *T. pfeilii* during various stages of mycorrhizal synthesis experiments and sales of the fruiting bodies as suggested in the study by Amicucci *et al.*, (2001).

Meanwhile, the utmost importance of such ECM specific primers lies in their ability to amplify from colonised roots of mycorrhizal plants. The use of this rapid molecular identification method was therefore applied to investigate the colonisation of root samples as well as evaluating the success of mycorrhizal synthesis involving *T. pfeilii* (Chapter V).

From the two phylogenetic analyses, the four sequences of *T. pfeilii* clearly form a monophyletic group (Group A quoted in both Fig. 3.5 and Fig. 3.6) which is well supported by a bootstrap value of 100% in both NJ and MP. Although *T. pfeilii* samples P and S were collected from South Africa, P is similar to sequences a (99%) and S to b (98%). The grouping of *T. claveryi, T. arenaria, T. bouldieri, T. leptodema* and *T. olbiensis* in Group A', which was well supported in a single clade with a bootstrap value of 89% is in agreement with the result obtained by Ferdman *et al.*, 2005. It was confirmed in their finding that *T. pfeilii* does not belong to *Terfezia* because of its distinct separation from the *Terfezia* clade as shown in the phylogenetic analysis (Fig. 3.5; Fig. 3.6; Fig; 3.7). Furthermore, it can be deduced that *C. venosus* and *C. alveolatus* (Group C) are as distantly related to other taxa as the outgroup. The two sequences of *C. echinulatus* form a well supported monophyleptic group (bootstrap value of 100%) in both analyses which is distantly located from other *Choiromyces* spp. in Group C which implies that this group is non-monophyletic. Therefore, *Choiromyces echinulatus* maintained the same position as it was in the trees constructed by Ferdman *et al.*, (2005) (Fig. 3.7). This suggests that a name change for *Choiromyces echinulatus* is also reasonable (Ferdman *et al.*, 2005).

T. pfeilii from South African and Botswana formed a well supported monophyletic clade in this study supporting the proposition of Ferdman *et al.*, (2005), that the representatives of *T. pfeilii* should be reclassified as *Kalaharituber pfeilii.* This also proved that both South African and Botswana samples are closely related. However, it would be advisable to study additional samples of *T. pfeilii* from South Africa, Botswana, Angola and Namibia before the reclassification is effected as this would assist in clarification of the differences between *T. pfeilii* and other South African desert truffles; *T. astroafricana* and *Choiromyces echinulatus*.

CHAPTER FOUR

4 Potential Mycorrhization Helper Bacteria Associated With the Ascocarp of *Terfezia pfeilii*

4.1 Introduction

Approximately 8.3 X 10^6 different bacteria species can be found in just 10g of unpolluted soil (Gans *et al.*, 2005). This huge presence in such a small quantity of soil is an indication of the well developed coexisting strategies of these microbes. Different roles of microorganisms in the ecosystem had been separately investigated. Examples of these are studies on the fuctioning of the bacteria such as *Pseudomonas* spp. in improving the health of plants by indirectly protecting the plants against diseases (Whipps, 2001), and the complex ecological roles of mycorrhizal associations (Smith and Read, 1997). For more than a decade, the overlap that exists between mycorrhizal associations and some bacteria has received considerable attention (Garbaye, 1994). This has eventually led to a proposed tripartite association between the plant, fungus and bacteria in the rhizosphere (Azcon-Aguilar and Barea, 1992; Frey-Klett and Garbaye, 2005). The fungus part of this association is referred to as mycorrhizal fungus while the bacterium is called Mycorrhization Helper Bacterium (MHB) (Smith and Read, 1997).

Rhizosphere is a term originally used by Hiltner in 1904, to define the root and its immediate zone of influence. Such influences include water and nutrient uptake, exudates and respiration (Suresh and Bagyaraj, 2002). Bacteria occupying the rhizosphere region are generally referred to as rhizobacteria (Azcon-Aguilar and Barea, 1992). The effects of rhizobacteria on plant growth can be positive, negative or neutral (Schroth and Weinhold, 1986). The MHB are also rhizobacteria and have been shown to have both direct and indirect beneficial effects on plants (Azcon-Aguilar and Barea, 1992; Garbaye 1994; Fitter and Garbaye, 1994).

4.1.1 Mycorrhization Helper Bacteria

In 1968, Oswall and Ferchau defined a section of the rhizosphere that is under the influence of both the plant root and the mycorrhizal fungus as the mycorrhizosphere. The mycorrhizosphere includes the hyphosphere, an area surrounding individual hyphae of the mycorrhizal fungus, and the rhizosphere. In the hyposphere, nutrients and compounds are being exchanged between the soil and the fungus (Smith and Read, 1997; Suresh and Bagyaraj, 2002) while root exudates containing carbon compounds, such as glucose and sucrose, are being released into the rhizosphere. These compounds support the growth of many microorganisms especially bacteria and mycoorhizal fungi in the soil (Smith and Read, 1997). Mycorrhizal formation leads to changes in the mineral composition and physiology of plant tissues and subsequently root exudates. These changes are accompanied by chemical and physical modifications of the mycorrhizosphere which invariably determines the microbial populations of the zone (Azcon-Aguilar and Barea, 1992). Bacteria that mediate by positively and selectively influencing their establishment (mycorrhizal associations) are referred to as MHB (Garbaye, 1994). Bacteria such as *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Rhodococcus*, and *Burkholderia* have all been identified as MHB (Garbaye, 1994; Poole *et al.*, 2001; Founoune *et al.*, 2002). The exact mechanisms through which these bacteria exert their influences to improve mycorrhizal association are not fully understood (Fitter and Garbaye, 1994).

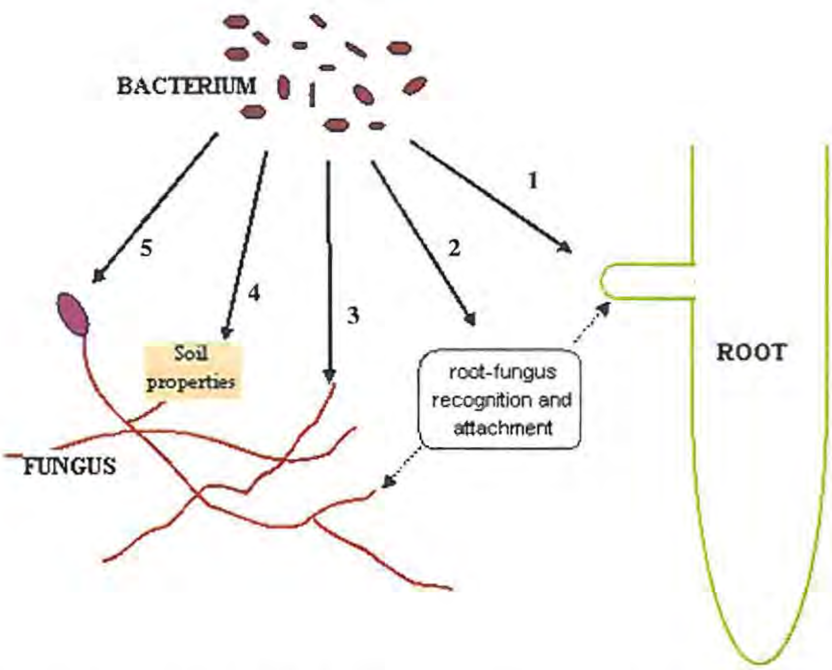


Figure 4.1: Different mechanisms through which bacteria exert stimulatory effects on mycorrhizal formation (Adapted from Garbaye, 1994)

4.1.2 Modes of action of Mycorrhization Helper Bacteria

In 1994, Garbaye, proposed different modes of interactions between MHB, mycorrhizal fungi and their host plants (Fig. 4.1). In one of his propositions, he suggested that prior to mycorrhizal formation, MHB may stimulate the root to hasten the reception of the mycorrhizal fungus. Under these circumstances, the rhizobacteria can make use of the substrates from the rich root exudates which are sometimes utilised by rhizobacteria to produce the secondary metabolites (such as Indole Acetic Acid IAA which is a plant growth promoting hormone (Strzelczyk and Pokojska-Burdziej, 1984; Rajasekhar, *et al.*, 1999). Several MHB have the capacity to produce indole acetic acid (IAA) from precursors such as L-tryptophan (Fig. 4.1-1) (Rajasekhar, *et al.*, 1999; Almonacid *et al.*, 2000). Plants generally respond to this external stimulus by increasing their short root formation. This eventually leads to the occupation of more soil volume by the root of the plant, thereby increasing the chances of the root becoming colonised by mycorrhizal fungi (Fig. 4.1). Some bacteria, such as *Azobacter* sp., have been shown to produce IAA in the presence or absence of tryptophan as a precursor (Ahmad *et al.*, 2005). In this case, the availability of IAA promoted short root formation in plants and the *Azobacter* sp. was shown to have the capacity to produce a concentration of IAA that significantly improved the health of *Sesbania aculeata* and *Vigna radiata* in the presence of tryptophan and as a consequence, more short roots were produced (Ahmad *et al.*, 2005). However, at higher concentrations of the precursor, 5mg/ml, IAA was found to be toxic to these plants (Ahmad *et al.*, 2005; Levau and Lindow, 2005).

The receptivity of the plant root is also enhanced by the capability of some MHB to produce enzymes that soften the cell walls and middle lamella of the root cortex of the host plant, thereby enhancing easy penetration by the fungal hypha (Fig. 4.1-1); a precondition for mycorrhizal formation. Such enzymes include pectase lyase, xylanase, endoglucanase and cellobiose hydrolase (Duponnois, 1992 - cited in Garbaye, 1994; Perotto *et al.*, 1997, Smith and Read, 1997). MHB may also act as an intermediate in the fugus-plant recognition process (Fig. 4.1-2) (Frey-Klett and Garbaye, 2005). This characteristic is attributed to the capability of some MHB to interact between the mycorrhizal fungi and the plant root by breaking down and transforming some mediating chemical subtances needed for the mycorrhizal interaction. A mechanical link between the partners was also suggested to play roles in this mechanism (Garbaye, 1994; Azcon-Aguilar and Barea, 1992). Production of phytohormones such as auxins that promote plant growth also allows physical attachment of bacteria to either of the symbionts, linking the phytobionts. In the study conducted by Azcon-Aguilar and Barea, (1978) a cell free extract of *Rhizobium* containing plant hormones significantly improved the mycorrhizal colonisation of *Medicago sativa* by *Glomus mosseae.*

MHB can directly influence the growth of the mycorrhizal fungus by producing metabolites such as malic and citric acids that promote the growth of the fungus (Fig. 4.1-3). These acids were reported to stimulate the growth of *Paxillus involutus* in a study by Duponnois and Garbaye (1990). The metabolites cause an increase in mycelial mass, thereby increasing the surface area of the fungus exposed to the root, and improving the chance of colonisation (Garbaye, 1994). This mode of action of MHB has been the well studied using an *in vitro* co-inoculaton technique (Aspray *et* *al.*, 2006b). Sbrana *et al.*, (2002), investigated the effects of pseudomonads, actinomycetes, and spore formers isolated from the mycorrhiza of *Tuber borchii*, on the growth of the mycelial culture *in vitro*. In the study, the spore formers, which were predominantly *Bacillus* spp., had up to 78% significant stimulatory effects on the growth of the fungus. Capabilities of some bacteria to produce utilisable forms of nitrogen for ECM fungi have also been reported (Azcon-Aguilar and Barea, 1992; Barbieri *et al.*, 2005). *Rhizobium* isolated from the fruit bodies of *Tuber borchii* by Barbieri *et al.*, 2005 was also suggested to have an important role in the fungal nutrition.

Additionally, the MHB could have an indirect effect on the growth of the fungus by detoxifying the mycorrhizosphere. This is achieved through the production of metabolites such as organic acids by some MHB. This was demonstrated by Duponnois and Garbaye, (1990), where bacterial metabolites (organic acids) were able to detoxify the polyphenols produced by *Paxillus involutus* both in liquid medium and during the formation of ectomycorrhizal with *Fagus silvatica*, thereby indirectly stimulating the growth of the fungus. Such compounds could also stimulate germination (Fig. 4.1-5). These bacteria were unidentified.

Lastly, some MHBs have the capacity to improve the physio-chemical properties of the soil, which could indirectly affects the growth of plants and mycorrhizal fungi coexisting in the soil as well as the mycorrhizas formed by these symbionts. This may be related to increased production of chelating ligands, such as siderophores (Garbaye, 1994; Smith and Read, 1997). Siderophores are agents of iron III transport, having a low molecular weight and can be produced by both mycorrhizal fungi such as some *Hymenoscyphus* spp. and bacteria (Smith and Read, 1997, Haselwandter *et al.*, 1992; Whipps, 2001). Azcon-Aguilar and Barea, (1992) suggested that siderophore-producing bacteria probably interact closely with mycorrhizal fungi in the rhizosphere for the purpose of supplying iron to the plants. According to Garbaye, (1994), lack of information on this mode of action may be due to the difficulty involved in monitoring other soil factors involved such as pH and movement of ions.

Other approaches to investigating the roles of MHB in the ecosystem have been considered and include the inhibition of some pathogenic microbes that inhabit the mycorrhizosphere. This mechanism affords the MHB opportunity to indirectly promote the healthy existence of the mycorrhizal symbionts through production of antimicrobial compounds. A study by Schelkle and Peterson, (1996), confirmed the inhibition effect of *Bacillus subtilis* on the growth of a *Fusarium* sp. and *Cylindrocarpon* sp. Similar inhibition was shown by Frey-Klett *et al.*, (2005) with *Pseudomonas fluorescens* inhibiting the growth of pathogenic fungi such as *Rhizoctonia solani* strains, *Fusarium avenaceum* and *Phytophthora* sp. as well as a suspected role of this bacteria in phosphate solubilisation. In another study by Nikolic *et al.*, 2006, a *Psseudmonas* sp. was found to inhibit the growth of the pathogenic fungus; *Fusarium circinatum* on MMN and also promoted the growth of *Boletus aestivalis* (Nikolic *et al.*., 2006).

The present study investigated the bacteria associated with the ascocarps of *T. pfeilii* presuming that these bacteria are beneficial, directly or indirectly to the mycorrhizal formation. Based on the performances of these bacterial isolates during investigation, some isolates were selected and identified using molecular methods. Beneficial effects, if present, would indicate the potential of these bacteria to be MHB. The various objectives set to achieve this aim were investigated by first isolating the bacteria from the ascocarp of *T. pfeilii*, followed by subsequent investigations into characteristics of these bacteria such as IAA production, *in vitro* assay of bacterial activity on mycelial growth and phosphate solubilising capability, which are all characteristics of MHB.

4.2 Materials and methods

4.2.1 Bacterial Isolation

Growths from isolations of *T. pfeilii* ascocarps, described in Section 2.2.1, were monitored daily for any contamination. Within 5 days, bacteria colonies were observed to grow out of the ascocarpic materials. Colonies were randomly selected and suspended in 1ml autoclaved double-distilled water. The suspension was serially diluted and the 10^{-2} dilution was plated out onto Nutrient Agar (NA) (Biolab Cat. # HG000C1.500) by spread plating each isolate selected. Distinct colonies were obtained after incubation at 37°C for 24 hours. Pure colonies were streaked onto NA as representatives of the individual bacterial isolates.

4.2.2 In vitro assay of bacterial activity on mycelial growth of *T. pfeilii*

Three different media types were used for this experiment. These included Fontana medium (FTN) (Appendix I), water agar (WA) and Fontana medium with reduced concentration of glucose (referred to as poor Fontana medium - PFTN). The carbon concentration of glucose in the PFTN was reduced from 6.5g to 2.25g. Water agar contained 15g of agar per litre water.

Bacterial samples were inoculated into Nutrient Broth (NB) (Biolab Cat. # 1024537) and grown at 37°C overnight. The bacterial culture was centrifuged at 13000 rpm after which the supernatant was discarded. The cells were then resuspended in autoclaved double-distilled water. Concentrations of all the bacterial isolates were adjusted (Sbrana *et al.*, 2002) with sterile water using a Beckman spectrophotometer (Du® 530) at OD level of 600λ to a range of 0.300 to 0.400 (Poole *et al.*, 2001).

Four different perpendicular wells equidistant from the centre of the Petri dish were created on Fontana medium with a core borer of 5mm diameter. An adjusted bacterial concentration volume of 20μl was inoculated into each well, giving a total volume of 80μl of each bacterial sample per plate at the same time. Plugs of *T. pfeilii* mycelial culture grown on Fontana medium was removed from the actively growing region of the culture and this was dual inoculated at the centre of each plate. Three replicates were prepared for each bacterial sample tested. The plates were incubated in the dark at 32°C, the optimum temperature for the growth of *T. pfeilii* (Section 2.3). Growth was recorded daily by measuring colony diameter of the fungus over a period of two weeks. The bacterial effects were evaluated by comparison to a control, which only contained the fungus inoculated onto the plates.

Two additional experiments were set up as described above but using PFTN and WA, The low level of nutrients in these media allowed the stimulatory effects of the bacterial isolates to be effectively monitored (Garbaye, 1994). The bacterial concentration was adjusted to a reduced OD reading of 0.03 to 0.04 because of the rapid growing nature of some of the bacterial isolates. All the inoculated plates were taped with parafilm to prevent contamination.

Further experimentation was conducted on an isolate that showed potential to be MHB by investigating the effects of different concentrations of the bacterial isolate on the fungal mycelial growth using different media. Two different concentrations of this isolate were used by adjusting the OD_{600} reading with sterile water to 0.05 and 0.50. FTN, PFTN and WA media were all coinoculated with both concentrations of the isolate and the fungus.

4.2.3 Indole acetic acid production of the bacteria samples and phosphate solubilising properties of the bacterial isolates

The ability of all the bacterial isolates to produce indole acetic acid was investigated using the method of Gordon and Weber, (1951). Liquid medium containing 1:1 ratio of NB and tryptone powder (Appendix I) was used for the culturing of the bacterial isolates. Tryptone powder is a source of L-trytophan which is a precursor for the formation of IAA (Ahmad *et al.*, 2005). Bacterial isolates were inoculated into the autoclaved liquid medium and incubated for 96 hours in the dark at 37°C. The culture was centrifuged at 4500 rpm for 20 min. The supernatant was used for the indole test by the addition of 2 times volume of the Salkowski reagent (Appendix I); 600µl of Salkowski reagent was added to 300µl of the supernatant. A colour change of the reagent from yellow to red of the mixture was an indication of indole production.

A selective medium for phosphate solubilising micorganisms (Mehta *et al.*, 2001) was modified (Appendix I) and used to detect bacteria that can solubilise the normally insoluble forms of phosphate The capability of the bacterial isolates to solubilise the insoluble phosphate ($CaHPO_3$) was determined by observing clearing zones around the colonies.

4.2.4 Gram staining

A loopful of water was placed on one side of a microscope slide, a wire loop was used to remove a little section of a bacterial colony and this was suspended in the drop of water on the slide. This preparation was heat-fixed by passing through the flame of a Bunsen burner. Gram staining was conducted by the addition of crystal violet (60s), which was later drained and washed-off with water. This was followed by addition of a mordant; iodine (30s) and destaining with 95% ethanol respectively. After the destaining, the slide was washed with water and counter-stained with safranin for 1 min. Slides were washed with water and air-dried (Madigan *et al.*, 1998).

4.2.5 Molecular Identification of selected bacterial isolates

Bacterial isolates were selected for molecular identification based on the stimulatory or inhibitory activities observed in dual culture. Other characteristics such as indole production and phosphate solubilising capability of the isolates were also considered when selecting isolates for molecular identification.

DNA extraction

Genomic DNA was extracted from overnight NB cultures of the bacterial isolates using the phenol/chloroform extraction method of Moore *et al.*, (1987). A volume of 1ml of the NB culture was centrifuged at 13000 rpm in a 1.5ml microcentrifuge tube for 2 min. The supernatant was discarded and the pellet resuspended in 567μl buffer that contained 10mM Tris HCl, 1mM Sodium ethylenediamene acetic acid (EDTA), at pH 8.0 (TE buffer) and 30μl of 10% sodium deodecyl sulphate (SDS). A volume of 3μl of a 20mg/ml solution of proteinase K was added and this was properly mixed by inverting the tubes before a 1hr incubation period in a heating block (ACCUBLOCK™ Digital Dry Bath) at 37°C. A 100μl volume of sodium chloride NaCl was added and mixed thoughly. This was followed by the addition of 80μl of Cetytrimethylammonium bromide/sodium chloride mix (1% CTAB / 0.7M $NaCl^-$) and another incubation period of 10 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24:1) was added and the mix was centrifuged for 5 min at 13000 rpm. The supernantant was transferred into a new 1.5ml microcentrifuge tube where an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the mixture centrifuged for another 5 min at 13000 rpm. The aqueous part of this was then precipitated with 0.6 volume isopropanol to obtain the DNA pellets. The pellets were then washed with 70% ethanol and dried in the lamina flow. After drying, the pellets were resuspended in 100μl TE buffer and stored at -20°C.

Polymerase chain reaction

The 16S rDNA bacterial genes was the target region for the PCR amplification using a universal pair of bacterial forward and reverse primers; GM5F and R907 (Muyzer, *et al.*, 1995) respectively (Table 4.1) (Myers, 1985). PCR was conducted in a 50μl volume which contains the following: 0.4μM of each of the primers, 1.25 units of *Taq* polymerase, 5ul of Promega 10X buffer (0.2mM) promega dNTPs, 1.75mM of Magnessium chloride and 2μl template DNA. The PCR was performed on a MJ Mini Personal Thermal Cycler (Bio-Rad) using the conditions listed in Table 4.2. The different annealing temperatures listed in decreasing order were due to the high variation in the Tm of the two primers (Table 4.1). PCR products were separated electrophoretically with ethidium bromide (0.1μg/ml) stained 1% agarose gel running at 120V for 60 min. DNA was visualised and photographed using a Uviprochem Transilluminator.

Table 4.1: Nucelotide sequences of primers used for the molecular identification of the bacterial isolates – R907 and GM5F. R- reverse primer; F- forward primer (Muyzer, *et al.*, 1995).

Primers	Nucelotide sequence (5'-3')	Tm (°C)
R907 (R)	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCGTCAATTCCTTTGAGTTT	81.8
GM5F (F)	CCTACGGGAGGCAGCAG	58.2

Table 4.2: PCR cyling conditions used for the amplification of DNA bacterial from the bacterial isolates.

Conditions	Tempreature (°C)	Time (s)	Cycles
Initial Denaturing	94	120	1
Denaturing	94	30	
Annealing	68	45	4
Elongation	72°C	120	
Above steps were repeated (excluding initial denaturing) with decreasing annealing temperature at 66, 64, 62, 60 and 58°C running at 4 cycles as above except at the annealing temperature of 58°C that ran at 12 cycles.			
Final Elongation	72°C	240	1

Sequencing conditions stated in chapter 2 (Section 2.2) were used for the final identification. The only modification was the use of bacterial primers (Table 4.1). Forward and reverse sequences obtained were then aligned using BioEdit and BLASTed on the NCBI website. The level of significance of the alignment was determined from the NCBI website through parameters such as e values. The e value is the number of different alignments where the scores are equivalent to or better than the raw scores (S) that are expected to occur in a database search by chance. The lower the e value, the more significant is the score. Another important parameter obtained from the NCBI website was the percentage identity. This helps in determining the extent to which the alignment of two nucleotide sequences is invariant (Wheeler *et al.*, 2006). This was set at >95% for the genus level and >98% for the specific level in the present study.

4.2.6 Statistical Analysis

Data obtained from *in vitro* assays of the bacterial activity on mycelial growth of *T. pfeilii* were analysed using a repeated measure ANOVA. This type of ANOVA is used when samples of the same characteristics are measured under different conditions (Statsoft, 2005). In this case, the fungal colony diameter is the measurement while day is the condition that changes. Fischer LSD was then selected to evaluate the level of significance. Statistica 7 software was used for the analysis.

4.3 Results

4.3.1 Bacterial isolation and gram staining reactions

Seventeen different bacterial isolates were obtained and coded (Table 4.6). In the Gram stain, a total of 10 Gram positive and 7 Gram negative bacteria were identified. Bacterial shapes observed under the microscope revealed that 7 of the Gram positive bacteria were rods while 3 were cocci. For the Gram negative, 4 were rods, 2 were cocci and 1 bacilli (Table 4.6).

4.3.2 *In vitro* assay of bacterial activity on mycelial growth of *Terfezia pfeilii*

All the plate readings were taken from the 4th day to the 14th day of the experiment. The bacterial isolates exhibited various effects ranging from neutral, inhibitory to stimulatory on the growth of the mycelial culture of *T. pfeilii.* On the Fontana medium, the statistical analysis of the result is graphically represented below (Fig. 4.2; Table 4.3). All the replicates for isolate KB06F1 promoted the growth of the fungus at the same rate giving equal colony diameters on the 11th day of the experiment, hence no standard error. Bacterial isolate KB06NF did not allow any growth of the fungus.

Table 4.3: Average growth (colony diameter) of *T. pfeilii* on Fontana medium under the influence of different bacterial isolates after 11days. Level of significant of values were estimated using LSD and represented with letters.(a-neutral, b-inhibitory, c-stimulatory ($P<0.05$, $F_{(136, 288)}=53.449$).

Isolate	**KB06A**	**KB06C**	**KB06D**	**KB06E**	**KB06F1**	**KB06F2**	**KB06G**	**KB06H**	**KB06I**
Colony diameter	50.33a	53.67a	29.33b	40.33b	76.00c	42.33b	46.33a	52.33a	38.00b
Standard Error	5.24	2.60	1.76	0.88	0.00	2.03	0.88	1.20	1.53
Isolate	**KB06J**	**KB06K**	**KB06L**	**KB06NA**	**KB06NC**	**KB06ND**	**KB06NE**	**KB06NF**	**CT**
Colony diameter	36.00b	68.00c	27.00b	16.67b	39.33b	37.00b	25.67b	0.00b	55.00a
Standard Error	0.58	2.52	1.00	1.20	2.60	0.58	2.03	0.00	2.89

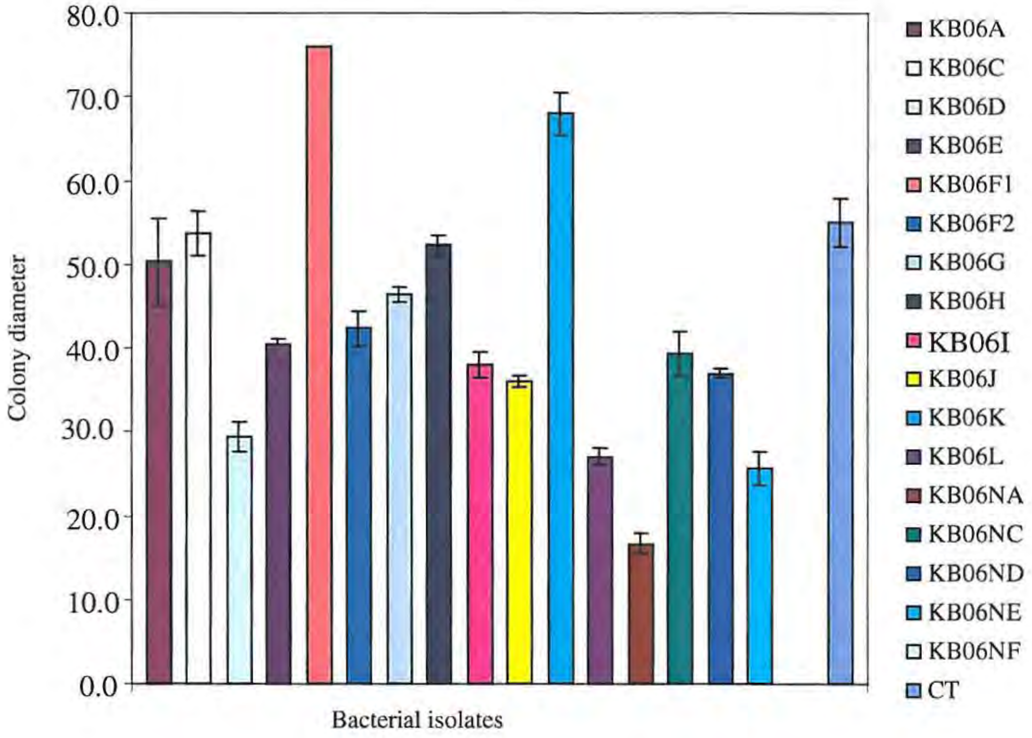


Figure 4.2: The *in vitro* effects of seventeen bacterial isolates on the growth of the mycelium of *T. pfeilii* on Fontana medium (11 days). Statistical analyses of the data are presented in Table 4.3.

The statistical analysis (Fischer LSD) of the data obtained from this experiment indicated that the stimulatory effect of bacterial isolate KB06F1 on the growth of the *T. pfeilii* mycelia culture was greater and most significant (Table 4.3; Fig. 4.2 and Fig. 4.3), fungal growth covered the Petri dish on the 11th day of the experiment. Isolate KB06K also had a significant stimulatory effects on growth of mycelial culture of *T. pfeilii in vitro* but the effect was slow at the beginning (Fig. 4.4).

Isolates KB06A, KB06C, KB06G, KB06H, KB06I and KB06J had neutral effects on the growth while KB06D, KB06E, KB06F2, KB06L, KB06NA, KB06NC, KB06ND, KB06NE and KB06NF inhibited the proliferation of the mycelial. KB06NC and KB06ND initially produced stimulatory effects on the growth of the fungus until the seventh and sixth day, when their effects gradually became inhibitory (Fig. 4.5 and

Fig. 4.6). It was observed at this point, that the rapid growth of these two bacteria isolates led to competition with the fungus for space on the Petri-dish.

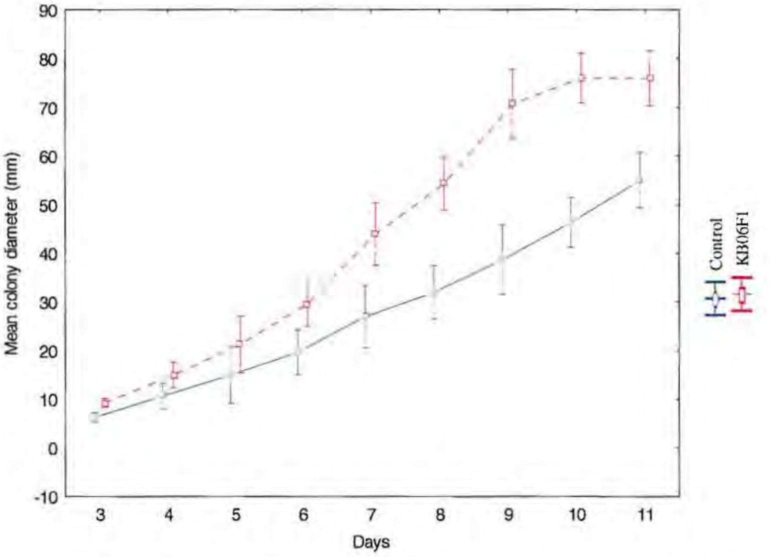


Figure 4.3: Daily effect of bacterial isolate KB06F1 on the growth of *Terfezia pfeilii* as measured by colony diameter observed for 11 days. The isolate produced a stimulatory effect on the growth of the fungus. Current effect: $F_{(8,32)}=40.984$, $P< 0.05$; vertical bars denote 0.95 confidence intervals

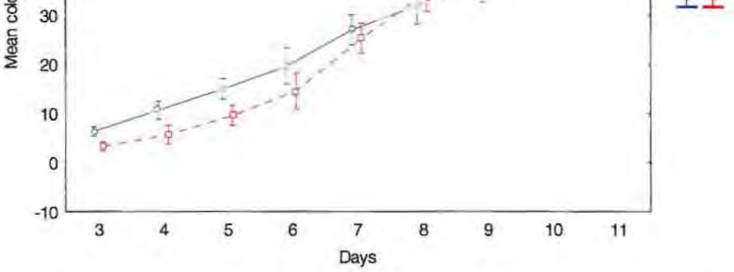


Figure 4.4: Daily effect of bacterial isolate KB06K on the growth of *Terfezia pfeilii* as measured by colony diameter observed for 11 days. The isolate produced a stimulatory effect on the growth of the fungus. $F_{(8,32)}=16.331$, $P< 0.05$; vertical bars denote 0.95 confidence intervals.

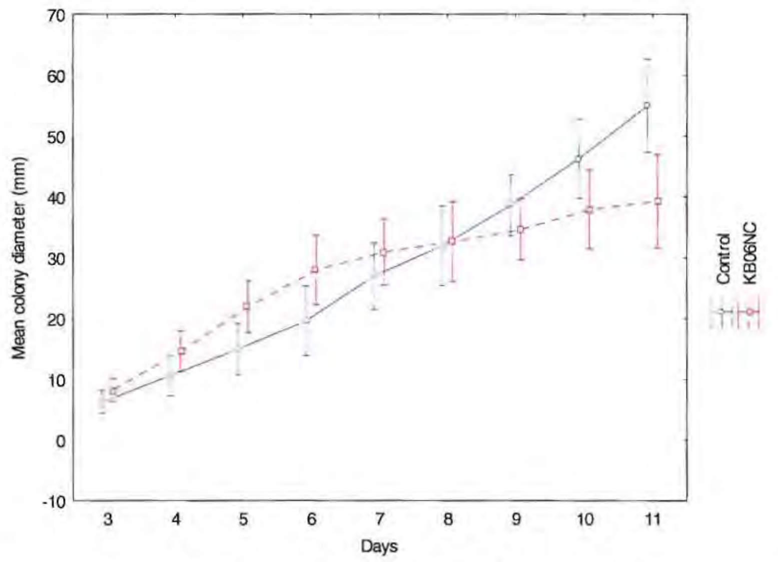


Figure 4.5: Daily effect of bacterial isolate KB06NC on the growth of *Terfezia pfeilii* as measured by colony diameter observed for 11 days. This isolate produced an initial stimulatory effect on the growth of the fungus which gradually declined with time and turned inhibitory after the eleventh day. $F_{(8, 32)}=25.527$, $p<0.05$; vertical bars denote 0.95 confidence

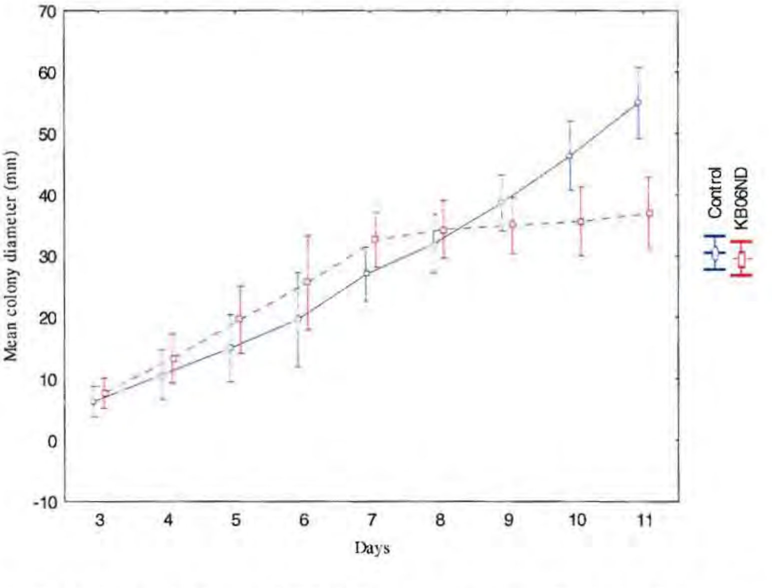


Figure 4.6: Daily effect of bacterial isolate KB06ND on the growth of *Terfezia pfeilii* as measured by colony diameter observed for 11 days. This isolate produced an initial stimulatory effect on the growth of the fungus which gradually declined with time and turned inhibitory after the tenth day: $F_{(8, 32)} = 34.655$, $p<0.05$; vertical bars denote 0.95 confidence.

Table 4.4: Average growths (colony diameter) of *T. pfeilii* on Poor Fontana medium under the influence of different bacterial isolates after 11 days. Level of significant of values were estimated using LSD and represented with letters (a-neutral, b-inhibitory) ($P<0.05$, $F_{(119, 252)}=8.8910$).

Isolate	**KB06A**	**KB06C**	**KB06D**	**KB06E**	**KB06F1**	**KB06F2**	**KB06G**	**KB06H**	**KB06I**
Colony diameter	41.00a	40.00a	22.67b	38.33a	41.00a	22.67b	17.33b	36.00a	54.00a
Standard Error	3.06	1.15	1.45	1.76	5.13	3.84	7.42	2.08	1.53
Isolate	**KB06J**	**KB06K**	**KB06L**	**KB06NA**	**KB06NC**	**KB06ND**	**KB06NE**	**KB06NF**	**CT**
Colony diameter	38.00a	46.67a	12.00b	59.33a	52.67a	55.67a	21.67b	24.00b	45.33a
Standard Error	7.09	2.33	3.06	2.40	2.19	0.33	5.84	5.51	1.76

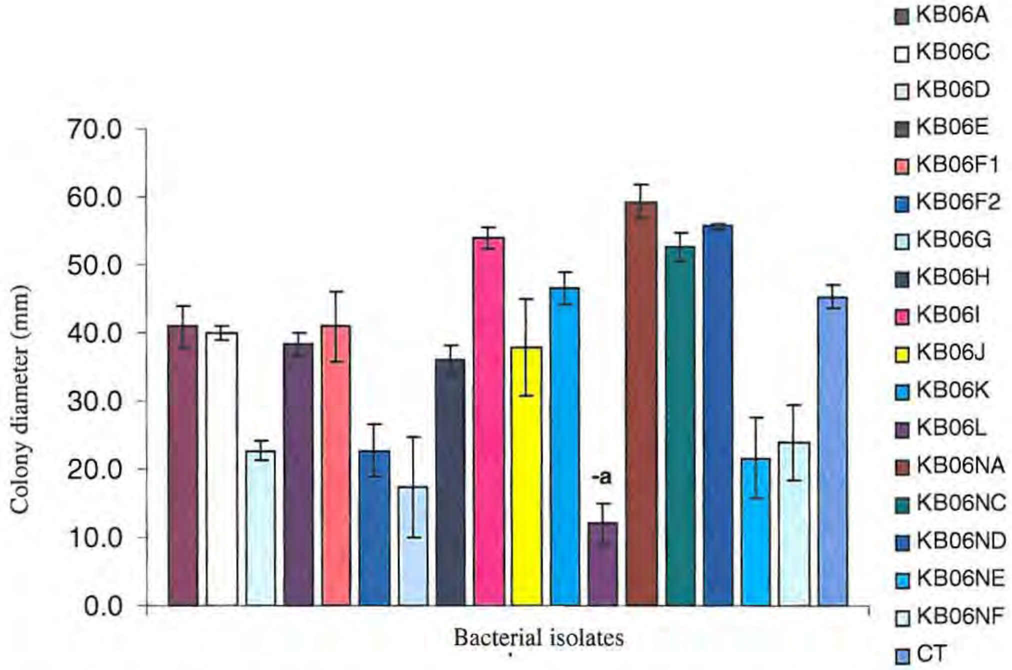


Figure 4.7: *In vitro* effects of seventeen bacterial isolates on the growth of the mycelium of *T. pfeilii* on poor Fontana medium (PFTN) after 11days. CT – control. Statistical analyses of the data are presented in the above Table 4.4

Four isolates; KB06I, KB06NA, KB06NC and KB06ND produced slight stimulatory effects (Fig. 4.7) during the treatment on PFTN, none of these isolates however exerted a significant stimulatory effect on the growth of the fungus when results were analysed statistically. Meanwhile 6 of the isolates; KB06D, KB06F2, KB06G, KB06L, KB06NE and KB06NF, produced significant inhibitory effects while 11 isolates; KB06A, KB06C, KB06E, KB06F1, KB06H, KB06I, KB06J, KB06K, KB06NA, KB06NC and KB06ND, produced neutral effects on the growth of the mycelia culture (Table 4.4 and Fig. 4.7).

Table 4.5: Average growths (colony diameter) of *T. pfeilii* on WA under the influence of different bacterial isolates (11days). Level of significant of values were estimated using LSD and represented with letters (a-neutral, b-inhibitory, c- stimulatory ($P<0.05$, $F_{(153, 324)}=15.705$).

Isolate	**KB06A**	**KB06C**	**KB06D**	**KB06E**	**KB06F1**	**KB06F2**	**KB06G**	**KB06H**	**KB06I**
Colony diameter	16.00b	19.00a	21.67a	23.67a	51.33a	16.67a	14.00a	47.33b	40.33a
Standard Error	1.53	2.65	0.33	2.03	8.82	2.19	2.00	2.40	1.76
Isolate	**KB06J**	**KB06K**	**KB06L**	**KB06NA**	**KB06NC**	**KB06ND**	**KB06NE**	**KB06NF**	**KB06J**
Colony diameter	38.67a	32.67a	33.00a	44.00a	76.00c	9.33b	25.67a	0.00b	38.67a
Standard Error	0.88	6.69	3.51	3.79	0.00	0.67	2.19	0.00	0.88

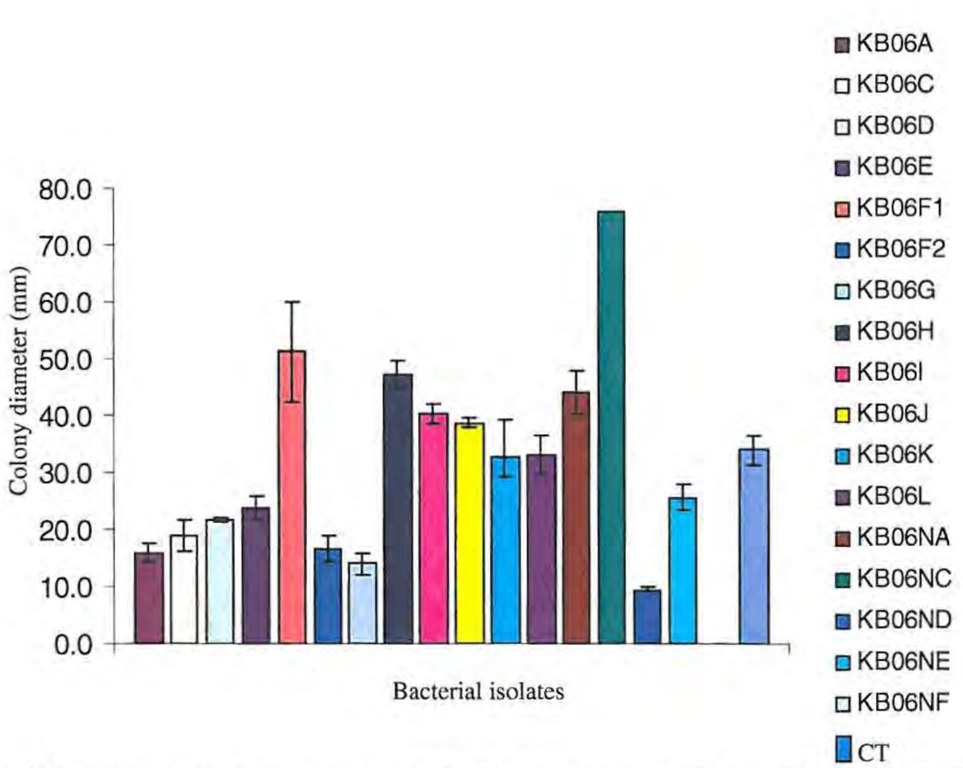


Figure 4.8: *In vitro* effects of seventeen bacterial isolates on the growth of the mycelium of *T. pfeilii* on WA; CT – control. Statistical analyses of the data are presented in the above Table 4.5.

On the WA, 13 out of 17 isolates produced neutral effects on the growth of the fungus while KB06NC was the only isolate that exerted a significant stimulatory effect on the growth of the fungus (Fig. 4.8; Table 4.5). Four isolates, KB06A, KB06G, KB06ND and KB06NF were inhibitory and KB06NF did not allow any growth of the fungus

(Table 4.5 and Fig. 4.8). In general, isolate KB06NF remained inhibitory to the growth of the fungus on all the media used.

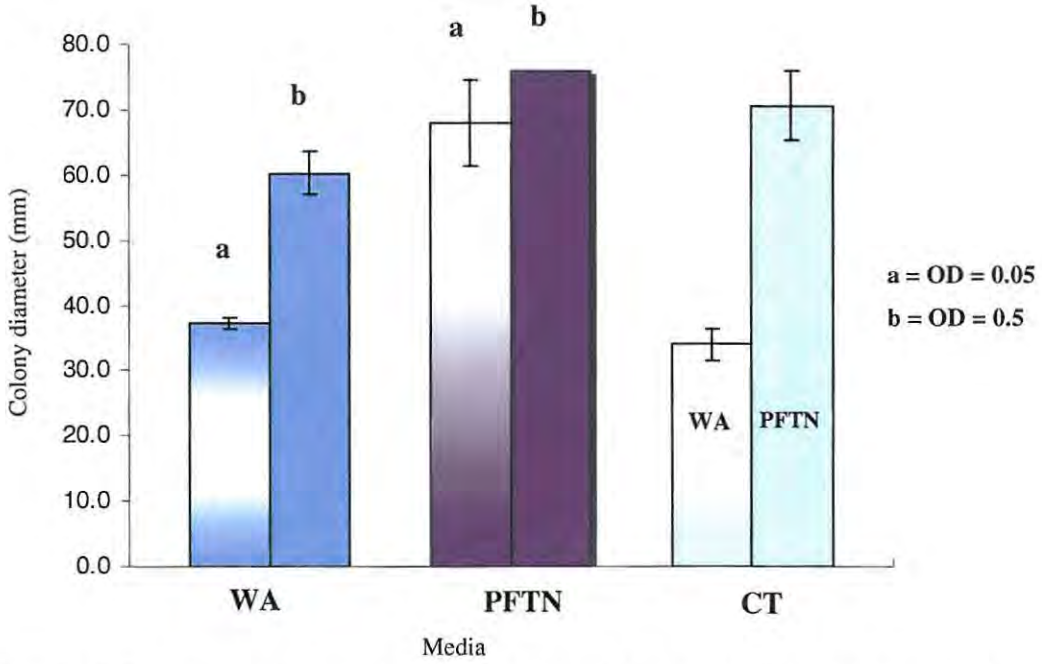


Figure 4.9: The concentration-dependent effects of bacterial isolate KB06F1 on the growth of the mycelium of *T. pfeilii in vitro* on WA and PFTN media. At high concentration (b), the stimulatory effects of the bacterial isolate were significantly greater than the lower concentrations (a) and the control (CT), therefore the stimulatory effects were concentration dependent.

The stimulatory effect of the isolate KB06F1 (Table 4.3) was further investigated by setting up different experiments at different concentrations on the three different media used. The result revealed that the stimulatory effect of this isolate is concentration-dependent. At a higher concentration (OD_{600} =0.5), the stimulatory effect was significantly greater than the effect at lower concentration on both WA and PFTN (Fig. 4.9).

4.3.3 Indole Test / Phosphate Solubilising Bacteria

A colour change from yellow to red which is an indication of indole production, was recorded in four of the bacteria isolates- KB06NC, KB06G, KB06A and KB06D (Table 4.6). This change was more intense in isolate KB06G. Isolate KB06NF was the only isolate that showed the capability to utilise the inorganic phosphate as indicated by a clearing zone of 13mm in diameter (Table 4.6).

Table 4.6: Summary of different tests conducted on bacterial isolates. + Positive result; - Negative result; 0 - Neutral result

Bacterial Isolate	***In vitro* effect on mycelial growth of *T. pfeilii***			**Gram Reaction**	**Indole test**	**Phosphate solubising property**
	FTN	PFTN	WA			
KB06A	0	0	-	- cocci	+	-
KB06C	0	0	0	+ cocci	-	-
KB06D	-	-	0	- cocci	+	-
KB06E	-	0	0	+ cocci	-	-
*KB06F1	+	0	0	+ rod	-	-
KB06F2	-	-	0	+ rods	-	-
*KB06G	0	-	-	- rods	+	-
KB06H	0	0	0	+ rods	-	-
KB06I	-	0	0	+ cocci	-	-
KB06J	-	0	0	- rods	-	-
*KB06K	-	0	0	+ rods	-	-
*KB06L	-	-	0	+ rods	-	-
KB06NA	-	0	0	+ rods	-	-
*KB06NC	0	0	+	- rods	+	-
KB06ND	0	0	-	-bacillus	-	-
KB06NE	-	-	0	+ rods	-	-
*KB06NF	-	-	-	- rods	-	+

* Selected for molecular analysis.

4.3.4 Molecular identification of selected bacterial isolates

Investigated characteristics such as stimulatory and inhibitory effects on the growth of the mycelium, IAA production and phosphate solubilising properties were used to select bacterial isolates for molecular identification. These were isolates KB06F1, KB06K, KB06NC, KB06ND KB06G, KB06L, and KB06NF (Table 4.6). The procedure used for the DNA extraction from all the selected isolates was successful as indicated in the PCR result (Fig. 4.10). The amplifed DNA products were between 600 and 700bp in length and the sequences obtained are as represented in Appendix II. BLAST values greater than 95% were chosen as the cut off for significant identification at the genus level (Table 4.7) and 98% at species level. Based on this, the identification of isolates KB06NF was not conclusive.

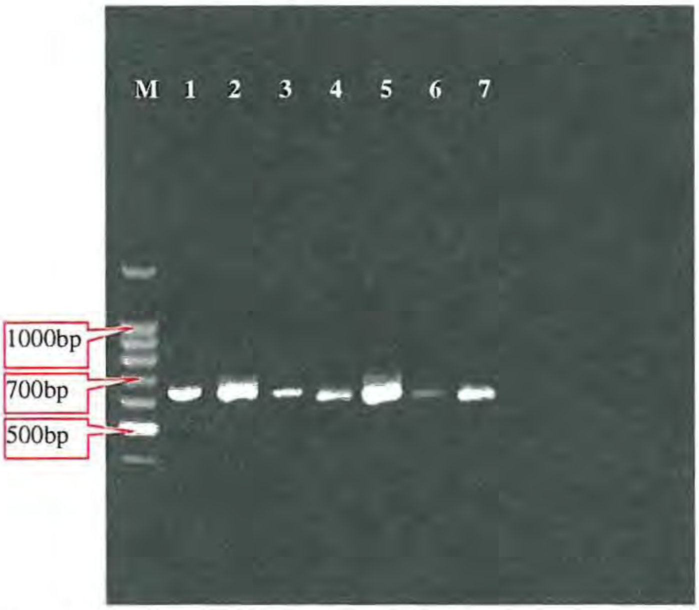


Figure 4.10: PCRproducts of the bacterial siolates on ethidium bromide stained 1% agarose gel. Lane M - Molecular marker (100 base pairs), Lane 1 - KB06F1, Lane 2 - KB06G, Lane 3 - KB06K, Lane 4- KB06L, Lane 5 - KB06NC, Lane 6 - KB06ND and Lane 7- KB06NF

Table 4.7: List of Identified bacterial isolates obtained from the analysis of the 16S rRNA using the National Center for Biotechnology Information (NCBI) website (Wheeler, *et al.*, 2006).

Bacterial Isolate	**NCBI Accession number***	**Most significant alignemnt**	**% Identity**	**e values**
KB06F1	D78473	*Paenibacillus lautus*	96%	0.0
KB06K	DQ993325	*Bacillus* sp.	98%	0.0
KB06G	U89832	*Rhizobium tropici*	98%	0.0
KB06L	AM396911	*Curtobacterium* sp.	99%	0.0
KB06NC	AF60275	*Rhizobium tropici*	99%	0.0
KB06ND	AY841799	*Strenotrophomonas* sp.	98%	0.0
KB06NF	EF120473.1	*Enterobacter cloacae* (inconclusive)	92%	3e -15

* Accession number of closest match to which isolate was compared

4.4 Discussion

Bacteria that exist in close association with *T. pfeilii* have not been previously reported. Culturable and non- culturable bacteria have been isolated and identified from truffle ascocarps and mycorrhizas of *Tuber borchii*. Sbrana *et al.*, (2002) isolated and identifed culturable bacteria such as pseudomonads, spore formers and actinomycetes from the ascocarp of *T. borchii* while (Barbieri *et al.*, 2000 and 2005) used molecular techniques to identify both culturable and non-culturable bacterial associates of *T. borchii* which included *Pseudomonas*, *Rhizobium* and *Sinorhizobium*. The absence of this type of study on *T. pfeilii* may be partly due to the attention being given to the investigation of the host plant of *T. pfeilii*. Although host plants are essential for the formation of mycorrhizas, it is also important to isolate bacterial associates of *T. pfeilii* from the ascocarp, and determine whether they could be potential MHB.

Isolated bacteria were studied for their MHB potential by investigating their various functional activities in the mycorrhizosphere which included their effects on the growth of mycelia culture of *T. pfeilii* which can be stimulatory or inhibitory, their capability to produce phytohormones (IAA) and their phosphate solubilising characteristics (Azcon-Aguilar and Barea, 1992; Frey-Klett *et al.*, 2005) were explored.

An initial *in vitro* assay of the effect of the bacterial samples on the growth of the fungi was conducted on FTN media. Dual inoculation on the FTN allowed the fungus to grow in the presence of basic nutrients in addition to the envisaged stimulatory effects the bacterial isolates may have on the growth of the fungus. However, *in vitro* experiments are better studied on nutrient poor media in order to deprive the fungus of one or more essential nutrients, which subsequently allows for the thorough observation of the aiding effects of the bacteria on the growth of the mycelium (Garbaye, 1994). Therefore, two nutrient poor media were used; one with a reduced concentration of glucose and the other with no nutrients. The concentrations of the bacterial isolates used on the poor media (PFTN and WA) were reduced in order to slow down the rate of growth of bacteria isolates KB06NC and KB06ND (Fig. 4.5 and Fig. 4.6).

Isolates KB06K was identified as a gram positive *Bacillus* sp. (Table 4.7). This isolate was able to produce a stimulatory effect on mycelial growth on FTN medium but only after an initial slow growth period (Fig. 4.4). This (slow growth) may be due to the preliminary adjustment of this isolate to the media conditions. Efforts to repeat the stimulatory effect of the *Bacillus* sp. were not successful on both the WA and PFTN. An increase in the concentration of this bacterial isolate (KB06K) inoculated on these two media did not produce any stimulatory effects possibly because bacteria are heterotrophic and cannot synthesize their own carbon needed for growth (Madigan *et al.*, 1998). This means that the 2.1g of glucose per L in the PFTN medium is not sufficient to support the metabolic activities of this bacterium and probably lacks the enzymes needed to break down other complex sources of carbohydrates in the medium. This is contrary to the report by Sbrana *et al.* (2002) who isolated a *Bacillus* sp. from *Tuber borchii* which produced a significant stimulatory effect on the *in vitro* growth of the fungus.

Isolate KB06F1, which was identified as a *Paenibacillus* sp. (Table 4.7 and Fig. 4.3) could not be replicated to produce the same stimulatory effects it initially exhibited on FTN medium on the other two media (PFTN and WA) (Fig. 4.3). Increase in the concentration of the *Paenibacillus* isolate interestingly re-established the stimulatory effects on both of these media (Fig. 4.9). This result may be due to the capability of this bacterium to hydrolyze the polysaccharide content of the agar, which rarely occurs (Sands and Bennett, 1966). This would allow the fungus to gain access to a more absorbable source of carbon which is more important for the growth of the fungus than any other nutrient. *P. lautus* is a gram-positive rod shaped bacterium, which is motile with round ends that are 0.5 μm to1.0μm wide by 4.0 to 7.0μm long and occurs singly and in pairs (Heyndrickx *et al.*, 1996). They are also facultatively anaerobic, catalase positive and spore producing bacteria (Heyndrickx *et al.*, 1996). *P.* spp. have been shown to be capable of degrading complex carbohydrates. Recently, the production of cellulolytic enzymes, such as xylanases and cellulases that degrade insoluble polysaccharides (cellulose and xylan) by *P. curdlanolyticus* was reported by Pason *et al.*, (2006). Additionally, some strains of the *P. lautus* have been reported to produce dextran-degrading enzyme (Finnegan *et al.*, 2004). These enzymes assist in softening the cell walls of the plant roots, thereby making fungal hyphal penetration easier to establish (Garbaye, 1994). *P. lautus* has been reported to occur in soil environments and in diesel sludge (Heyndrickx *et al.*, 1996; Vrdoljak *et al.*, 2005) where they participate in the degradation of complex carbohydrates such as cellulose and dextran (Hansen *et al.*, 1992; Finnegan, 2000). It can be deduced that a *Paenibacillus* sp. (KB06F1) isolated in this study exhibited stimulatory effects on the growth of *T. pfeilii* mycelia through the degradation/hydrolysation of complex carbohydrates on the media thereby making them available in simple and more absorbable forms such as glucose and sucrose to the fungus (Fontana, 1968; Smith and Read, 1997). This characteristic is very useful under natural conditions where complex sources of carbon can be easily made available for fungal nutrition. This result also revealed that the *in vitro* stimulatory effect produced by a *Paenibacillus* sp. on the growth of *T. pfeilii* is concentration dependent. These characteristics are consistent with those of Aspray *et al.*, (2006b), where a strain of *Burkholderia* sp. EJP67 was found to be concentration dependent in its role as MHB. Mycorrhizal formation was only stimulated at a concentration range of 10^7 to 10^9 CFU mL^{-1} *in vitro*. In contrast, Bending *et al.* (2002), reported a relationship between the mycorrhizal formation on *Pseudotsuga menziesii* (Douglas fir) and *Pseudomonas fluorescens* BBc6, where there was a decrease in mycorrhizal colonisation with increasing bacterial doses.

Isolates KB06NA, KB06NC and KB06ND produced stimulatory effects, though not statitiscally significant on *PFTN medium*. Only isolate KB06NC had stimulatory effects on WA. Isolates KB06NC and KB06ND were identified with molecular analysis as *Rhizobium tropici* and *Strenotrophomonas* sp. respectively (Table 4.7). The initial stimulatory effects of these isolates (KB06NC and KB06ND) on *FTN medium* (Fig. 4.4 and Fig. 4.5) was terminated by rapid growth of the bacteria isolates which competed for space and nutrients with the fungus on the Petri-dishes. Therefore bacterial concentrations were reduced for subsequent experiments. This led to the reduction in the bacterial concentrations used for PFTN and WA media.

Strenotrophonas maltophilia is an aerobic, non-fermenting, gram-negative bacilli found predominantly in the rhizosphere associating with plants and has been reported as a human pathogenic bacterium causing some nosocomial infections (Bollet *et al.*, 1995; Mehta *et al.*, 2000). The stimulatory effect of this bacterium, though not statistically significant, is in agreement with the discovery of Hoflich *et al.*, (2001), where an isolate of this bacterium, in conjuction with an ectomycorrhizal fungus stimulated the growth of *Pinus* sp. growing on post mining soil.

Rhizobia are nitrogen fixing gram negative rods that usually form symbiotic relationships with leguminous (Berkum *et al.*, 1998) and non-leguminous (Antoun *et al.*, 1998) plants. Close associations of *Rhizobium* with mycorrhizal plants was also noted by Garbaye (1994), indirectly influencing mycorrhizal formation. The isolation of *Rhizobium* from an ascocarp harvested in this study might not be unconnected to the presence of leguminous trees such as *Acacia erioloba*, which have been previously identified as potential hosts of *T. pfeilii* (Taylor *et al.*, 1995). The isolation of *Rhizobium* from *Tuber borchii* ascocarps was also recorded by Barbieri *et al.* (2005). The significant stimulatory effect of *Rhizobium tropici* on the growth of *T. pfeilii* mycelium on WA could probably be attributed to their capability to supply the nitrogen requirement of the fungus, which is absent on the water agar. The other two media (FTN and PFTN) contained a nitrogen source which the fungus could access for growth (Hayman, 1983). The positive effects of *Rhizobium* on mycorhizal formation have been reported to be a result of improved plant health caused by the phytohormone production of this bacterium. However, the high demand of nitrogen by mycorrhizal fungi for the formation of chitin, the main constituent of their cell wall, is more likely the driving force behind the reliability of the fungus on the bacteria for nitrogen (Azcon-Aguilar and Barea, 1992; Xie *et al.*, 1995). Interestingly, another strain of *Rhizobium tropici*, KB06G (Table 4.7), produced inhibitory effects on the PFTN and FTN media (Table 4.3, Table 4.4, and Table 4.5). This genetical differences between these isolates of the same species indicates that the 98% identity chosen as the "cut-off " for the species level identification may probably be low since one of the *R. tropici* isolates is 98% and the other is 99% (Table 4.7).

The sequence obtained for isolate KB06NF (Appendix II) contained many unknown nucleotides which could have been errors from the DNA extraction or PCR or sequencing procedures. Therefore only 92% identity to *Enterobacter sp.* was given, which was below the standard set for this study (Table 4.7). The molecular identification of this isolate is therefore regarded as inconclusive. However, this isolate had the most inhibitory effect on the mycelial growth among the isolates. The isolate is a Gram negative rod and non-indole producer. Another isolate (KB06L) that had an inhibitory effect on the growth of the fungal mycelium was identified as *Curtobacterium* sp. This is a gram positive, spore producing bacterium (Tang *et al.*, 2000). Although Barriuso *et al.* (2005) isolated a strain that was a phosphate solubiliser, this isolate did not compare (Table 4.6).

The large proportion of Gram positive (10 out of 17) bacteria isolated in this study agrees with the finding of Barbieri *et al.*, (2005) where most of the bacteria associates of *Tuber borchii* were Gram positive. However, as the majority of these showed little or no effect on mycelial growth, they were not identified.

IAA production observed in four of the isolates (Table 4.4) indicated that they could be indirectly involved in mycorrhizal formation by improving the short root formation of the plants. However, only two of the isolates were identified, KB06NC and KB06G. Both isolates are different strains of the same bacterial species, *Rhizobium tropici* (Table 4.7), but *R. tropici* (KB06G) produced more indole (according to the intensity of the colour change) than *R. tropici* (KB06NC). Bacterial isolate *R. tropici* KB06NC exhibited another potential to be a MHB by stimulating the growth of the fungus on WA. In contrast, KB06G, was significantly inhibitory to the growth of the fungus, on both PFTN and WA media. Inability of this isolate to stimulate the growth of the fungus does not disqualify it from being a MHB. Positive effects on plant growth due to the high phytohormone production could indirectly influence the mycorrhizal formation.

Apart from isolates KB06A and KB06D that produced indole, other unidentified isolates did not exhibit any significant MHB characteristics. Since not all the MHB characteristics have been investigated in this study, there is a possibility that the remaining bacterial isolates could be exhibiting one or more of the other roles such as inhibition of pathogenic microorganisms in the rhizosphere (Azcon-Aguilar and Barea, 1992).

The bacterial isolate (KB06NF) that was most inhibitory to the growth of the fungus, was inconclusively identified as an *Enterobacter* sp. This was the only phosphate solubilising bacteria isolated from the ascocarp of *T. pfeilii*. No other bacterial isolate showed any sign of phosphate solubilisation. This is also an indication that this bacterial isolate could be involved in mycorrhizal formation by solubilising the immobile phosphorus in the soil and passing it on to the fungus for eventual transfer to the plant. This hastens and improves the communication between the plant and the fungus, thereby encouraging early mycorrhizal formation (Garbaye, 1994).

The characteristics exhibited by isolates of *R. tropici* (KB06G and KB06NF) raise concerns about the use *in vitro* co-inoculation to assess the MHB potential of bacteria as reported by Bowen and Theodore (1979). Some bacteria that are not stimulatory nor are inhibitory to the growth of the fungus could still be classified MHB, especially under natural conditions. It can therefore be deduced from this study that the ascocarp of *T. pfeilii* harbours many potential MHB that are capable of positively influencing mycorrhizal formation both directly and indirectly. It seems that the mycorrhizosphere of *T. pfeilii*/host plant has a way of selectively influencing and structuring the microbial diversity of the region to encourage the survival of those bacteria that are directly or indirectly beneficial to the mycorrhizal association or any of its partners (symbionts) (Frey-Klett *et al.*, 2005).

It was difficult at this stage to confirm the inhibitory and stimulatory effects of these bacteria *in vivo*. A tripartite experiment involving a host plant, *T. pfeilii* vegetative inoculum, and the bacterial isolates was set up *in vivo* to confirm the host plant species (Chapter V). Additional investigation into the tripartite association *in vivo* may probably provide more insights into the identification of the host plants of *T. pfeilii*.

This study has reported the potential beneficial roles of bacteria associated with the fruiting bodies of *T. pfeilii*. This is a step towards unravelling the complexity involved in the biology of *T. pfeilii* and may assist in the discovery of potential host plants of *T. pfeilii*. The emphasis was essentially on the culturable bacterial population associated with *T. pfeilii*. Considering that 95% of soil bacteria are yet to be cultured (Amann *et al.*, 1995), there are likely to be many more unidentified bacterial associates. Furthermore, it will be very difficult to prove the mycorrhizal helper roles of non-culturable bacteria because they cannot be isolated on bacterial media. As already suggested by Frey-Klett and Garbaye, (2005), present methods used in the investigation of the effects of beneficial microorganisms on mycorrhizal symbiosis are time consuming and very laborious. Development of good molecular techniques to identify possible genetic markers that are representative of these microorganisms will go a long way to reducing the time and resources currently required for the investigation, identification and confirmation of these ecologically important bacteria. For example, Barbieri *et al.*, (2000), used molecular techniques to detect some, yet-to-be cultured *Cytophaga-Flexibacter-Bacteroides* in ECM mycelium of *Tuber borchii*. These are soil associated bacteria that were recorded from truffles for the first time. Their roles in relation to the mycorrhizal formation are yet to be determined (Barbieri *et al.*, 2000). Recently, some fungal regulated genes of *Candida albicans* were discovered in *Pseudomonas aeruginosa* by Hogan *et al.* (2004) during their investigation of the pathogenicity of the bacterium on this fungus. Such techniques could be applied to identify MHB from natural environments (Frey-Klett and Garbaye, 2005).

Another focus area that can possibly improve the knowledge about MHB is investigating the overlap that exists between the PGPR and MHB. Garbaye, (1994) suggested possible modes of action of MHB in relation to the mycorrhizal symbiosis but the connection between MHB, PGPR and the host plant has not been well investigated. As suggested by Fitter and Garbaye (1994), some MHB can actually be PGPR because bacteria in both groups mostly belong to the same genera (*Pseudomonas* and *Bacillus*). The overlap between the characteristics of these two groups was also highlighted by Frey-Klett *et al.*, (2005). An example of the common features of both groups is the role of PSB. These bacteria were proposed to be capable of solubilsing phosphate in the mycorrhizosphere thereby able to release phosphate ions which can then be easily taken up by mycorrhizal hyphae for eventual transfer onto the plant, which subsequently influences the mycorrhizal establishment and their roles in this region (Azcon-Aguilar and Barea, 1992). In this instance, such bacteria are referred to as MHB but if the solubilised phosphorus is immediately available to the plants, then the bacteria are called PGPR. The question here is: If the solubilised phosphorus is available to both the plant and the the fungus at the same time, should the bacteria be referred to as MHB + PGPR? Similar questions arise from the phytohormone production. Plants can benefit directly from the IAA production by some bacteria and this indirectly influences the mycorrhizal formation. Should all IAA producing bacteria be referred to as MHB or PGPR? Investigations into the related attributes of these two groups will definitely help to provide more understanding of the exact roles of MHBs in the ecosystem. Based on these findings, this study agrees with the suggestion of Barriuso *et al.*, (2005) that there are more benefits to be derived from mycorrhiza-MHB-PGPR associations than mycorrhizal-MHB associations.

The *Paenibacillus* sp. seems to be the most significant bacterial isolate obtained from this study as it was able to produce stimulatory effects on the growth of *T. pfeilii* on all the media tested at the right concentration. This is reasonable evidence that this isolate is a MHB considering that in previous studies where *in vitro* stimulation of the mycelial growth of mycorrhizal fungi by MHB was experienced a corresponding ability of the same bacteria to stimulate the mycorrhizal formation was noted. The stimulatory results obtained by Duponnois, (1992) (cited in Garbaye, 1994), from the use of nutrient poor media to investigate the MHB activities of some bacterial isolates *in vitro*, significantly correlated with the results obtained on mycorrhizal formation *in vivo* under the aiding effects of the same set of bacteria. Similarly, in the study conducted by Frey-Klett *et al.*, (1997), *Pseudomonas fluorescens* BBc6 promoted ectomycorrhizal formation between *Laccaria bicolour* and *Pseudotsuga menziesii* through the improved growth of the fungus trigerred by the bacterium. This was consistent with the initial *in vitro* stimulation of the fungal growth. Caution must however be exercised in extrapolating this type of result to the actual establishment of mycorrhizal association as it has been shown that not all mycelial growth inhibition caused by bacteria *in vitro* prevents mycorrhizal establishment. Thus, the bacterial inhibitory experiment should always be confirmed with a follow-up experiment to check the effect of the bacteria on the establishment of mycorrhizal association *in vivo* (Bowen and Theodore, 1979; Sbrana *et al.*, 2002).

Considering the effects of these bacterial isolates on the growth of the mycorrhizal fungi, the results obtained by Sbrana, *et al.*, (2002) indicated that spore-formers (*Bacillus*) enhanced the growth of the *Tuber borchii* mycelium *in vitro*. In another study, Citerrio *et al.*, (2001) reported an intimate association of *Pseudomonas* and *Bacillus* from the ascocarp of *Tuber borchii* and noted the activity of these two bacteria in chitin and cellulose degradation.

It is interesting to imagine a possible scenario in the mycorrhizosphere where *Paenibacillus lautus* and *Rhizobium tropici* (KB06NC) stimulates the hyphal proliferation of *T. pfeilii*, *Rhizobium tropici* (KB06G and KB06NC) producing indole for promotion of short root formation of the host plant and another isolate solubilises immobile and insoluble P, thereafter transferring this for easy absorption by *T. pfeilii* mycelium which subsequently transfer P to the host plant. However, it would be difficult to replicate this in the field because of many unpredictable influencing biotic and abiotic factors (Smith and Read, 1997). It is therefore necessary to simulate the effects of these bacteria on mycorrhizal formation through mycorrhizal synthesis experiments by inoculating them separately or individually alongside host plant and truffle inoculum.

CHAPTER FIVE

5 Identification of Host Plants for *Terfezia pfeilii* Using Molecular and Morphological Methods

5.1 Introduction

Over 2500 edible mushrooms have been reported, of which the most pricey and hunted belong to the mycorrhizal group (Chandra, 1989). The most sort after are the truffles which have been well adored as a source of food for both animals and humans (Claridge, 2002). The dramatic decrease recorded over the last century in the occurrence of truffles has spurred more interests in their commercial cultivation in truffle plantations (Brundrett *et al.*, 1996; Yun and Hall, 2004).

Important improvements have been recorded in the science of mycorrhizas over the years. For example, ability to grow the ECM fungi *in vitro* has become routine for species and broadens the scope of study in this group of fungi. ECM pure culture is now one of the most frequently used inocula for mycorrhizal synthesis experiments.

Though efforts to artificially grow truffles have not been totally successful (Yun and Hall, 2004), considerable progress has been recorded in the cultivation of some truffle species, such as *Tuber melanosporum*, which was successfully introduced to Israel using *Quercus* as the host plants (Pinkas *et al.*, 2000).

The initial stage of artificial truffle cultivation requires mycorrhizal synthesis (Brundrett, *et al.*, 1996; Pinkas *et al.*, 2000). Mycorrhizal synthesis has been described as the act of exposing seedlings of host plants to inoculum of mycorrhizal fungi under favourable environmental conditions (Brundrett *et al.*, 1996). These synthesis experiments are most useful in checking the compatibility of the host plant and the fungus, and also for structural and physiological investigations of the mycorrhizal root material (Brundrett *et al.*, 1996). Purposes of mycorrhizal inoculation of plants include increasing the yield of plants grown in forestry and agriculture, production of fruit bodies, the use as biological control mechanisms and improving the diversity and stability of ecosystems (Riffle and Maronek, 1982; Brundrett *et al.*, 1996).

Marx and Kenney, (1982) highlighted different methods that can be used to inoculate plants undergoing mycorrhizal synthesis. These include soil inoculum from natural environment that contain one or more mycorrhizal fungi, mycorrhizal seedlings and roots, sporocarps and spores and pure culture of ECM fungi. The use of most of these inoculation methods are restricted by one or more disadvantages. For instance, inoculation with soil inoculum carries a risk of introducing other competing soil microorganisms especially pathogens, as well as transportation problems associated with the bulkiness of soil inoculum. There is also the possibility of the absence of the desired mycorrhizal fungus in the soil inoculum. Spore inoculum can slow down the development and formation of mycorrhiza due to poor germination or reduced viability of the spores. The best time for the application of these inocula has been reported to be before or when the seeds of the host plants are being sown as this requires minimum amount of inoculum to obtain the necessary level of mycorrhization (Riffle and Maronek, 1982).

In mycorrhizal synthesis experiments, good identification methods are necessary to avoid the misidentification of mycorrhiza formed and to prevent the introduction of unwanted mycorrhizal fungi into new areas (Brundrett *et al.*, 1996; Pinkas *et al.*, 2000; Yun and Hall, 2004). The most common identification method is the morphological method, but with the advent of molecular methods, the focus is gradually shifting away from morphological methods of identifcation. The use of molecular analyses has been widely applied to confirm the efficiency of various mycorrhizal synthesis experiments (Mello *et al.*, 1999; Pinkas *et al.*, 2000; Amicucci *et al.*, 2001; Iotti and Zambonelli, 2006). Most of the molecular techniques involved in mycorrhizal studies are PCR-based. Recent studies have focussed on ways of improving these molecular techniques by developing protocols that reduce time and cost (Lanfranco *et al.*, 1998; Amicucci *et al.*, 2001; Mello *et al.*, 2006; Baciarelli-Falini, 2006).

Available knowledge about the symbiotic characteristics of truffles has been used to manipulate events leading to the artificial cultivation and production of fruiting bodies. Just like other mycorrhizal fungi, truffles cannot survive naturally in the absence of the host plants (Delmas, 1978; Smith and Read, 1997). It has however, been shown that with the provision of necessary nutrients in the right proportion, some ECM can actually produce fruit bodies in the absence of host plants *in vitro* (Ohta, 1997).

Studies have confirmed the capability of *Terfezia* spp. to form ectendomycorrhizal, ectomycorrhizal and endomycorrhizal associations with a variety of host plants (Fortas and Chevalier, 1992; Kagan-Zur 1998; Gutierrez *et al.*, 2003). Formation of these different types of mycorrhizal associations has been largely attributed to nutrients supply to the fungus (Fortas and Chevalier, 1992). *Terfezia* spp. are generally known for their preference to associate with the Cistaceae family particularly *Helianthemum* spp. (Giovanneti and Fontana, 1982; Gutierrez *et al.*, 2003). The absence of members of this plant family in South Africa (Taylor *et al.*, 1995) has apparently thrown more challenges into the invesigation of the host plants of *T. pfeilii*. Previous assumptions about the association of *T. pfeilii* with plants such as *Acacia erioloba*, *Acacia haemotoxylon* and *Cynodon* grass has never been confirmed (Taylor *et al.*, 1995; Kagan-Zur *et al.*, 1999). In addition, a potential host, *Citrullus vulgaris*, discovered in the field by Kagan-Zur *et al.*, (1999) may require further studies through mycorrhizal synthesis experiments before it can be confirmed.

This study therefore focussed on the detection of potential phytobionts for *T. pfeilii* through mycorrhizal synthesis experiments and investigation of suspected host plants from the field. To achieve this goal, mycorrhizal synthesis experiments were set up using both *in vivo* and *in vitro* methods. The success of the synthesis was assessed through light microscopic observations, which included staining of the root samples and observation, under the microscope. Molecular techniques, already discussed in chapter 2, were modified in order to resolve the complications involved in PCR-amplification from the root samples. This was then used for the rapid identification of *T. pfeilii* colonised roots of plants from the field and those undergoing mycorrhizal synthesis experiments.

5.2 Materials and methods

As earlier described (Chapter 2), truffle ascocarps were collected from Upington, South Africa and material from plant species that were found in the area were also collected for use in the mycorrhizal synthesis experiments. The roots of these plants were examined for possible colonisation and some of them were propagated from their seeds for mycorrhizal synthesis experiments. Some plants that were suggested as possible phytobionts by Taylor *et al.*, (1995) and Kagan-Zur *et al.*, (1999) but not found in this study site, were also used in the mycorrhizal synthesis experiments.

5.2.1 Suspected host plants from the field

Since there has not been any report on host specificity of *T. pfelii*, it was difficult to select plants for the mycorrhizal synthesis experiments. Therefore, indigenous plants that grow in the Kalahari desert where the fruiting bodies were collected, and those that have been previously suggested (no scientific evidence) to be phytobionts of *T. pfeilii* were selected for the experiments.

A total of 10 different plants (roots and seeds) collected around the areas where ascocarps of *T. pfeilii* were harvested (Table 5.1) were identified using a plant identitfication book (van Rooyen, 2001). The sampled plant roots were transported in paper bags and processed within 24 hours of collection. Root samples were further processed to investigate their mycorrhizal status and possibility of colonisation by *T. pfeilii*. The roots were thoroughly washed with distilled water to remove any traces of soil and kept in sterile water. Approximately 5-10cm long, young newly formed parts of the root samples were cut and subjected to both light microscopic and molecular analysis as explained in Section 5.2.9 and Section 5.2.10.

Table 5.1: List of plants collected in the vicinity of *T. pfeilii* ascocarps at Spitskop Nature Reserve, South Africa

Common name	**Scientific name**	**Family name**	**Sections of Plants Collected**
Stinkwitgat; Stink bos	*Boscia foetida subsp foetida* Schinz	Capparaceae	Roots, seeds
Bushmans grass	*Stipagrostis ciliata* L. de winter	Poaceae	Roots, seeds
Wild green-hair tree	*Parkinsonia africana* Sond.	Fabaceae	Roots, pods
Shappards tree; witgat	*Boscia albitrunca* Gilg & Benedict	Capparaceae	Roots
Springbokopslag	*Indigofera alternans* DC.	Fabaceae	Roots, seed pods
Drie doring	*Rhigozum trichotomum* Burch.	Bignoniaceae	Roots
Tsamma melon	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	Cucurbitaceae	Roots; seeds; melon
Suurgrass	*Schmidtia kalihariensis* Steud.	Poaceae	Roots; seeds
Brosdoring	*Phaeoptilum spinosum* Radlk.	Nyctaginaceae	Roots
White buffalo grass	*Panicum coloratum* Kunth	Poaceae	Roots; seeds?

Table 5.2: List of plants inoculated with mycelia culture for the mycorrhizal synthesis experiment

Common name	**Scientific name**	**Family name**
Bushmans grass	*Stipagrostis ciliata* L. de winter	Poaceae
Pine tree	*Pinus patula* Schlecht.et chem.	Pinaceae
Suur grass	*Schmidtia kalihariensis* Steud.	Poaceae
Tsamma melon	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	Cucurbitaceae
Water melon	*Citrullus vulgaris* Schrad.	Cucurbitaceae
White buffalo grass	*Panicum coloratum* Kunth	Poaceae
Camel thorn	*Acacia erioloba* E.Meyer	Fabaceae
Couch grass	*Cynodon dactylon* L.Pers.	Poaceae
Bastard camel thorn	Acacia erioloba X *haematoxylon* Willd.	Fabaceae
Grey camel thron	*Acacia haematoxylon*	Fabaceae
Candle thorn	*Acacia hebeclada* DC.	Fabaceae

Table 5.3: List of plants inoculated with ascocarp for the mycorrhizal synthesis experiment

Common name	**Scientific name**	**Family name**
Camel thorn	*Acacia erioloba* Steud.	Fabaceae
Corol	*Erythrina caffra*	Fabaceae
Olive	*Olea* L.	Oleaceae
Couch grass	*Cynodon dactylon* L.Pers.	Poaceae

5.2.2 Experimental designs of mycorrhizal synthesis

Three methods were used to initiate studies on artificial mycorrhizal association between suspected host plants and *T. pfeilii*. The first method involved the *in vitro* use of mycelial cultures of *T. pfeilii* as an inoculant of seedlings on Petri dishes under complete aseptic conditions. The other two methods involved the use of pots and torpedo tubes. These were set up in a plastic tunnel under semi-aseptic conditions and certain environmental conditions using mycelial culture and ascocarp slurry as inocula.

5.2.3 Preparation of seeds

Seeds of different plants (Table 5.2 and Table 5.3) were surfaced sterilised in 3% sodium hypochlorite (NaOCl) for 15 min and later washed 3-5 times with distilled water. The seeds with the exception of the grasses (*Cynodon datylon, Stipagrostis ciliata* and *Schmidta kalihariensis*), were soaked in autoclaved double-distilled water for 24 hours before inoculation onto 15% water agar plates where they were pregerminated. This was done to obtain maximum germination of all the seeds that were to be used for the synthesis. This process also reduced risk of contamination as the seeds were germinated under sterile conditions. Germinants were considered ready for when radicles were 1 to 2cm long. Seeds of the grasses were surface-sterilised in 3% NaOCl for 5 min and thoroughly washed with distilled water.

5.2.4 Petri dish method for *in vitro* mycorrhizal synthesis

Petri-dishes of 90mm in diameter were filled with 25ml FTN medium (Section 2.2) with a reduced concentration of glucose (2.1g) in a slightly slanting position. Plants used in this experiment included *Acacia erioloba, Erythrina caffra, Pinus patula* and *Citrullus lanatus*. A 6mm cork-borer was used to cut plugs of the mycelial culture of *T. pfeilii* from the actively growing edge of 7 day old culture. This was inoculated on Fontana medium with reduced glucose concentration and grown at 32°C for 5 days. A hot spatula was used to cut and make a slit into the side of the Petri-dishes and their covers after which the seedlings were introduced through the slit. The seedling was held in place by autoclaved petroleum jelly that also sealed the slit. Cleaned parafilm was used to hold the Petri dish and the cover together, this also prevents contamination. Petri dishes with seedlings were inoculated and kept incubated in upright positions at 28°C for a period of 3 to 5 weeks. As from the second week, development of the seedling roots towards the mycelium of the fungus was monitored by examining with the aid of the dissecting microscope. Preparation of seeds used for this experiment is as stated below (Section 5.2.5). Harvesting of the whole plants was done at the fifth week when some of the plants started showing signs of low nutrients (leaves turning yellow).

5.2.5 Preparation of plant and fungal materials for *in vivo* mycorrhizal synthesis

After germination, healthy seedlings that showed no sign of contamination were selected for the mycorrhizal synthesis experiment.

Ascocarps and mycelia cultures were used as the two sources of inocula for inoculation. Ascocarps were surfaced sterilised with 50% ethanol and crushed with a blender that has been initially washed with 80% alcohol and rinsed thoroughly with sterile water.

The truffle inoculum from the ascocarp (slurry) was prepared by blending an average weight of 15g of dried ascocarpic material in a volume of 50ml autoclaved milli-Q water. The inoculum from the mycelia culture was grown in Fontana broth for a period of 3 to 5 weeks after which the culture was filtered through an autoclaved filter paper (90mm in diameter) placed in a Buchner funnel under aseptic conditions and washed with sterile water to remove any left-over medium. The mycelia mass was then homogenised using a sterile blender in a ratio of 1g of the mycelium to 10ml of sterile water.

5.2.6 Inoculation of plant seeds and seedlings with the mycorrhizal fungal inocula

Torpedo tubes and plastic pots were filled with an autoclaved mixture of soil and vermiculite (2:1). Planting holes big enough to hold seedlings were created in the growth medium and this was half-filled with the prepared spore or mycelial inocula. Root sections (radicle) of all the healthy seedlings were coated with the spore slurry or the mycelial culture under aseptic conditions to ensure perfect contact with the inoculum just before they were planted in the torpedo tubes. Washed seeds of the grass were mixed with the inoculum before planting.

5.2.7 Inoculation of plant seedlings with mycorrhizal helper bacteria

The ability of the *Paenibacillus* sp. as a potential MHB (Chapter 4) to replicate the stimulatory effects on the mycelial growth of *T. pfeilii* was investigated *in vivo*. This experiment was restricted only to some of the plants inoculated with the mycelial culture (Section 5.2.6). Pure culture of *Paenibacillus* isolate was grown overnight in NB after which the concentration was adjusted to 0.500 at 600λ. After 3 weeks of plant growth in the plastic tunnel, few of the seedlings already inoculated with mycelial culture were further inoculated with the adjusted concentration of the bacterial culture (80ml). Plants inoculated were *Cynodon dactylon*, *Stipagrostis ciliata*, *Acacia erioloba*, *Acacia haematoxylon* and *Acacia hebeda*.

5.2.8 Watering, nutrient supply and other environmental conditions

All planted seeds and seedlings were watered by an automatic irrigation system in the plastic tunnel which runs for 5 min every day. All irrigation water was UV treated to reduce water-borne contamination. The temperature of the tunnel ranges between a maximum of 35°C to a minimum of 19°C everyday. Long Ashton's (Hewitt, 1996) low phosphorus nutrient solution (Appendix IV) was used to supply the plants some basic nutrients once a week.

5.2.9 Harvesting

Plants were harvested at the 1st, 3rd, 5th, 8th, 12th and 15th month. The first step in harvesting included the careful separation of the seedling from the growth medium, ensuring that the roots remained intact. The roots were thereafter severed from the shoot system, washed free of the substrate and transported to the laboratory for further processing. In the laboratory, the root samples were thoroughly washed again with distilled water to remove any trace of the growth medium and then divided into two equal parts for microscopic and molecular investigations.

5.2.10 Detection of mycorrhizal formation (microscopy and DNA analysis)

Light microscopy and root staining

All root samples collected (Table 5.1) from the field and those from mycorrhizal synthesis experiments were examined microscopically. Root samples for observation under the dissecting microscope were placed into cleaned Petri-dishes and covered with distilled water to prevent dehydration (Molina and Palmer, 1982). The root samples were observed under the dissecting microscope (Leica S4E) for a hyphal sheath, partially or totally covering the root surfaces as an indication of mantle formation.

Root materials that were not immediately processed for staining were preserved in 50% ethanol and stored in the fridge (4°C) for staining at a later period of time. Washed root samples were stained using the method described by Smith and Dickson, (1997). This method involved the initial clearing by treating the root samples with 5% KOH for 45 min at 90°C a water bath, which removes the cytoplasm and all coloured material from the plant cells. The KOH solution was discarded and the root samples were rinsed with distilled water. This stage was followed by bleaching in alkaline H_2O_2 (Appendix IV) for 30 min. The Bleach was poured off and the roots rinsed with distilled water. Bleached roots were acidified with 0.1M HCl solution for 3 hours to ensure proper binding to the dye. After the acid was poured off, staining was conducted with 0.5% trypan blue in lactoglycerol (Appendix IV) and placed in water bath at 90°C for 45 min. The stain was poured off and the roots covered with lactoglycerol (Appendix IV) overnight to destain.

Stained roots were carefully cut into pieces of 5 – 7cm length and 3 to 5 pieces placed onto microscope slides with cover slip and were mounted on a compound microscope (Nikon YS10) for observation to check for evidence of intra- or intercellular root penetration and the type of mycorrhizal formed. The presence of intra or intercellular septate hyphal colonisation of the root was recorded as an indication of colonisation by *T. pfeilii* (Fortas and Chevalier, 1992; Kagan-Zur, 1999).

5.2.11 Molecular analyses

The rapid identification method developed earlier was modified for the molecular detection and confirmation of *T. pfeilii* (Chapter 3) in the root of the plants collected from the field and resynthesis experiment.

DNA Extraction from the root samples

Two methods were employed for the DNA extraction. These were the CTAB method and Qiagen extraction kit. Both methods were slightly modified. The CTAB method was used to extract DNA from ascocarps and the mycelial cultures as described in Section 2.2.2. The Qiagen DNeasy® Plant Mini Kit (Cat.# 69104) was used for the extraction of DNA from mycorrhizal root samples harvested from the mycorrhizal synthesis experiments. The protocol was as follows; 100mg of root fragments were placed inside a mortar to which liquid nitrogen was added. The liquid nitrogen was allowed to evaporate and the sample was crushed with a pestle to break up the cell tissue. AP1 extraction buffer (750μl) was added and the pestle was used again to crush and homogenise the mixture. The homogenate was transferred to 1.5ml microcentrifuge tubes where 7.5μl of RNaseA that contains ribonuclease A was added to catalyse the breakdown of RNA that could later compete with DNA during binding reaction. The sample was then incubated at 65°C for 15 min to enhance proper lysing of the cells. During the incubation, the tube was inverted 2 to 3 times to ensure proper lysing of the cells. The cells were centrifuged at 10,000 rpm for 2 min. A 500μl aliquot of the mixture was transferred to a clean 1.5ml microcentrifuge tube and added to this was 150μl of buffer AP2, which contains acetic acid that precipitates proteins and polysaccharides. The solution was mixed well by tube inversion and incubated on ice for 5 min. The lysate was then applied to the purple 'QIAshredder' column sitting in a 2ml collection tube. This was centrifuged for 2 min at 13,000 rpm. The clear lysate (400μl) was transferred into a new 1.5ml microcentrifuge tube where 600μl of buffer AP3 was added and mixed by inverting the tube gently. The binding reaction was conducted with AP3 that contains guanidine hydrocholoride for binding the DNA. Half of the mix (500μl) (to avoid spillage during spining) was transferred to the clear 'DNeasy' column sitting in a 2 ml collection tube. The column was centrifuged for 1 min at 13,000 rpm and the liquid flow through was discarded. This step was repeated with the other half (500μl) of the mix and the liquid flow through also discarded. To the column was added 500μl of AW buffer which is the washing solution and the column was centrifuged again for 1 min. The liquid flow through was discarded. To completely dry the column, the column was centrifuged again for another 1 min at 13,000 rpm. The column was then transferred to a clean 1.5ml microcentrifuge tube and 100μl of the buffer AE (heated to 65°C) was transferred onto the membrane to release the DNA into the collection tube. This was left to stand for 20 min, after which it was centrifuged at 13,000 rpm for 1 min. The column was discarded and the extracted DNA stored at -20°C.

Amplification of genomic DNA

The non-specificity of TPF1 and TPR1 to amplify effectively from the root samples led to the adjustments of the initial molecular protocol developed earlier (Chapter 3).

Because of the non-specificity of the primer pair TPF1 / TPR1 when used on root samples, another forward primer was designed and named TPF2. Subsequently, another two primers were designed using the computer software programme, Primer3. The primers were named TPF3 and TPR2.

The efficiencies of the designed primers were first tested by using them to amplify from both ascocarps and mycelial cultures of *T. pfeilii* at different concentrations while their specificity was tested by using them to amplify from DNA obtained from contaminant fungi (Chapter 2) during the isolation process.

PCR conditions

The PCR conditions were optimised in a final volume of 50μl that consisted of Promega *Taq* DNA polymerase- 1.25units, 5μl of Promega 10x buffer-, 0.875mM of Promega $MgCl_2$, 0.2mM of Promega dNTP mix, 32.25μl of autoclaved double distilled water, 0.4μM each of both forward primer and reverse (new) primer, genomic DNA (2μl) and BSA-3μl of 3mg/ml. Different concentrations of the template were tested to determine which quantity of the DNA would be optimum for amplification. The PCR was performed with a BIO-RAD MJ Mini Personal Thermal cycler that allows temperature optimisation of the PCR conditions. The cycling conditions used were: initial denaturing at 94°C for 3 min, followed by 30 cycles of 1 min at 94°C (denaturing), 30s at 50°C (annealing), 2 min at 72°C (elongation) and final elongation period of 8 min at 72°C The PCR products were separated electrophoretically and visualised on ethidium bromide-stained 1% agarose gel.

Purification of the PCR product from the root samples and nested PCR

The PCR product obtained from the root samples was purified with Promega Wizard SV Gel and PCR VClean-up System purification kit (Cat.# A9280). A volume of 30μl membrane binding solution was added to 30μl of the PCR product inside an autoclaved 1.5ml microcentrifuge tube to bind the DNA. The mixture was transferred to the column provided and allowed to stand for 1 min at room temperature. This was centrifuged for 1 min at 6,000 rpm to pellet the DNA and the flow through liquid discarded. Seven hundred microliters of the wash buffer solution was added to the column and spun for 1 min at 13,000 rpm. After discarding the liquid flow through, another 500μl of the wash buffer was added and centrifuged for 5 min. The liquid flow through was discarded and the column spun for another 1 min to dry the column. A 50μl volume of nuclease free water was then added to the column in a clean collection tube and spun for 1 min to collect the DNA. The cleaned product was stored at -20°C.

The PCR condition for the nested PCR using the purified DNA is as stated above using ITS primers in the first PCR and the newly designed primers for the second stage. PCR products obtained from the root samples were "double-checked" through sequencing procedure for further confirmation of the efficiency of the newly designed primers.

5.2.12 Testing of newly designed primers on preserved samples

The newly designed primers were also used to investigate if they could amplify from the DNA obtained from preserved (dried) ascocarps of *T. pfeilii* and *C. echinulatus*. Prior to this, these samples were morphologically identfed by Mr. Schalk van Wyk (personal communication, 2006).

5.3 Results

5.3.1 Petri dish method for *in vitro* mycorrhizal synthesis

The Petri dish method was not suitable for the grasses as their structure would not allow growth through the slit created on the Petri dish. All other selected plants and the fungi were able to grow very well on the FTN medium; however, no initial contact was observed under the dissecting microscope between any plants and the fungus growing on the medium (Fig. 5.1). Further analysis of the root samples through staining and subsequent observation under the compound microscope did not show any sign of root colonisation.

Figure 5.1: Mycorrhizal synthesis experiment (on Petri-dish) involving Coral tree and myclia culture of *T. pfeilii*. The plant root did not form any mycorrhizal association with the fungus.

5.3.2 *In vivo* mycorrhizal synthesis

Most of the plants in the plastic tunnel grew very well under the environmental conditions earlier mentioned (Fig. 5.2; Fig. 5.3). At all the intervals, light microscopy and molecular analyses of these samples did not yield any result until the 8th month when light microscopic analyses of the roots of *Cynodon dactylon* revealed hyphal penetration of the root (Fig. 5.5). The hyphae observed were septate and appeared to colonise and move between cortical cells. No Hartig net or mantle formation was observed.

None of the plant inoculated with the mycelial culture and mycelial culture with bacteria showed any sign of root colonisation.

Figure 5.2: *Cynodon dactylon* plants inoculated with mycelial culture of *Terfezia. Pfeilii* and grown in the plastic tunnel.

Figure 5.3: *Acacica erioloba* plants inoculated with ascocarp slurry of *T. pfeilii* and grown in the plastic tunnel.

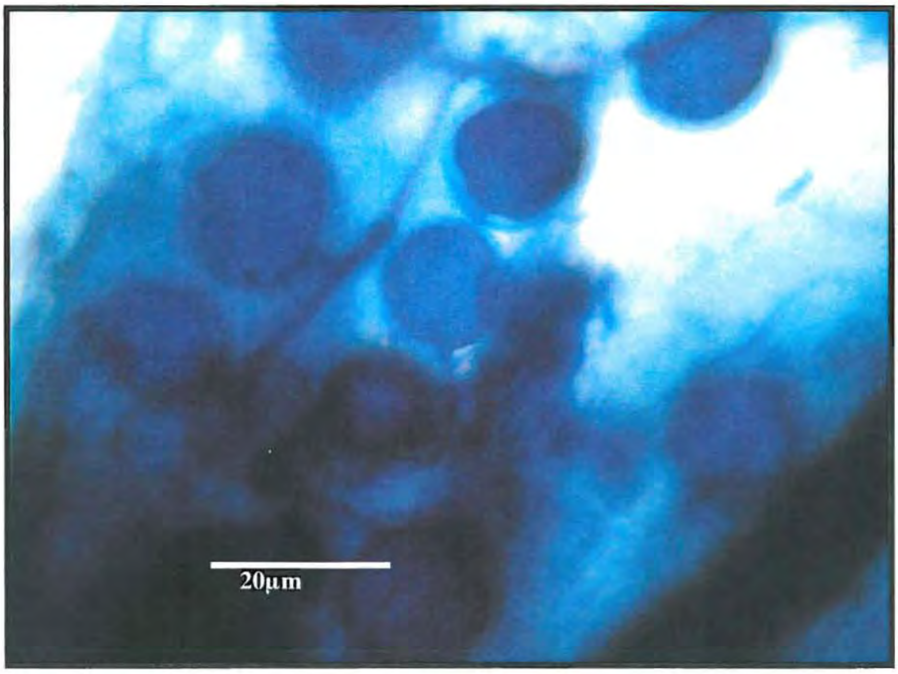


Figure 5.4: AM colonised roots of one of the plants collected from Upington. Roots of plant harvested in the Kalahari Desert were mostly colonised by of Arbuscular Mycorrhizal fungi as indicated in the presence of vesicles and intracellular hyphae.

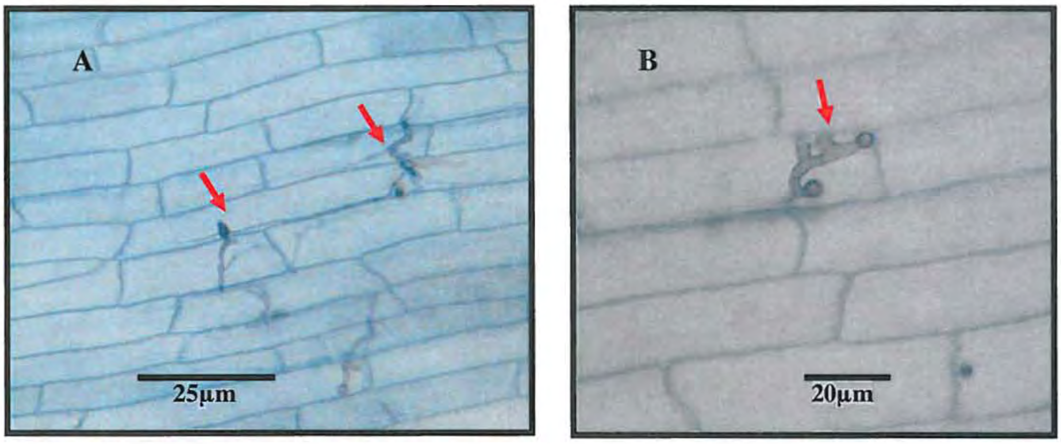


Figure 5.5: Colonised roots of *Cynodon dactyon* showing an endomycorrhizal type of association with intercellular hyphal growth at 32nd week.

5.3.3 Molecular detection of *Terfezia pfeilii*

Molecular analyses of plants collected from the field through amplification with ITS primers ITS1F and ITS4, showed that the plants roots were colonised by fungi, these were later confirmed not to be *T. pfeilii* through the use of specific primers. Light microscopic examinationof the root samples also revealed that the roots were mostly colonised by AM fungi (Fig. 5.4). This is probably due to the typical vegetation of the area where grasses predominate as, grasses are mostly colonised by AM fungi (Table 5.8).

Molecular analysis was used to confirm the colonisation of roots by *T. pfeilii*. The Qiagen DNeasy® Plant Mini Kit was effective in the extraction of DNA from the root samples.

Development of new primers

Although the designed set of primers; TPF1 (forward) and TPR1 (reverse) were able to amplify from both the ascocarp and mycelial cultures (Chapter 2), they could not amplify specifically from the root samples as there were amplifications from other fungi colonising the root, even after optimisation of the PCR conditions. Using the same Gene Runner software that was used to design TPF1 and TPR1, another forward primer TPF2 (Table 5.4) was designed and synthesised. Unfortunately, the same problem of non-specificity of the primer was encountered when it was used alongside TPR1 for amplification of genomic DNA from the root samples. A change in software to Primer3 provided a faster way of designing the primers. Primer3 is a software programme designed to automatically suggests a list of potential primers after the submission of the sequence that the primers would amplify. The software suggested five pairs of primers of which the first pair was selected. The primers are the forward primer with a sequence of 5'-TAGCAAGTGAGCCCTCTGGT-3' and the reverse with a sequence of 5'- GCAATTCACTGCGTTCTTCA-3'. These suggested primers were then modified by the addition of more nucleotides to allow for thorough binding to the target sequence. After modifications, the two primers were named TPF3, a forward primer and TPR2, a reverse primer (Table 5.4).

Table 5.4: List of newly designed primers using Gene Runner and Primer3 (Hastings Software, 1994, Rozen and Skaletsky, 2000).

Primer name	Tm(°C)	Primer sequence
TPF1	**54.6**	5^1 –TACCTGTTGCTCCACTG-3^1
TPF2	**46.5**	5^1– TTGCGATAAGTAATGTGAA-3^1
TPF3	**60.8**	5^1 – CAATTGTAGGCAAGTGAGCC CTCTGGT-3^1
TPR1	**51.7**	5^1 – ACCTTCTTATCCAATGAGTCC-3^1
TPR2	**60.6**	5^1GCATCGATGAAGAACGCAGTGAATTGC- 3^1

The efficiency of these primers was assessed by successfully using them to amplify from genomic DNA extracted from the ascocarp and the pure culture at different concentrations using an annealing temperature that was optimised to 55°C (Fig. 5.6).

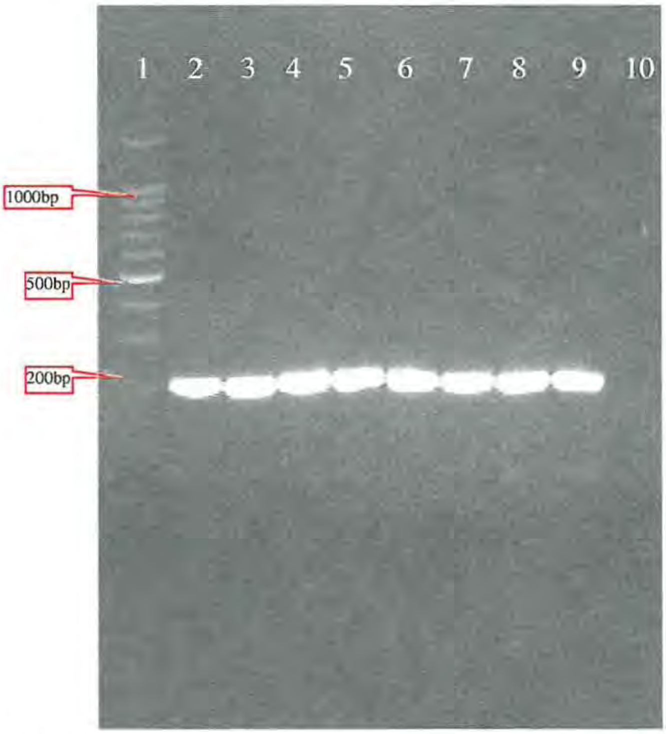


Figure 5.6: Ethidium bromide stained 1% agarose gel showing PCR products from ascocarp and pure culture of *Terfezia pfeilii* at different dilutions using the newly designed universal primers- TPF3 and TPR2. Lane 1-Molecular marker; Lanes 2- (10^{-1}), 4- (10^{-2}), 6- (10^{-3}), 8- (10^{-4}) amplified DNA from ascocarp. Lanes 3- (10^{-1}), 5 (10^{-2}), 7- (10^{-3}), 9- (10^{-4}), amplified DNA from the pure culture of *T. pfeilii*, Lane 10- negative control with no DNA added.

The newly designed TPF3 and TPR2 primers were also tested on the contaminant fungi obtained during the isolation of the pure culture. In this case, ITS1F and ITS4 were used as positive controls to amplify from both the pure culture of the contaminant fungi and that of *T. pfeilii* as well as the ascocarp of *T. pfeilii* (Fig. 5.7). The primers selectively amplified from only the DNA extracted from the ascocarp and pure culture of *T. pfeilii* leaving out the two contaminant *Fusarium equiseti* and *Phoma glomerata* (Section 2.3)

Figure 5.7: Ethidium bromide stained 1% agarose gel showing the selective amplification by the primer pair TPF3 and TPR2. Lane 1 -1kb ladder, Lane 2 - amplification by the universal primers; ITS1F and ITS4 from the ascocarp of *T. pfeilii*, Lane 4 - pure culture of *T.pfeilii*, Lane 6 - *Fusarium equiseti* (isolated contaminant fungus) and Lane – 8 *Phoma glomerata* (isolated contaminant fungus), these primers are not specific for amplifying *DNA from T. pfeilii.* However, the newly designed specific primers TPF3 and TPR2 selectively amplified DNA only from the ascocarp Lane 3 and pure culture – Lane 5 of *T. pfeilii* and could not amplify DNA from *Fusarium equiseti* Lane 7 and *Phoma glomerata* - 9, 10 is the control with water substituted for genomic DNA.

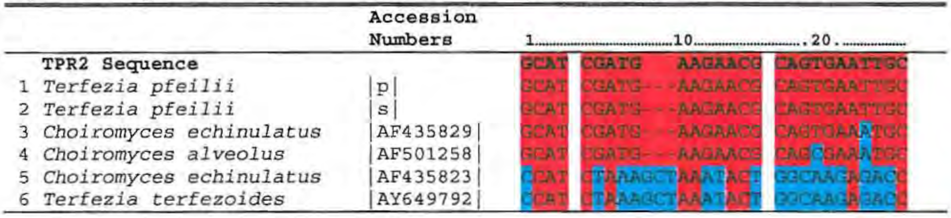
After the eighth month, successful amplification of *T. pfeilii* from the roots of *Cynodon dactylon* was demonstrated. The colonisation of the root of the same plant under the microscope was observed at the same time. However, the PCR amplification was weak and this could be due to PCR inhibitors present in the root samples affecting the quality of the extracted DNA (Sejalon-Delmas *et al.*, 2000). The PCR amplification of the ITS region of the sample was then purified and a nested PCR was conducted. The result of the nested PCR (as shown in Fig. 5.8) had the expected length of base pairs (199bp).

Figure 5.8: PCR confirmation of mycorrhizal formed by *T. pfeilii* with Cynodon grass using TPF3 and TPR2 primers. Lane (1) -1kb base pair molecular marker), Lanes (2-4) -amplified *T. pfeilii* sequence from the roots, and Lane (5) -the control, water was used instead of DNA template.

Testing of newly designed primers on preserved samples

Having used the primer pair TPF3 and TPR2 successfully for identification of the ascocarp, mycelial culture and the root samples, efforts were made to test these primers on any of the closely related members of the family Terfeziaceae, most especially *C. echinulatus*. The presence of *C. echinulatus*, which is also hypogeous, has been previously reported in southern Africa (Marasas and Trappe, 1973). In an effort to predict the level of specificity of the primers, homologous sequences of closely related species such as *Choiromyces echinulatus, Choiromyces alveolus* and *Terfezia terfezoides* were obtained from GenBank (Wheeler *et al.*, 2006; Tamara *et al.*, 2006) and aligned with the sequence of *T. pfeilii.* As stated by van Tuinen *et al.*, (1998), when using specific primers, mismatches at the 3′ of a primer end are needed to prevent amplification of closely related sequences. The result of the alignment indicated that *C. echinulatus* (AF435829) is homologous to the primer TPR2 except at the position 24 (T to A). Though the substitution is at the 3′ end, amplification is still possible since it is only one nucleotide different (Table 5.5). Unlike the *C. echinulatus* (AF435829) and *C. alveolus* (AF501258), other aligned sequences are not homologous to this primer (*C. echinulatus* -AF435823 and *T. terfezoides* – AY649792) (Table 5.5).

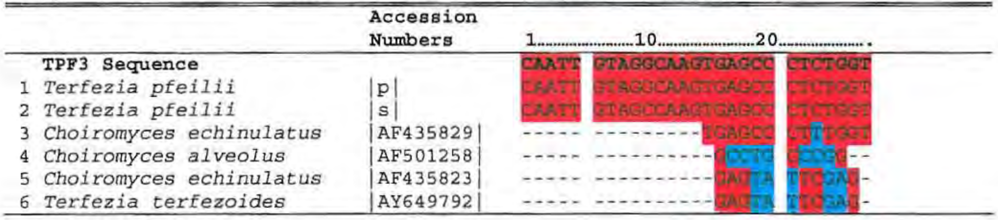
Table 5.5: Partial alignment of primer sequence of TPR2 of six closely related fungal species. Alignment was carried out with CLUSTAL W (Thompson *et al.*, 1994).



	Accession Numbers	1..........10..........20.
TPR2 Sequence		**GCAT CGATG AAGAACG CAGTGAATTGC**
1 *Terfezia pfeilii*	|p|	GCAT CGATG---AAGAACG CAGTGAATTGC
2 *Terfezia pfeilii*	|s|	GCAT CGATG---AAGAACG CAGTGAATTGC
3 *Choiromyces echinulatus*	|AF435829|	GCAT CGATG---AAGAACG CAGTGAAATGC
4 *Choiromyces alveolus*	|AF501258|	GCAT CGATG---AAGAACG CAGCGAAATGC
5 *Choiromyces echinulatus*	|AF435823|	CCAT CTAAAGCTAAATACT GGCAAGAGACC
6 *Terfezia terfezoides*	|AY649792|	CCAT CTAAAGCTAAATACT GGCAAGAGACC

However, similarity of TPF3 to the sequence of *C. echinulatus* was not significant as there were gaps at the 5′ which extended halfway down the primer sequence, there is also a substitution at position 23 (C to T) which is around the 3′ end (Table 5.6). Using the correct annealing temperature, this primer is not likely to amplify the sequence of *C. echinulatus*.

Table 5.6: Partial alignment of primer sequence of TPF3 of six closely related organisms Alignment was carried out with CLUSTAL W (Thompson *et al.*, 1994).

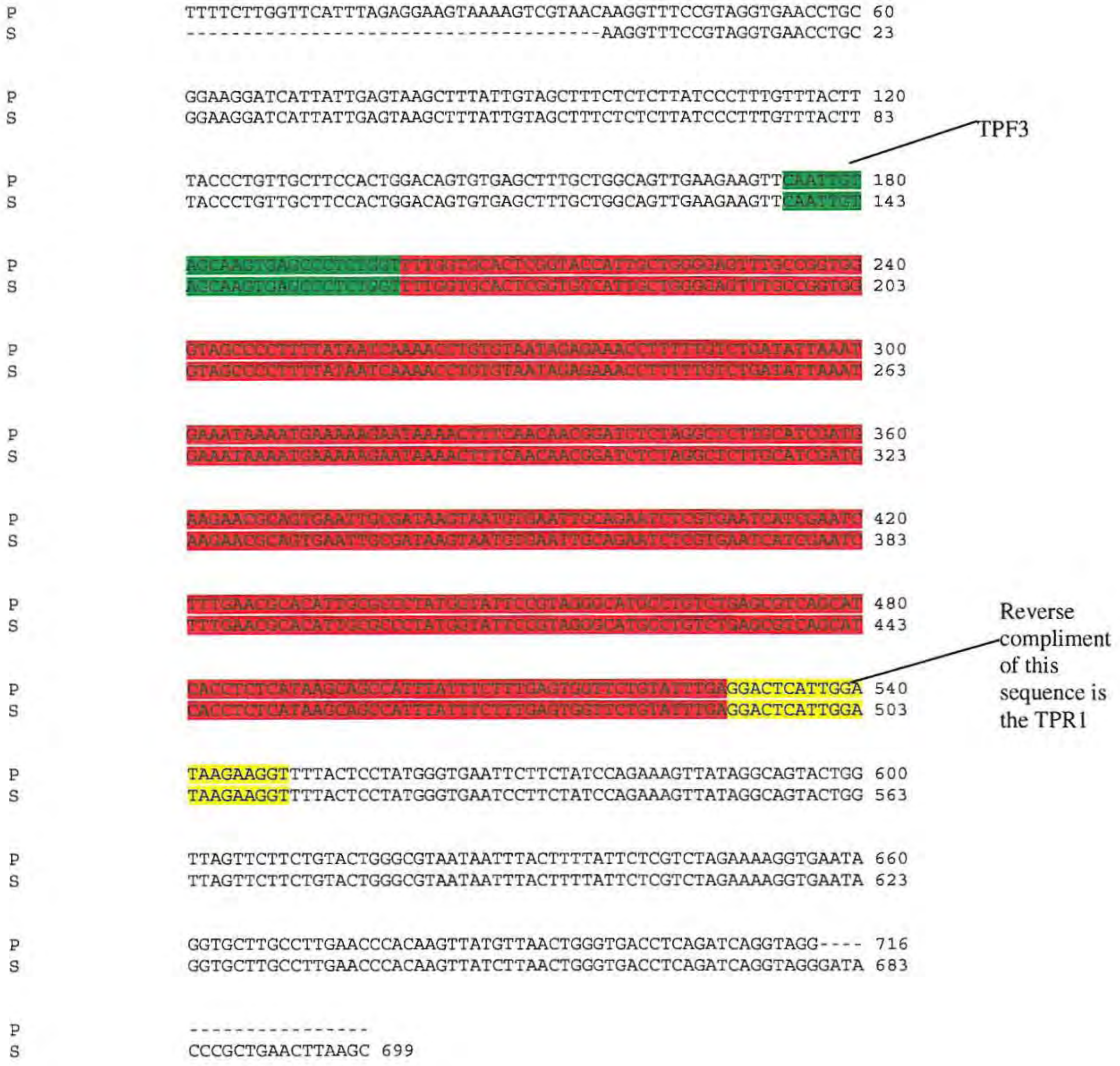


	Accession Numbers	1..........10..........20..........
TPF3 Sequence		**CAATT GTAGGCAAGTGAGCC CTCTGGT**
1 *Terfezia pfeilii*	|p|	CAATT GTAGGCAAGTGAGCC CTCTGGT
2 *Terfezia pfeilii*	|s|	CAATT GTAGCCAAGTGAGCC CTCTGGT
3 *Choiromyces echinulatus*	|AF435829|	----- ---------TGAGCC CTTTGGT
4 *Choiromyces alveolus*	|AF501258|	----- ----------GCCTG GCCGG--
5 *Choiromyces echinulatus*	|AF435823|	----- ----------CAGTA TTCGAG-
6 *Terfezia terfezoides*	|AY649792|	----- ----------CAGTA TTCGAG-

The sequence of the previously designed reverse primer TPR1 was checked as a possible replacement for TPR2. TPR1 was not homologous to the aligned sequence of *C. echinulatus*, which contained gaps and substitutions (A to T and A to G) at the 3′ end (Table 5.7). Having confirmed the absence of this primer sequence in the aligned sequences of *C. echinulatus* and other related species of the family, the combination of TPR1 and TPF3 was expected to give an amplicon of 376bp (Fig. 5.8; Fig. 5.9).

Table 5.7: Partial alignment of primer sequence of TPR1 of six closely related organisms. Alignment was carried out with CLUSTAL W (Thompson *et al.*, 1994).

	Accession Numbers	1.........10..........20.
TPR1 Sequence		GGACTCATTGGATA AGAAGGT
1 *Terfezia pfeilii*	|p|	GGACTCATTGGATA AGAAGGT
2 *Terfezia pfeilii*	|s|	GGACTCATTGGATA AGAAGGT
3 *Choiromyces echinulatus*	|AF435829|	----ACTTTGGAGA AGTGG--
4 *Choiromyces alveolus*	|AF501258|	GGTTCCCAGCACGG AGGATCT
5 *Choiromyces echinulatus*	|AF435823|	---CACTCGGTTGT AGGTGGG
6 *Terfezia terfezoides*	|AY649792|	---CACTCAACTAT GTGTGGG



```
P          TTTTCTTGGTTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC 60
S          -------------------------------------AAGGTTTCCGTAGGTGAACCTGC 23

P          GGAAGGATCATTATTGAGTAAGCTTTATTGTAGCTTTCTCTCTTATCCCTTTGTTTACTT 120
S          GGAAGGATCATTATTGAGTAAGCTTTATTGTAGCTTTCTCTCTTATCCCTTTGTTTACTT 83

P          TACCCTGTTGCTTCCACTGGACAGTGTGAGCTTTGCTGGCAGTTGAAGAAGTTCAATTGT 180
S          TACCCTGTTGCTTCCACTGGACAGTGTGAGCTTTGCTGGCAGTTGAAGAAGTTCAATTGT 143

P          AGCAAGTGAGCCCTCTGGTTTTGGTGCACTCGGTACCATTGCTGGGGAGTTTGCCGGTGG 240
S          AGCAAGTGAGCCCTCTGGTTTTGGTGCACTCGGTGTCATTGCTGGGGAGTTTGCCGGTGG 203

P          GTAGCCCCTTTTATAATCAAAACTTGTGTAATAGAGAAACCTTTTTGTCTGATATTAAAT 300
S          GTAGCCCCTTTTATAATCAAAACCTGTGTAATAGAGAAACCTTTTTGTCTGATATTAAAT 263

P          GAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATG 360
S          GAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATG 323

P          AAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGTGAATCATCGAATC 420
S          AAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGTGAATCATCGAATC 383

P          TTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCAT 480
S          TTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCAT 443

P          CACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATTTGAGGACTCATTGGA 540
S          CACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATTTGAGGACTCATTGGA 503

P          TAAGAAGGTTTTACTCCTATGGGTGAATTCTTCTATCCAGAAAGTTATAGGCAGTACTGG 600
S          TAAGAAGGTTTTACTCCTATGGGTGAATCCTTCTATCCAGAAAGTTATAGGCAGTACTGG 563

P          TTAGTTCTTCTGTACTGGGCGTAATAATTTACTTTTATTCTCGTCTAGAAAAGGTGAATA 660
S          TTAGTTCTTCTGTACTGGGCGTAATAATTTACTTTTATTCTCGTCTAGAAAAGGTGAATA 623

P          GGTGCTTGCCTTGAACCCACAAGTTATGTTAACTGGGTGACCTCAGATCAGGTAGG---- 716
S          GGTGCTTGCCTTGAACCCACAAGTTATCTTAACTGGGTGACCTCAGATCAGGTAGGGATA 683

P          ----------------
S          CCCGCTGAACTTAAGC 699
```

Figure 5.9: Aligned nucleotide sequences of isolates P and S showing the position of primers TPF3 and TPR1.

Forward primer (TPF3) -5'–*CAA TTG TAG CAA GTG AGC CCT CTG GT*-3' 🟩

Reverse primer (TPR1) – 5' –*ACCTTCTTATCCAATGAGTCC*–3' 🟨

Expected sequence region to be generated 🟥

TPF3 and TPR1 primers were then applied in different PCR (with annealing temperature of 63°C) on all the samples of *T. pfeilii* that were previously amplified with TPF3 and TPR2. The amplification with TPF3 and TPR1 primer starts from the ITS1 region, covering the entire 5.8S rDNA and a small section of the ITS2 region. The expected amplicon was 376bp and this was obtained after running the PCR and the nested PCR products on 1% agarose gel (Fig. 5.10; Fig. 5.11).

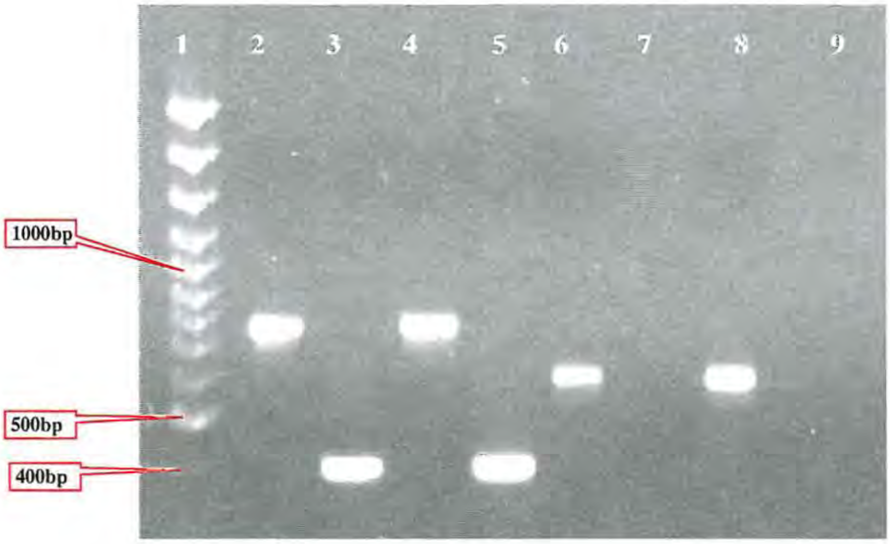


Figure 5.10: Selective amplification of the ITS region of *T. pfeilii* by TPF3 and TPR1 primers. Lane 1- 100bp ladder, the universal primers; ITS1F and ITS4 amplified DNA from the ascocarp of *T. pfeilii* - Lane 2, pure culture of *T.pfeilii* - Lane *4, Fusarium equiseti* (isolated contaminant fungus) - Lane 6 and *Phoma glomerata* (isolated contaminant fungus) - Lane 8. The new primers selectively amplified DNA only from the ascocarp- Lane 3 and pure culture - Lane 5 of *T. pfeilii* and could not amplify DNA from *Fusarium equiseti* - Lane 7 and *Phoma glomerata* - Lane 9.

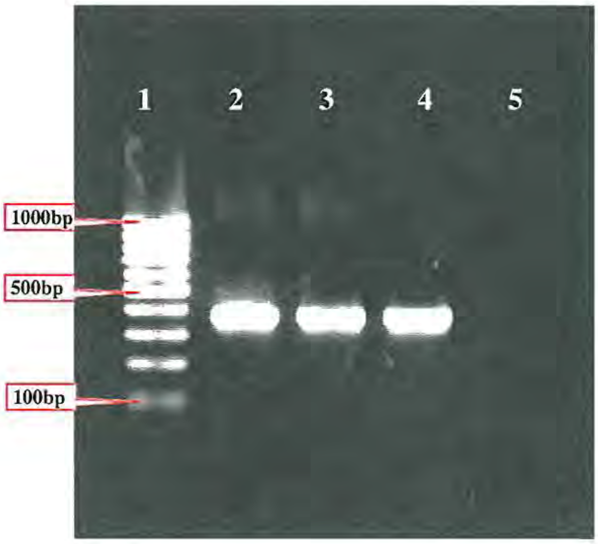


Figure 5.11: Ethidium bromide stained 1% agarose gel showing the amplification from the root samples of *Cynodon datylon* (Lanes 2-4) by TPF3 and TPR1 primers. Lane 1- 100bp ladder, Lane 5- Control with no template.

Both the light microscopy and molecular analyses of all the other plants inoculated continued until the 15th month when the nested PCR amplification of the genomic DNA extracted from the root of *Acacia erioloba* (Camel thorn) confirmed presence of the truffle in the root samples (Fig. 5.12).

Regardless of mycorrhizal inoculation technique used, no other plant root was colonised by *T. pfeilii.* Therefore, the only successful method of inoculation where the plant roots showed signs of colonisation was the use of spore slurry. Plants inoculated with the mycelial culture showed no sign of root colonisation when the roots were subjected to light microscopic and molecular analyses.

Figure 5.12: : Ethidium bromide stained 1% agarose gel showing the amplification from the root samples of *Acacia erioloba* (Lanes 2-4), Lane 1- 100bp ladder, Lane 5- Control with no template.

Table 5.8: Results of Mycorrhizal Synthesis (MS) Experiment (Wang and Qui, 2006). (+ Successful; 0 Not successful); EEM-Ectendomycorhizas, MS-Mycorrhizal Synthesis.

Scientific name	**Germination of seeds**	**MS using ascocarp slurry as inoculant**	**MS using mycelial culture as inoculant**	**MS using Petri dish method**	**Reported Mycorrhizal status of family members** (Wang and Qiu, 2006)
Poaceae:					
Stipagrostis ciliate	+	0	0		Mostly AM, <1% ECM
Schmidtia kalihariensis	+	0	0		
Panicum coloratum	0				
Cynodon dactylon	+	+	0		
Fabaceae:					
Acacia erioloba	+	+	0	0	86% AM, 7%, ECM, 7% ECM+EEM,,0.3% AM+EEM+ECM
Acacia erioloba **X** *haematoxylon*	+	0	0		
Acacia haematoxylon	+	0	0		
Acacia hebeclada	+	0	0		
Erythrina caffra	+	0	0	0	
Pinaceae:					
Pinus patula	+	0	0	0	Mostly ECM, rarely AM+ECM
Cucurbitaceae:					
Citrullus lanatus	+	0	0	0	AM
Citrullus vulgaris	0				
Oleaceae:					
Olea	0				Mostly AM, few ECM

5.4 Discussion

Knowledge about the host plants of *T. pfeilii* is still vague as there are few studies that have attempted investigations into the mycorrhizal status and the host plants. A study by Kagan-Zur *et al.* in 1999 that concentrated on the host plant and the mycorrhizal status was informative but failed to identify the exact phytobionts of *T. pfeilii*. Their study was not confirmatory because of the inability to reproducibly form mycorrhizas with any of the putative hosts. A reliable way of identifying a plant as a potential host of *T. pfeili* would be to inoculate and then isolate and identify the formed mycorrhiza (Pinkas, 2000).

The aims of artificial mycorrhizal synthesis are to produce mycorrhizal plantlets that can be transferred to the field where they can be grown to produce fruiting bodies, or for various research purposes such as investigation of the host plant / mycorrhizal status and some biochemical activities that occur during the process of mycorrhizal formation as well as to improve plant growth and health (Molina and Palmer, 1982). The most widely used method for truffle inoculation of plant seedlings is the use of spore inoculum (Iotti, 2002) while the mycelia obtained from the pure culture are mostly used for research purposes to investigate events during the preliminary colonisation period as well as biochemical response of the mycorrhizal fungus during colonisation (Giomaro *et al.*, 2005). The use of mycelial culture could also provide an opportunity of selecting strains that are better suited to various soil and environmental conditions as well as investigating compatibility with some host plants. However, the quickest method is the use of mother plant-technique because it requires neither spores nor mycelial culture, just an already colonised root (Giomaro *et al.*, 2005). This method is not very popular because of the high risk of contamination and the likelihood of the plant becoming colonised by the wrong fungus. Lack of knowledge about the host plants of *T. pfeilii* ruled out this option for this study.

Plants collected from the field and examined microscopically showed signs of colonisation by AM fungi with characteristic inter and intracellular colonisation and vesicle formation. There is no evidence to indicate that any truffle species can form vesicles or intra cellular hyphae, although intercellular hyphae would be a common feature of an endomycorrhizal relationship. Confirmation of the occurrence of AM funagal species was provided by Mycoroot (Pty) Ltd, Grahamstown Unit, South Africa. AM fungal spores of several AM fungi species (including *Glomus* and *Gigaspora*) were extracted from the soil collected around the area where *T. pfeilii* was harvested. It is not inconceivable that these host plants may be colonised by more than one mycorrhizal type (AM and the truffle). For instance, Chilvers *et al.*, (1987) reported the simultaneous colonisation of the root of a *Eucalyptus dumosa* by AM and ECM fungus. In addition, other plants such as *Acacia linifolia, Acacia ulicifolia* and *Afzeli bipidensis* have been reported to also form such (AM + ECM) associations (Wang and Qiu, 2006). In such cases, the morphological identification could be very difficult or even impossible if the truffle also forms an endomycorrhizal association. As a result, the design of specific primers for the molecular detection of *T. pfeilii* was essential.

Plants, such as *Citrullus vulgaris*, *Panicum coloratum* and *Olea* sp. that failed to germinate during this study require pregermination treatment. However, this was not investigated further as it was outside the scope of this study. *Pinus patula* was included as it is a well recognised host of several ECM fungi (Smith and Read, 1997). Inability of *T. pfeilii* to form mycorrhiza with *Pinus* could be an indication that this truffle exhibits specificity in forming mycorrhizal association.

As stated by Molina and Palmer (1982), positive synthesis results are conclusive and it is a confirmation of the host-fungus relationship while negative results are not confirmatory but raise doubts about the host-fungus association. The poor results (mycelial inoculation and petri-dish method) obtained from the mycorrhizal synthesis experiments could be attributed to several reasons. The Petri dish technique for mycorrhizal syntheses provided a method that enabled the frequent observation of the development of both the root and the fungi under the dissecting microscope (Gutierrez *et al.*, 1996). One of the possible reasons for the inability to establish a mycorrhizal association between the plants and the mycelial culture of *T. pfeilii* may be the attenuation of the mycelial culture used for the inoculation. Prolonged growth of ECM fungi on agar media has been indicated to affect the efficiency and colonising capability of ECM fungi (Thomson *et al.*, 1993). Another reason could be the strain compatibility of the mycelial culture with the plants in question. Possible existence of different strains of *T. pfeilii* (Roth-Bejerano *et al.*, 2004) means that some strains may lack the capability to initiate a successful colonisation of the root of host plants. This, however, may not be an important factor since both monokayotic and dikaryotic hyphae of *Terfezia* spp. have been reported as being able to colonise the root of the host plants (Roth-Bejerano *et al.*, 2004). The limited space offered by this method results in nutrients becoming rapidly depleted and the accumulation of toxic waste products. The synthesis experiments therefore cannot be conducted over a prolonged period of time. This may hamper the visual detection of any mycorrhizal root formation which could take 12 weeks (Molina and Palmer, 1982), All these factors may have also contributed to the failure of the *Paenibacillus* sp. to stimulate the mycorrhizal formation during the *in vivo* mycorrhizal synthesis / MHB experiment.

Cynodon dactylon roots are fine cortical tissue. This made observation of any external hyphal development such as mantle difficult to ascertain. The light microscopic observation of intercellular septate hyphae colonising the root of *Cynodon dactylon* (Fig. 5.5) was an indication that *T. pfeilii* can form an endomycorrhizal association. This is in agreement with the findings of Kagan-Zur *et al.*, 1999 where *Citrullus vulgaris* formed an endomycorrhizal association with *T. pfeilii*. In order to confirm this relationship, it was necessary to employ a more reliable molecular method. The use of other microscopy procedures such as Scanning Electron Microscope or Transmission Electron Microscope would have revealed more details about the type of mycorrhizal formation and development of the mycorrhizas. However, these techniques were not conducted in this study. Previously, Taylor *et al.*, (1995), observed *Cynodon* plants growing very close to *T. pfeilii* ascocarps, but could not confirm the mycorrhizal association. Generally, grasses (Poaceae) are considered to be endomycorrhizal, with a very few species of the grass family Poaceae, forming ECM associations (Table 5.8) (Wang and Qiu, 2006). ECM fungi are, in most cases, found colonising trees and some shrubs (Molina *et al.*, 1992; Wiensczyk *et al.*, 2002), but they can also associate with grasses as reported by Manske (2004), where unnamed ectomycorrhizal fungi colonised the root of some grasses, characterised by sheath formation but no penetration of the host tissue. Therefore, the colonisation of the *Cynodon* root by *T. pfeilii* in the synthesis experiments was unusual but not totally unexpected.

The investigated *in vivo* mycorrhizal synthesis under semi-aseptic conditions has provided the opportunity to supply nutrients under standard environmental conditions for the optimal growth of both symbionts. This type of experimental set up could also retard mycorrhizal formation as specific requirements for temperature, water and nutrients were not investigated although some soil characteristics such as pH and nutrient contents were analysed (Table 1.2). The data for these analyses were not varied to determine the optimal growth conditions in the tunnel.

The specific primers designed for the identification of *T. pfeilii* allowed for the establishment of a detection method which amplified *T. pfeilii* from root samples, thereby aiding in the search for the phytobionts. The problems associated with the determination of phytobionts of *T. pfeilii* (Kagan Zur *et al.*, 1999) could be associated to the lack of good identification methods. Morphological methods are not specific enough for the proper identification of mycorrhizas formed by *Terfezia* spp. as they may form endomycorrhiza or ectendomycorrhiza with or without mantle and Hartig net (Gutierrez, *et al.*, 1996).

The protocol developed earlier for the molecular identification of the ascocarps (Chapter 3) and the pure culture was a simple, single PCR step of identification using the newly designed primers TPF1/TPR1. Unfortunately, due to the inability of these primers to specifically amplify DNA from the root samples, other primers were designed. TPF2 was designed with Gene Runner software programme. This primer, when used alongside TPF1 could also not amplify specifically from the root samples. The two types of software used for the design of the primers provided good checks for the primer characteristics. The irrational binding of the primer pairs of TPF1 / TPR1 and TPF2 / TPR1 (Table 5.4) to some contaminant fungi in the root samples necessitated the use of another software programme, Primer3, which was more effective. This software programme also made use of the ITS regions of the aligned nucleotide sequences of both the pure culture and ascocarp (Fig. 5.9).

TPF3 and TPR2 were successfully used to amplify DNA from ascocarp and mycelial cultures of *T. pfeilii* (Fig. 5.6). The direct amplification of the DNA extracted from the root samples of *Cynodon dactylon* was weak due probably to PCR inhibitors present in the root samples (Sejalon-Delmas, 2000; Iotti and Zambonelli, 2006). This led to the design of additional techniques such as purification of the PCR products and a second PCR stage. These new techniques were more cost effective than sequencing when used to detect *T. pfeilii* mycorrhizas. This nested PCR approach with specific pair of primers (TPF3 / TPR2) amplified an inner target of the ITS region obtained from the first round (Martin and Rygiewicz, 2005). The purification step was introduced to eliminate the possible carry over of contamination from the first round PCR product (Diaz and Fell, 2005). The nested PCR was effective (Fig. 5.8), thereby signifying the success of the new rapid detection method for *T. pfeilii*. These primers were therefore most useful in the mycorrhizal synthesis experiments carried out because of its rapidity and affordability which allows for many root samples to be examined molecularly within a reasonably short period of time.

Three species of Terfeziaceae were initially reported in South Africa. Marasas and Trappe, (1973) referred to these as South African tuberales which included *Terfezia astroafricana, Terfezia pfeilii* and *Choiromycetes echinulatus*. Only the sequences of *T. pfeilii* and *C. echinulatus* were found in the GenBank of NCBI and EMBL (Wheeler *et al.*, 2006; Tamara *et al.*, 2006). The occurrence of *T. austroafricana* is rare species (van Wyk, personnal communication), further collection and identification is therefore necessary to substantiate the existence of this species and its relationship to *T. pfeilii*. Based on their close molecular and morphological characteristics, both *T. pfeilii* and *C. echinulatus* could be incorrectly identified. The degree of the specificity of the primers selected was tested in order to differentiate between samples of both *T. pfeilii* and *C. echinulatus*. Sequences of TPF3 and TPR2 primers were partially aligned with the ITS sequences of the 2 isolates of *T. pfeilii* earlier obtained in this study (Section 2.3.2), two different nucleotide sequences of *C. echinulatus* and other closely related desert truffles such as *C. alveolatus* and *T. terfezoides* were obtained from GenBank. The homology recorded between the sequences revealed that the sequence of TPR2 has a 97% identity to the sequence of *C. echinulatus* but this was not significant (Table 5.5) especially at the 3' end of the sequences, which is important for the annealing of primers. This discovery led to the consideration of the reverse primer TPR1. The sequence of TPR1 has an identical region on *C. echinulatus* sequence but not significant (Table 5.7). It is most unlikely that these primers will amplify successfully from *C. echinulatus* because of the fact that a good oligonucleotide sequence must have a minimum of 18 nucleotides (van

Tuinen *et al.*, 1998). However, van Tuinen *et al.*, (1998), pointed out that the efficiency of a good primer is best known when used in PCR reactions. Therefore to check for the efficiency of these primers, they were tested on preserved samples of both *T. pfeilii* and *C. echinulatus.*

At an annealing temperature of 63°C, TPF3 and TPR1 successfully amplified from only *T. pfeilii* samples but did not amplify from *C. echinulatus.* Below this annealing temperature (60°C), both the DNA from *T. pfeilii* and *C. echinulatus* were amplified, though of the bands of *C. echinulatus* samples were less intense. This emphasises the importance of establishing the correct annealing temperature in improving the efficiency of specific primers (van Tuinen *et al.*, 1998). Meanwhile, there is need to repeat these findings on more samples of *C. echinulatus* to confirm this result.

The efficiency of the primer pair TPF3 / TPR1 was further assessed on all the samples previously tested with TPF3 / TPR2. Separation on ethidium bromide stained 1% agarose gel showed that the expected length of 376 amplicon (Fig. 5.9; Fig. 5.10; Fig. 5.11; Fig. 5.12) were obtained, a confirmation that TPF3 and TPR1 are a good primer pair for the rapid detection of *T. pfeilii.*

The amplification of *T. pfeilii* from the root samples of *Acacia erioloba* (Fig. 5.12) obtained from the mycorrhizal synthesis experiment at the 15th month is a confirmation of the speculation that *Acacia erioloba* is a host plant of *Terfezia pfeilii.* The reason for the long duration of the mycorrhizal association to develop between *T. pfeilii* and the *Acacia* plants may be due to different environmental conditions that both organisms were subjected to in the growing tunnel, as compared to what is obtainable in the field. Another reason for the delayed formation of mycorrhiza could also be due to the concentration of inoculum used for the synthesis experiment (Brundrett *et al.*, 1996). It has been noted that inadequate application of the inoculum does affect the mycorrhizal formation. There was however, no microscopic confirmation of this result.

The molecular protocol developed in this study is very efficient, rapid and cost effective. However in future, efforts can be made to amplify directly from the mycorrhizal tissues of *T. pfeilii.* This has been done by Iotti and Zambonelli, (2006) where they amplified DNA directly from mycorrhizal tissues taken from the mantle formed by some *Tuber* spp. on the root of *Corylus avellana*. In their study, BSA was added to bind the PCR inhibitors. The efficiency of the exact protocol they used may not be possible with the mycorrhiza of *T. pfeilii* because of the absence of a mantle.

Due to the importance of these mycorrhizal synthesis experiments in the determination of a phytobiont of *T. pfeilii*, the success of this new protocol was verified through the sequencing of the nested PCR products. A sequencing result of 99% identity to *T. pfeilii* isolate of accession number AF301422 was obtained with BLAST on the NCBI website (Wheeler *et al.*, 2006) and was the climax of the investigations conducted in this study as this confirmed the phytobionts (*Cynodon dactylon* and *Acacia erioloba*) of *T. pfeilii* and the reliability of the new molecular method developed.

Terfezia spp. have have been reported to have preference for the members of the Cistaceae family (Gutierrez et al, 2003). Absence of this family in southern African where the Kalahari truffle thrives may be an indication of how distinct these truffles are from their counterparts. As already shown in the phylogenetic analyses of the desert truffles (Chapter 2), *T. pfeilii* does not belong to the same phylogenetic group as other *Terfezia* spp. Additionally, if truffles are to be classified according to their hosts, *T. pfeilii* would not belong to the *Terfezia* genus. This study has indeed challenged the knowledge base of the biology of *T. pfeilii.*

With the success recorded by Kagan-Zur *et al.*, (2001) in the introduction of a *Tuber* sp. to Israel, it would be interesting to investigate the possible cultivation of *T. pfeilii* in South Africa as a potential cash crop for rural communities. Collection could ensure reliable production and steady supply of *T. pfeilii* which would result in it being more appreciated throughout the world; making it a potential viable source of income for these impoverished communities.

CHAPTER SIX

6 Overall Discussion and Conclusion

6.1 General Discussion

6.1.1 Isolation and optimisation of physiological growth parameters of *T. pfeilii in vitro*

Laboratory isolation of the mycelial pure culture of *T. pfeilii* and subsequent optimisation of the growth parameters of the culture were essential to all the other experiments carried out in this study.

The present study focussed on an isolation technique that relied on mycelial growth from fresh ascocarps. In spite of the few problems associated with this method, such as the growth of contaminant microbes and the initial slow growth of the explants on FTN medium, it was interesting to follow how the FTN medium was able to support growth of this truffle. Growth comparison of *T. pfelii* on FTN medium with other popular fungal media used (PDA and MMN), showed that FTN medium had the best nutrient formulation to support growth of *T. pfeilii*.

The ascocarps of *T. pfeilii* were identified morphologically in the field. The morphological identification was only preliminary because of the existence of closely related, morphologically similar species previously reported in South Africa (Marasas and Trappe, 1973). Also for a complete identification of mycelial cultures, morphological identification was far from sufficient. The molecular method identification of plate cultures using species specific ITS primers proved to be a more reliable method of identification. The use of both morphological and molecular methods of identification is recommended.

Of all the physiological growth parameters tested, only the nitrogen source significantly increased the growth of the *T. pfeilii in vitro*. The optimal temperature for mycelial growth proved to be 32°C. This temperature correlates well with the temperature of the natural environment (Upington) of these samples. Considering the two methods (colony diameter and biomass) used to evaluate the effects of the tested parameters on the growth of *T. pfeilii*, it was difficult to give one preference over the other. High values of colony diameter is an indication that the truffle may spread faster to colonise the root of a host plant (Garbaye, 1994), while high quantity of biomass is required during artificial inoculation of plants. Only BSA added as a N source successfully stimulated the growth of *T. pfeilii* both in terms of biomass and colony diameter on FTN medium. This is an indication that *T. pfeilii* can produce the enzyme necessary to release inorganic N from the BSA. Compounds similar to BSA could be present in soil in the form of decaying organic matter from animals. This was reported by Yamanaka (1999) where the growth of *Hebeloma* spp. was enhanced on both BSA *in vitro* and soil containing decomposed dead bodies and faeces of animals. The ability of BSA to promote the growth of *T. pfeilii* is of great interest in respect to the nutrient acquisition from the soil but the chief importance of this process, is how much of this nitrogen will the fungus release to the host plant to enhance its role in the mycorrhizal association (Abuzinadah *et al*,1986).

6.1.2 Development of a rapid molecular identification method and molecular phylogenetics investigation of the classification of *T. pfeilii*

The usefulness of molecular methods to compliment and sometimes validate results from the traditional morphological methods of identification (Giomarao *et al.*, (2005) has once again been proven. This is the first report of such a rapid and the cost-effective molecular technique for *T. pfeilii*. The ability of the designed primers to amplify from a variety of *T. pfeilii* ascocarps, pure cultures and mycorrhizal root samples is an indication that the intraspecific variability of this truffle is low. Therefore there is a low level of polymorphism in this species (Mello *et al.*, 1999). The newly designed primers were specific enough to detect and amplify from the root samples in the presence of other contaminant fungi. Addition of BSA was very effective in binding the PCR inhibitors and increasing the success of the amplification.

Elimination of the post PCR stages especially nucleotide sequencing significantly reduced the cost and accelerated the process of identification, therefore making it easy to examine or identify more samples within a shorter period of time.

The Kalahari truffle is not as economically important as forest truffles (*Tuber*), but it can still be mistaken for other members of the same family which are not edible especially *Choiromyces echinulatus*, as reported by Ackerman *et al.*, (1975). The development of this rapid method will definitely assist in investigating any cases of food poisoning that may result in the consumption of similar species of this truffle. Furthermore, it will also aid in combating fraud especially in the form of substituting similar species for the Kalahari truffle and selling them on the open markets. This fraudulent activity is currently being experienced with some *Tuber* spp. (Douet *et al.*, 2004).

The proposed molecular methods cannot be used to identify intraspecific variations that exist within species of some truffles. In such situations, RFLP can be a better alternative and sequencing will reveal most of the information needed to confirm the variation. The capability of methods such as RFLP to detect intraspecific variations could lead to confusing results when used for the identification of *T. pfeilii*. Different patterns of enzymatic digest can easily be misinterpreted as different organisms rather than the strains of the same species as was reported by Kagan-Zur *et al.*. (1999).

The result obtained after the addition of South African samples for the molecular phylogenetic reconstruction is strong evidence that this truffle does not belong to the same genus as other *Terfezia* spp. as previously reported by Ferdman *et al.* (2005).

6.1.3 Potential Mycorrhization Helper Bacteria associated with the ascocarp of *T. pfeilii*

In the last decade or so, especially after Garbaye (1994) suggested possible modes of action of MHB, mycorrhizal helper bacteria have received increasing attention which has led to the hypothesis that mycorrhizal associations do not just involve the fungus and plant but bacteria as well, forming a tripartite association. This study has isolated and identified different bacterial associates of *T. pfeilii*, which could be capable of enhancing mycorrhizal formation. The *Paenibacillus* sp. isolated has shown the greatest potential to be a MHB having significantly stimulated the growth of the fungus on FTN, PFTN and WA media. This is not the first time that *Paenibacillus* spp. have been reported as a MHB, it has also been previously isolated from ectomycorrhizal associations (Poole *et al.*, 2001). The discovery of other potential MHB such as *Bacillus* sp. and *Rhizobium tropici* is an indication of a possible co-existence of these bacterial isolates in the mycorrhizosphere. Though their roles could be affected by other factors in the field, nevertheless, they possess good features capable of stimulating mycorrhizal formation. Association of such bacteria to the ascocarp of *T. pfeilii* could be interpreted as an indirect proof that *T. pfeilii* can form a mycorrhizal association.

6.1.4 Identification of host plants of *Terfezia pfeilii* using molecular and morphological methods

Knowledge about host plants of *T. pfeilii* has eluded scientists for a long time. Concerted efforts to investigate the host plants of this truffle by Taylor *et al.*, (1995) and Kagan-Zur *et al.* (1999) has provided background knowledge, which enormously benefited this study. The design of specific primers to identify possible host plants from the field and to monitor the success of mycorrhizal synthesis experiments increased the number of samples that could be investigated within a shorter period of time. The expectation that the mycorrhizal formation of *T. pfeilii* would be ECM made it difficult to confirm that the endomycorrhizal colonisation observed in the root of *Cynodon dactylon* observed microscopically was actually *T. pfeilii* or a combination of AM and another ECM. This necessitated a confirmatory identification method of these mycorrhizas. The molecular identification of the endomycorrhizas on *Cynodon* root tips, using species specific ITS primers, confirmed that (i) *T. pfeilii* may form endomycorrhiza with some host species (ii) *Cynodon* species is a mycorrhizal symbiont of *T. pfeilii* as was earlier suspected by Taylor *et al.* (1995). This molecular method was also successful in proving *Acacia erioloba* as a host plant. *A. erioloba* could possibly be a better host plant for *T. pfeilii* because it is a perennial plant compared to the *Cynodon* grass that is an annual, but this would require further investigation.

The hypothesis that *T. pfeilii* forms an ECM association could not be confirmed in this study, *T. pfeilii* mycorrhizas were identified from *Cynodon dactylon* and *Acacia erioloba,* both belonging to plant families that are generally regarded as being AM.

6.2 Summary

This study has successfully investigated and identified two host plants of *T. pfeilii*. The traditional morphological method of identifying truffles cannot be disregarded as initial identification in the field is necessary. This, together with molecular methods enabled a good starting point for the whole study. The accurate identification of the ascocarp confirmed the identity of the pure culture of *T. pfeilii*. Pure cultures obtained could also be produced within a shorter period of time as confirmed through the investigation of physiological growth parameters. It has become clear that temperature, pH and source of nitrogen are very important factors in the *in vitro* growth of *T. pfeilii*.

The need to always confirm the identity of the starter culture for all the experiments carried out in this study was recognised. This was especially important in investigating the host plants through mycorrhizal synthesis experiments. This, therefore, necessitated the development of a reliable and rapid PCR-based molecular method of identification.

The inclusion of the South African harvested ascocarps of *T. pfeilii* in the phylogenetic reconstruction of MP and NJ trees of selected desert truffles confirmed that South African *T. pfeilii* does not belong to the *Terfezia* genus. This study therefore supports the proposed reclasssification of *T. pfeilii* as *Kalaharituber pfeilii* by Ferdman *et al.* (2005).

Bacteria that are beneficial to mycorrhizas have been reported to exist in close proximity to them and can be isolated from their fruiting bodies (Garbaye, 1994). A number of bacteria isolates were obtained from the ascocarps of *T. pfeilii* and some showed one or more characteristics of being MHB, such as the ability to improve the mycelial growth, production of indole and the ability to solubilise the complexed inorganic phosphate *in vitro*. There was a strong emphasis on the assumption that MHB exhibit their aiding effects on mycorrhizal formation by directly promoting the growth of the fungus. The investigation of this characteristic of MHB *in vitro*, indicated that some of the bacteria, especially *Paenibacillus* sp., are capable of stimulating the growth of *T. pfeilii* in culture. Other bacterial isolates such as *Bacillus* sp. and *Rhizobium tropici* also produced stimulatory effects on the growth of *T. pfeilii* cultures. A phosphate solubilising and four indole producing bacterial isolates were also obtained from the ascocarps. This study has therefore highlighted the roles of such bacteria in the ecosystem and the significant effects they could have on mycorhizal formation and development of the fruiting bodies. These bacteria are all potential MHB that needs further investigation.

Many host plants have been suggested for *T. pfeilii* (Taylor *et al.*, 1995) but none of them has been confirmed. Although this can be attributed to many problems, this study has made use of three different inoculation techniques using both mycelial culture and spore inoculum to inoculate the plants. The inability of the mycelial culture to form any mycorrhizal association could be due to loss of viability during sub-inoculation of the pure culture. The use of freshly isolated pure culture could probably solve this problem. Ascocarp inocula used as a slurry was most successful in the mycorrhizal synthesis experiment of *T. pfeilii*. A combination of both morphological and molecular techniques facilitated the confirmation of *Cynodon dactylon* and *Acacia erioloba* as host plants of *T. pfeilii*.

Some of the results obtained and presented in this study were mostly carried out under controlled or semi-controlled conditions. This did not allow for adjustment of environmental factors that can influence the growth and development of mycorrhizal fungi. Investigations into how various biotic and abiotic factors in the environment affect the development of this mycorrhizal relationship requires further investigation.

6.3 Recommendations and future work

It is pertinent to mention that, although this study has provided more insights into the biology of *T. pfeilii*, replication of such results in entirely natural conditions will require further studies to cater for both biotic and abiotic factors that are uncontrollable under natural conditions.

There is a need to extend the investigation of the physiological growth parameters of *T. pfeilii* to the field especially the effects of pH and nitrogen requirements on this species. Though it has been reported that field pH does not always correspond to the pH at which ECM fungi optimally grow *in vitro* (Hung and Trappe, 1983), the exact factors responsible for this have not been ascertained. It would therefore be useful to investigate, in conjunction with other factors such as nutrient utilisation, the optimal conditions for growth that may directly or indirectly be influenced by the pH. It is also important to intensify efforts in confirming the existence of different strains of *T. pfeilii*. If these strains do exist, they are most likely to have different nutrient requirements (Daza *et al.*, 2006) and may be important for the successful inoculation of the host plants.

It is difficult to confirm the identity of truffles with similar morphology as this requires an in-depth knowledge to discern between species. However, morphological identification is necessary for the initial sorting of species, especially in the field and has over the years provided a foundation for other methods of identification, especially molecular methods. Failure to amplify *T. pfeilii* from the plant root samples collected from the field does not rule out possibility of any of these plants to be symbionts of *T. pfeilii*. There is a possibility of DNA degradation of fungal tissue during transportation of root samples from the field to the laboratory. It would therefore be recommended to transport the root samples by storing in liquid nitrogen (freezing temperature) in order to prevent the possibility of degradation of DNA in the colonised root samples. Alternatively, root samples could be processed immediately on site. Additionally, time length and number of stages involved in the extraction protocols could also be reduced to eliminate possibility of DNA degradation (Iotti *et al.*, 2002). The rapid molecular technique developed in this study could be improved by eliminating the DNA extraction stage and amplify directly for the fungal tissues.

Furthermore, it is necessary to extend the phylogenetic analysis of *T. pfeilii* to include more species of South African truffles and a larger number of different *T. pfeilii* sequences as this will increase the reliability of the results. Use of more analyses such as MrBayes (Hall, 2001) rather than only NJ and MP may also help in this regard. However, this study recommends the adoption of the new classification of *T. pfeilii* as *Kalaharituber pfeilii* as proposed by Ferdman *et al.*, (2005).

Though the spore inoculation method used in this experiment was successful, this might not be the best method to use as it was difficult to maintain semi-aseptic conditions which led to contamination. The presence of microrganisms that always associate with the ascocarps of varioustruffles (Barbieri *et al.*, 2005) may also introduce contamination. However, inoculation with the correct strain of mycelial culture, capable of colonising the plant could be used to establish a faster colonisation. Plants that were colonised by *T. pfeilii* should be transferred to the field and monitored and investigated for their ability to produce fruiting bodies. There is need to allocate more attention to microscopy in the area of identification to ensure successful determination of the mycorrhizal type it forms with other plants. SEM and TEM of both *Cynodon* and *A. erioloba* roots will definitely reveal more information about the type of mycorrhizal association that *T. pfeilii* formed with these plants. Additionally, molecular methods designed in this study could be used to investigate the environmental fate of inoculated plant seedlings.

Finally, the cultivation and exportation of *T. pfeilii* would increase its popularity and acceptance worldwide as indicated in this quote from an article in Los Angeles Times- "Kalahari truffles are cheap in Africa because it is Africa: The collectors are often poorly paid or ask little for their goods. But if the Kalahari truffle ever found its way into the exotic street markets of Paris or Rome, it would doubtless create ripples of excitement, curious buyers and higher prices — if not the sky-high prices of European black or white truffles" (Dixon, 2006).

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APPENDICES

Appendix I: Media and Reagents

Fontana medium (Fontana, 1968)

D-glucose	6.5
peptone	65g
casein hydrolysate	0.33g
potassium dihydrogen orthophosphate, KH_2PO_4	0.33g
Magnessium sulphate heptahydrate, $MgSO_4.7H_2O$	0.15ml of 1% solution
Iron (II) chloride, $FeCl_2$	0.15ml of 1% solution
Zinc sulphate, $ZnSO_4$	0.15ml of 1% solution
Magnessium sulphate -$MnSO_4$	0.15ml of 1% solution
Calcium Chloride, $CaCl_2$	1.65ml of 1% solution).

Phosphate Solubising Medium (Mehta *et al.*, 2001)

This medium consisted of the following per liter:

glucose	10g
$CaHPO_3$	5g
magnessium chloride hexahydrate $MgCl_2.6H_2O$	5g
magnesium chloride heptahydrate ($MgCl_2.7H_2O$)	0.25g
potassium chloride (KCl)	0.2g
$(NH_4)_2 SO_4$	0.1g and
Agar	15g

Modified Melin-Norkrans Agar (MMN) (Marx, 1969)

KH_2PO_4	0.5g
$MgSO_4.7H_2O$	0.15g
$CaCl_2.2H_2O$	0.05g
NaCl	0.025g
$(NH_4)_2HPO_4$	0.15g
Tiamindichloride	5mg/300ml
$FeCl_3.6H_2O$	1.2cm-3 / l
Glucose	2.5g

Malt Extract	10g
Agar	15g

Indole acetic acid (IAA) test media

Nutrient broth	8g / l
Tryptone powder	8g / l

Salkowski Reagent

$FeCl_3$	12g / l

in 7.9 M H_2SO_4

10% SDS (sodium dodecyl sulphate)

10g SDS

100 ml distilled water

Warm to 65°C to allow SDS to dissolve.

TE (Tris/EDTA) buffer pH 8.0

Tris/HCl pH 8.0	10mM
EDTA pH 8.0	10mM

Appendix II: Nucleotide Sequences of Bacterial Isolates and the *T. pfeilii* Detected in the Root of *Acacia Erioloba*

>***Strenotrophomonas*** sp.

```
CTGTCTTGCGACCGTACTCCCCAGGCTTCGAACTTAACGCGATAGCTTCG
ATACTGCGTGCCAAATTGNACCCAACATCCAGTTCGCATCGTTTAGGGCG
TGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTC
AGTGTCAATGTTGGTCCAGGTAGCTGCCTTCGCCATGGATGTTCCTCCTG
ATCTCTACGCATTTCACTGCTACACCAGGAATTCCGCTACCCTCTACCAC
ATTCTAGTCATCCAGTATCCACTGCAGTTCCCAGGTTGAGCCCAGGGCTT
TCACAACGGACTTAAATAACCACCTACGCACGCTTTACGCCCAGTAATTC
CGAGTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTA
GCCGGTGCTTATTCTTTGGGTACCGTCATCCCAACCAGGTATTAGCCGGC
TGGATTTCTTTCCCAACAAAAGGGCTTTACAACCCGAAGGCCTTCTTCAC
CCACGCGGTATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCA
CTGCTGCCTTCCCGTANG
```

>***Rhizobium tropici*** **(KB06G)**

```
ACGTAGGNATGGGCGCAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAG
GCCTTCGGTCNNGTAAAGCCCTTTNGTTGGGAAAGAAATCCAGCCGGCTA
ATTCCTGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCG
TGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACT
GGGCGTAAAGCGTGCGTAGGTGGTTATTTAAGTCCGTTGTGAAAGCCCTG
GGCTCAACCTGGGAACTGCAGTGGATACTGGATGACTAGAATGTGGTAGA
GGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGA
ACATCCATGGCGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGNAGTCCACGCCCTAA
ACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCNAAGCTA
ACGCGTTAAGTTCGCCGCCT
```

>***Rhizobium tropici*** **(KB06NC)**

```
AATTCTTGCGACCGTACTCCCCAGGCTNCTNTGTTTAATGGGAAAGCTGC
GCCACCGAACAGTATACTGTTCCNACGGCTAACATNCATCGTTTACGGCG
TGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTC
AGCGTCAGTAATGGACCAGGGAGCCGCCTTCGCCACTGGTGTTCCTCCNA
ATATCTACGAATTTCACCTCTACACTNGGAATTCCACTCACCTCTTCCNT
ACTCCAGATCGACAGTATCAAAGGCAGTTCCNGGGTTGAGCCCTGGGATT
TCACCCCTGACTGATCGATCCGCCTACGTGCGCTTTACGCCCAGTAATTC
CGAACAACGCTNGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTA
GCCGGGGCTTCTTCTCCGGATACCGTCATTATCTTCTCGGGTGAAAGAGC
TTTACAACCCTAAGGNCTTCATCACTCACGCGGCATGTTT
```

>***Curtobacterium sp.***

```
GCATGGGCGAAGCCTGATGCAGCAACNCCGCGTGAGGGATGACGGCCTTC
GGCCTTGTAAACCTCTTTTAGTAGGGAAGAAGCGAAAGTGACGGTTCCTG
CNGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GTGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGNTT
GTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTCGGGCTTGCAGTGGGT
ACGGGCAGACTANAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGG
TGGAATGCGCAGATATCANGAGGAACACCGATGGCGAAGGCAGATCTCTG
GGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGTTGGGCGCTAGATGTAGGGACCT
```

TTCCACGGTTTCTGTGTCGNAGCTAACGCAAGTAAGCGCCCCGCCTGGGG
AGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGAC

>Enterobacter cloacae
GAAGGATGNTGNGCAGNNCTGAATGCANNTANAACCTTTGGCGGCCGNTA
CTCCCCAGGTCGTNTCGTACTTAACGACGATTAGGCTCCGAGGAAGCCAC
GACCTCAAGGANTNCNACCTCCAAGTTCGNACATCGCTTTACGGAAGNGG
CACTACCAGGNCTATCTAATCCTGATTTGCTCCCCACGCTTNCGNANCTG
AGCGTNANCCTNNNTACAGGGGGNCGACTTCGCCACCGGTATTCNTCCAG
ATCTATNCGAANTNCNCNGNTNCANCNNGAANNCTNCCNCCCNCNANGAG
ACNCTTGACTGTCAGTATACGGATGCAGAANNCNAGGTGAACCCGGGAAN
TNCACATACAACTNGACAGAANNCCCTGNGNGAACNTTNCCCCCNGGANT
AACAATNANCNNNNGGNNCCTNNGNATNACCGAGCAANGNTGATTAGATA
CCCTGNCNAGTGCTTCTTCTNNANGAAANCTAATTNGACGCTGTGATNAN
TGAGGTNTGCCTTNCTCNACGTTAAAAGTANNTTANACCCCNNNGGNNTT
CTTGATACANGGTNAATGNTNNAATCAANCTTGCGCCCATTGTGGCATTA
TTCCCCACTGCTGCCTCCCGTAGNAGA

>*Bacillus* sp.
CAGCCTTGCGGCCGTACTCCCCAGGCGGAG
TGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTT
AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTT
CGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGA
ATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTC
CACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCG
CGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCG
CGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAG
GTGCCAGCTTATTCAACTAGCACTTGTTCTTCCCTAACAACAGAGTTTTA
CGACCCGAAAGCCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCG
TCCATTGCGGAAGATTCCCTACTGTGGCCTCCCGGTAGGA

>Paenibacillus lautus
AAGTCTGACGGAGCAACGCCGCGTGAGNGATGAAGGTTTTCGGATCGTAA
AGCTCTGTTGCCAGGGAAGACGTCTTCTAGAGTAACCGCTAAGAGAGNGA
AAATACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGNA
ATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGC
AGGCGGGTCTTTAAGTTGGTGTTTAACCCGAGGCTCAACTTTGGGTCGCA
TTGTAAACTGGGGGACTTGAGTGCAGAAGAGGAGAGGGGAATTCCACGTG
TAGCCGGTGAAATGCGTACATATGTGGAGGAACACCAGTGGCGAAGGCGA
CTCTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGCAAACGATGAATGCTAGGTGTT
AGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCATTCCGCC
TGGGGA

>Nucleotide sequence obtained from the root sample of *Acacia erioloba*
TGGTGCTCGGTCCATTGCTGGGGAGTTTGCCGGTGGGTAGCCCCTTTTAT
AATCAAAACCTGTGTAATAGAGAAACCTTNTTGTCTGATATTAAATGAAA
TAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCA
TCGATGAAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCT
CGTGAATCATCGAATCTTTGNACGCACATTGCGCCCTATGGTATTCCGTA
GGGCATGCCTGTCTGAGCGTCAGCATCACCTCTCATAAGCAGCCATTTAT
TTCTTTGAGTGGTTCTGTATTTGAGGACTCATGGGAATAAGAAGGTA

Appendix III: Nucleotide Sequence Alignments Used in the Phylogenetics Analysis of *T. pfeilii*

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[                                10         20         30         40         50         60
T. pfeilii-P             GGATCATTAT TG-------- -AGTAA-GCT TTATTGTAGC TTTCTCTCTT ATCCCT-TTG   [49]
T. pfeilii _a            GGATCATTAT TG-------- -AGTAA-GCT TTATTGTAGC TTTCTCTCTT ATCCCT-TTG   [49]
T. pfeilii _b            --ATCATTAT TG-------- -AGTAA-GCT TTATTGTAGC TTTCTCTCTT ATCCCT-TTG   [47]
T. pfeilii _S            GGATCATTAT TG-------- -AGTAA-GCT TTATTGTAGC TTTCTCTCTT ATCCCT-TTG   [49]
T. claveryi _3           GGATCATTAC TG-------- -AAAAACTAT ATCAAGT--- --TTTTTTAT ACCCTT-TTG   [45]
T. claveryi _4           ------TAAC CG------GA GGACAA---- -----GT--- --TTTTTTAT ACCCTA-TTG   [33]
T. arenaria _5           GGATCATTAC TG-------- -AAAAACTAT TTTTAAG--- --TTTTTTAT ATCCCA-TTG   [45]
T. arenaria _6           GGATCATTAC TG-------- -AAAAACTAT TTTTAAG--- --TTTTTTAT ATCCCA-TTG   [45]
T. boudieri _7           GGATCATTAC TG-------- AAAAAGTATT TTTAAG---- --TTTTTTAT ATCCCA-TTG   [45]
T. boudieri _8           GGGTCATTAC TG------AA AAAGTATTTT TTTAAGT--- ---TTTTTAT ATCCCA-TTG   [47]
T. boudieri _9           GG-TCATTAC TG-------- -AAAAACAAT TTTAAG---- --TTTTTTAT ATCCCA-TTG   [43]
T. boudieri _10          GGATCATTAC TG------AA AAACTATTTT TTTAAGT--- ---TTTTTAT ATCCCA-TTG   [47]
T. leptoderma _11        GGATCATTAC TG------AA AAAAAAC--T ATCTAGAT-- ---TTTTTAT ATCCCA-TTG   [46]
T. leptoderma _12        GGATCATTAC TG------AA AAAAAAC--T ATCTAGAT-- ---TTTTTAT ATCCCA-TTG   [46]
T. olbiensis _13         ---------- ---------- ---AAACTCT TTCAAGT--- ---TTTTTAT ATCCCA-TTG   [30]
T. olbiensis _14         ---------- ---------- ---AAACTCT TTCAAGT--- ---TTTTTAT ATCCCA-TTG   [30]
T. pinoyi _15            GGATCATTAA TG-------- -AAAAACTAC TACAAGT--- ---TTTTTAT ATCCCA-TTG   [44]
T. nivea _16             GGATCATTAA AG-------- -AAAAACTAC AAGTTTT--- ----TTTTAT ATCCCA-TTG   [43]
C. echinulatus-12        GGATCATTAC TG-------- AAAAAACTAT CTCTTAGT-- ---TCTTCAT ATCCTA-TTG   [46]
C. echinulatus-44894     GGATCATTAC TG-------- AAAAAACTAT CTCTTAG--- --TTCTTCAT ATCCTA-TTG   [46]
M. terfezoides _19       GGATCATTAA TG---AAAAA AAAAAATGCT ATTTTCATTG GCCTATCAAA ATCCCTCTTG   [57]
M. terfezoides _20       GGATCATTAA TG---AAAAA AAAAAATGCT ATTTTCATTG GCCTATCAAA ATCCCTCTTG   [57]
Tuber melanosporium      GGATCATTA- TG--AATGCT TTGGGATACC GTGGTGTATT CCCGAACACA AACCTCTCTG   [57]
C. venosus _24           GGGGTCGCCC GACGGT---A TC-AAA---- ---------- --------CA A-CCCCCC-G   [32]
C. alveolatus _25        GGGGACGCCT GACGGTGTTA TC-AGA---- ---------- --------CA --CCCCACTG   [35]


[                                70         80         90        100        110        120
T. pfeilii-P             TTTACTTTAC CCTGTTGCTT CCAC-TGGAC AGTGTGAGCT TTGCTGGCAG -TTGAAGAAG   [107]
T. pfeilii _a            TTTACTTTAC CCTGTTGCTT CCAC-TGGAC AGTGTGAGCT TTGCTGGCAG -TTGAAGAAG   [107]
T. pfeilii _b            TTTACTTTAC CCTGTTGCTT CCAC-TGGAC AGTGTGAGCT TTGCTGGCAG -TTGAAGAAG   [105]
T. pfeilii _S            TTTACTTTAC CCTGTTGCTT CCAC-TGGAC AGTGTGAGCT TTGCTGGCAG -TTGAAGAAG   [107]
T. claveryi _3           TTTACC-TAC CCTATTGCTT CCAC-TGGAC AG-----G-T TT-----CAC CTT-------   [85]
T. claveryi _4           TTTACC-TAC CCTATTGCTT CCAC-TGGAC AG-----G-T TT-----CAC CTT-------   [73]
T. arenaria _5           TTTACC-TAC TCTGTTGCTT CCAC-TGGAC AG-------- ---------- ----------   [75]
T. arenaria _6           TTTACC-TAC TCTGTTGCTT CCAC-TGGAC AG-------- ---------- ----------   [75]
T. boudieri _7           TTTACT-TAC CCTGTTGCTT TCAC-TGGAC AG------AT TT-----CAC CTT-------   [85]
T. boudieri _8           TTTACT-TAC CCTGTTGCTT TCAC-TGGAC AG------AT TT-----CAC CTT-------   [87]
T. boudieri _9           TTTACT-TAC CCTGTTGCTT TCAC-TGGAC AG------AT TT-----CAC CTT-------   [83]
T. boudieri _10          TTTACT-TAC CCTGTTGCTT TCAC-TGGAC AG------AT TT-----CAC CTT-------   [87]
T. leptoderma _11        TTTACT-TAC CCTGTTGCTT CCAC-TGGAC TG------AT TT-----CAC CTT-------   [86]
T. leptoderma _12        TTTACT-TAC CCTGTTGCTT CCAC-TGGAC TG------AT TT-----CAC CTT-------   [86]
T. olbiensis _13         TTTACT-TAC CCTGTTGCTT CCAC-TGGAC AG------AT TT-----CAT CTT-------   [70]
T. olbiensis _14         TTTACT-TAC CCTGTTGCTT CCAC-TGGAC AG------AT TT-----CAT CTT-------   [70]
T. pinoyi _15            TCTTACCTAC CCTGTTGCTT CCAC-TGGAC A-TG-----T TTT-----AC CTTT------   [86]
T. nivea _16             TTTTACCTAC CCTATTGCTT CCAC-TGGAC A-TG-----T TTT-T----C CTTTT-----   [86]
C. echinulatus-12        TTAACT-TAC CATGTTGCTT CCACGTGGAC A--G-G---- ---CT--CAC CCT-------   [86]
C. echinulatus-44894     TTAACT-TAC CATGTTGCTT CCACGTGGAC A--G-G---- ---CT--CAC CCT-------   [86]
M. terfezoides _19       TCATACTTAC CTTGTTGCTT CCAC-TGGAC TA-G------ TTG-G--CAT TCTT------   [100]
M. terfezoides _20       TCATACTTAC CTTGTTGCTT CCAC-TGGAC TA-G------ TTG-G--CAT TC--------   [98]
Tuber melanosporium      CGTATCACTC CATGTTGCTT CCAC-AGGTT AA-GTG---- ---------- ----------   [91]
C. venosus _24           TGTACCTCTC CACGTTGCTT CCCC-AGG-- --------C- ---C-GG--- -------CAG   [66]
C. alveolatus _25        CGTACCTCTC CATGTTGCTT CCCC-AGG-- ---------- ----T--CA- ---G---CAG   [69]


          [                     130        140        150        160        170        180
T. pfeilii-P             TTCAATTGTA G-CAAGTGAG CCCTCTGGTT TTGG-TG-CA CTC----GGT ACC-ATTGCT   [159]
T. pfeilii _a            TTCAATTGTA G-CAAGTGAG CCCTCTGGTT TTGG-TG-CA CTC----GGT ACC-ATTGCT   [159]
T. pfeilii _b            TTCAATTGTA GGCAAGTGAG CCCTCTGGTT TTGG-TG-CA CTC----GGT ACC-ATTGCT   [158]
T. pfeilii _S            TTCAATTGTA G-CAAGTGAG CCCTCTGGTT TTGG-TG-CA CTC----GGT GCC-ATTGCT   [159]
T. claveryi _3           -----GTGTG G--AA----- -CCTCTGGCT TGTG-TGAAA AGC----ACC TG------CC   [121]
T. claveryi _4           -----GTGTG G--AA----- CCCCCTGGCT TGTG-TGAAA AGC----ACC TA------CC   [110]
T. arenaria _5           -----GTTTT --CA------ CCCTTTGGCT TATG-TGAAA AAC----ATC TG------CC   [111]
T. arenaria _6           -----GTTTT --CA------ CCCTTTGGCT TATG-TGAAA AAC----ATC TG------CC   [111]
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T. boudieri _7            -----GTGTG --------GG TCCTTTGGCT TGTG---TGA AAT----ACC A---CTTGCC   [122]
T. boudieri _8            -----GTGTG G----GTGGG TCCTTTGGCT TGTG--TAAA AAT----ACC A---CCTGCC   [129]
T. boudieri _9            -----GTGTG --------GG TCCTTTGGCT TGTG---TGA AAT----ACC A---CTTGCC   [120]
T. boudieri _10           -----GTGTG G----GTGGG TCCTTTGGCT TGTG--TAAA AAT----ACC A---CCTGCC   [129]
T. leptoderma _11         -----GTGTG -------GGA TCTTTTGGCT TGTG-TGAAT A--------- ACA-CCTGCC   [123]
T. leptoderma _12         -----GTGTG -------GGA TCTTTTGGCT TGTG-TGAAT A--------- ACA-CCTGCC   [123]
T. olbiensis _13          -----GTGTG -------GAA TCTTTTGGCT TGTG-TGAAT AAT----A-- ----CTCGCC   [107]
T. olbiensis _14          -----GTGTG -------GAA TCTTTTGGCT TGTG-TGAAT A--------- ATA-CTCGCC   [107]
T. pinoyi _15             -----GTGTA A-------GG ACCTTTGGCT TGTG-TGAAT AAC----T-- ----CATGCC   [123]
T. nivea _16              -----GTGTG -------AGA ACCTTTGGCT TGTG-TGAAT AAC----T-- ----CAGGCC   [123]
C. echinulatus-12         -----GAGTG --------AG CCCTTTGGTA TAGTGTGAAT ATT----TT- CATACATTCC   [128]
C. echinulatus-44894      -----GAGTG --------AG CCCTTTGGTA TAGTGTGAAT ATT----TT- CATACATTCC   [128]
M. terfezoides _19        -----ATG-- TTT--TATAG AATGTTGACT CTCG---AGC GTC------G TTTG-GGGCT   [141]
M. terfezoides _20        -----TTATG TTT--TATAA AATGTTGACT CTCG---AGC GTC------G TTTG-GGGCT   [141]
Tuber melanosporium       ---------- ---------A CCATGCTGG- CA-------- ---------- ----------   [103]
C. venosus _24            CCATCGCCTG ---------- CCC---GGCC CACG--ACCC ATC-CGGGGC GTG--TGGCC   [108]
C. alveolatus _25         CCTTTGCCTG ---------- CCC---GGC- -ATGC---CG ---------- ----------   [91]


[                                190        200        210        220        230        240
T. pfeilii-P              GGGGAGTTTG CCGGTGGGTA G----CCCCT TTTATAATCA AAACCTGTGT AATAGAGAAA   [215]
T. pfeilii _a             GGGGAGTTTG CCGGTGGGTA G----CCCCT TTTATAATCA AAACCTGTCT AATAGAGAAA   [215]
T. pfeilii _b             GGGGAGTTTG CCGGTGGGTA G----CCCCT TTTATAATCA AAACCTGTGT AATAGAGAAA   [214]
T. pfeilii _S             GGGGAGTTTG CCGGTGGGTA G----CCCCT TTTATAATCA AAACCTGTGT AATAGAGAAA   [215]
T. claveryi _3            AGAGAGTT-G CTGGTGGGAA GAA--C---T ATTT--ACTA AAACTTG-AT TTTTAAAAAA   [172]
T. claveryi _4            AGAGAGTT-G CCGGTGGTAA GAAA-C---T ATTT--ACCA AAACTTG-AT TTTTTAAAAA   [162]
T. arenaria _5            AAAGAGTT-G CCAGTGGTAA GA---CCA-- -TTT--ACCA AAACTTG-AT TTACTGAAAA   [161]
T. arenaria _6            AAAGAGTT-G CCAGTGGTAA GA---CCA-- -TTT--ACCA AAACTTG-AT TTACTGAAAA   [161]
T. boudieri _7            AAAGAGTT-G CCAGTGGTAA GA---CC--T ATTT--ATCA AAACTTG-A- GTTTCAATAA   [172]
T. boudieri _8            AAAGAGTT-G CCAGTGGTAA GA---CC--T ATTT--ATCA AAACTTTTTA GTTTCAATAA   [181]
T. boudieri _9            AAAGAGTT-G CCAGTGGTAA GA---CC--T ATTT--ATCA AAACTTG-A- GTTTCAATAA   [170]
T. boudieri _10           AAAGAGTT-G CCAGTGGTAA GA---CCTA- -TTT--ATCA AA-G------ ----------   [164]
T. leptoderma _11         AAAGAGTTTG CCAGTGGCAA GAA-----TT TTTTTACCAA AAACTTGACT ------GAAA   [172]
T. leptoderma _12         AAAGAGTTTG CCAGTGGCAA GAA-----TT TTTTTACCAA AAACTTGACT ------GAAA   [172]
T. olbiensis _13          AAAGAGTT-G CCAGTGGCTA GA---CCTTT TTTT-AACCA AAACTTGATT -------AAA   [155]
T. olbiensis _14          AAAGAGTT-G CCAGTGGCTA GA---CCTTT TTTT-AACCA AAACTTGATT -------AAA   [155]
T. pinoyi _15             AGAGAGTT-G CCGGTGGGAA GA---CCA-T CT---ACCAA AACCTTGATT ------GAAA   [169]
T. nivea _16              AGAGAGTT-G CCAGTGGTAA GA---CCAA- -TC-TA-TCA AAACTTGATT T-----GAAA   [170]
C. echinulatus-12         AGAGAGTTTG CCCGTGGTAA GA---CCTCC AAA----CCA AAACTTGAAG AAAACC-AAA   [180]
C. echinulatus-44894      AGAGAGTTTG CCCGTGGTAA GA---CCTCC AAA----CCA AAACTTGAAG AAAACC-AAA   [180]
M. terfezoides _19        GGGGAGT--G CCAGTGG--- -A--TAGCCC AAACTAAAAA ACTCTTTTGT TTGAAA--TT   [191]
M. terfezoides _20        GG-G-GAGTG CCAGTGG--- -A--TAGCCC AAACT--AAA AAACTCTTTT GTTTGAAATT   [191]
Tuber melanosporium       ---------- CCTGTGGGA- GA--T-CTCT ATGTT--AAC GGAGTATTTG ATT-GT-GCT   [145]
C. venosus _24            G--GAGGT-G CCCGGGGGAG GACCAACCCC AACCTCCAAC CATCCAGCGG AAGAGAA---   [162]
C. alveolatus _25         ---GAGGC-G CCTGGGGGAG GACCCACACC AATCTCGCCT TGTCCAGCGG AAGAGAA---   [144]



[                                250        260        270        280        290        300
T. pfeilii-P              CCTTTTTG-- ----TCTGAT ATTAAAT-GA AATAAAATGA AAAAGAATAA AA-CTTTCAA   [267]
T. pfeilii _a             CCTTTTTG-- ----TCTGAT ATTAAAT-GA AATAAAATGA AAAAGAATAA AA-CTTTCAA   [267]
T. pfeilii _b             CCTTTTTG-- ----TCTGAT ATTAAAT-GA AATAAAATGA AAAAGAATAA AA-CTTTCAA   [266]
T. pfeilii _S             CCTTTTTG-- ----TCTGAT ATTAAAT-GA AATAAAATGA AAAAGAATAA AA-CTTTCAA   [267]
T. claveryi _3            ATACAATG-- ----TCTGAA --TAA-T--- ---TTCCTTT AATGAAATAA AA-CTTTCAA   [216]
T. claveryi _4            AAAATACA-- -ATGTCTGAA --TAA-T--- ---TTCCTTT AATGAAATAA AA-CTTTCAA   [209]
T. arenaria _5            ACACAATG-- ----TCTGAC TGTT------ ---TTCCTTT AATGAAATAA AA-CTTTCAA   [205]
T. arenaria _6            ACACAATG-- ----TCTGAC TGTT------ ---TTCCTTT AATGAAATAA AA-CTTTCAA   [205]
T. boudieri _7            ACACAATTG- ----TCTGAA TATTTTT--- ---TTTCTTT AATGAAATAA AA-CTTTCAA   [220]
T. boudieri _8            ACACAATTG- ----TCTGAA TATTTTTTG- --TTTTCTTT AATGAAATAA AAACTTTCAA   [233]
T. boudieri _9            ACACAACTG- ----TCTGAA TATTTTT--- ---TTTCTTT AATGAAATAA AA-CTTTCAA   [218]
T. boudieri _10           --ACAATTG- ----TCTGAA TATTTTTTG- --TTTTCTTT AATGAAATAA AAACTTTCAA   [214]
T. leptoderma _11         ACACAATTG- ----TCTGAA TATTTTTT-- -----CCTTT AATGAAATAA AA-CTTTCAA   [219]
T. leptoderma _12         ACACAATTG- ----TCTGAA TATTTTTT-- -----CCTTT AATGAAATAA AA-CTTTCAA   [219]
T. olbiensis _13          ACACA-TTG- ----TCTGAA TATATTT--- ------CTTG AATGAAACAA AA-CTTTCAA   [199]
T. olbiensis _14          ACACA-TTG- ----TCTGAA TATATTT--- ------CTTG AATGAAACAA AA-CTTTCAA   [199]
T. pinoyi _15             ACAAA-TTG- ----TCTGAA TCTTTT--G- ------CTTC AATGAAATAA AA-CTTTCAA   [213]
T. nivea _16              -CA-AATTG- ----TCTGAA TCTTTT--G- ------CTTC AATGAAATAA AA-CTTTCAA   [213]
C. echinulatus-12         ACAG------ ----TCTGAC AATTTATTG- --------CA AATGAAATAA AA-CTTTCAA   [220]
C. echinulatus-44894      ACAG------ ----TCTGAC AATTTATTG- --------CA AATGAAATAA AA-CTTTCAA   [220]
M. terfezoides _19        TGCTGATTTG -A-GTCTGAA AATG-GTTG- -----TCAAA ATCG--ATAA AA-CTTTCAA   [239]
M. terfezoides _20        TGCTGATTTG -AT-TCTGAA AATG-GTTG- -----TCAAA ATCG--ATAA AA-CTTTCAA   [239]
Tuber melanosporium       GTCTGAGCTG ---G-CCATG TGTCA---G- -----ATTTA GTAAAGTAAA AA-CTTTCAA   [191]
C. venosus _24            GTCTGACCGG G-CCACGTGC CATC--T-G- -----TATTA ACGTACTTAA AA-CTTTCAA   [211]
C. alveolatus _25         GTCTGACTTG G-CCACGCGC CATCA---G- -----TATTA ACGTACTTAA AA-CTTTCAA   [193]
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[                                    310        320        330        340        350        360
T. pfeilii-P                  CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAATTGCGAT AAGTAATGTG   [327]
T. pfeilii _a                 CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAATTGCGAT AAGTAATGTG   [327]
T. pfeilii _b                 CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAATTGCGAT AAGTAATGTG   [326]
T. pfeilii _S                 CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAATTGCGAT AAGTAATGTG   [327]
T. claveryi _3                CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [276]
T. claveryi _4                CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [269]
T. arenaria _5                CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [265]
T. arenaria _6                CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [265]
T. boudieri _7                CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [280]
T. boudieri _8                CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [293]
T. boudieri _9                CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [278]
T T. boudieri _10             CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [274]
T. leptoderma _11             CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [279]
T. leptoderma _12             CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [279]
T. olbiensis _13              CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [259]
T. olbiensis _14              CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [259]
T. pinoyi _15                 CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [273]
T. nivea _16                  CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [273]
C. echinulatus-12             CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [280]
C. echinulatus-44894          CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT AAGTAATGTG   [280]
M. terfezoides _19            CAACGGATCT CTAGGCTCTT GCATCGATGA AGAACGCAGT GAAATGCGAT ACGTAATGTG   [299]
M. terfezoides _20            CAACGGATCT CTAGGCTCTT GCATCGATGA AAAACGCAGT GAAATGCGAT ACGTAATGTG   [299]
Tuber melanosporium           CAACGGATCT CTTGGCTCTC GCATCGATGA AGAACGCAGC GAAATGCGAT AGGTAATGTG   [251]
C. venosus _24                CAACGGATCT CTTGGTTCTC GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG   [271]
C. alveolatus _25             CAACGGATCT CTTGGTTCTC GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG   [253]
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[                                    370        380        390        400        410        420
T. pfeilii-P                  AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [386]
T. pfeilii _a                 AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [386]
T. pfeilii _b                 AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [385]
T. pfeilii _S                 AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [386]
T. claveryi _3                AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TTTGGTATTC   [335]
T. claveryi _4                AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TTTGGTATTC   [328]
T. arenaria _5                AATTGCAGAA TTCTCGTGAA TCATTGAATC TTTGAACGCA CATTGCGCGC TTTGGCATTC   [325]
T. arenaria _6                AATTGCAGAA TTCTCGTGAA TCATTGAATC TTTGAACGCA CATTGCGCCC TTTGGCATTC   [325]
T. boudieri _7                AATTGCAGAA T-CTCGTGAA TCATTGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [339]
T. boudieri _8                AATTGCAGAA T-CTCGTGAA TCATTGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [352]
T. boudieri _9                AATTGCAGAA T-CTCGTGAA TCATTGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [337]
T. boudieri _10               AATTGCAGAA T-CTCGTGAA TCATTGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [333]
T. leptoderma _11             AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [338]
T. leptoderma _12             AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [338]
T. olbiensis _13              AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [318]
T. olbiensis _14              AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [318]
T. pinoyi _15                 AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [332]
T. nivea _16                  AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [332]
C. echinulatus-12             AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [339]
C. echinulatus-44894          AATTGCAGAA T-CTCGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [339]
M. terfezoides _19            AATTGCAAAA T-CTCGTGAA TCATTGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [358]
M. terfezoides _20            AATTGCAAAA T-CTCGTGAA TCATTGAATC TTTGAACGCA CATTGCGCCC TATGGTATTC   [358]
Tuber melanosporium           AATTGCAGAA TTC-AGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TTTGGTATTC   [310]
C. venosus _24                AATTGCAGAA TTC-AGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TTTGGTATTC   [330]
C. alveolatus _25             AATTGCAGAA TTC-AGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC TTTGGTATTC   [312]
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[                                    430        440        450        460        470        480
T. pfeilii-P                  CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CATCAC CT--CTCATA AGCAGCCATT   [437]
T. pfeilii _a                 CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CATCAC CT--CTCATA AGCAGCCATT   [437]
T. pfeilii _b                 CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CATCAC CT--CTCATA AGCAGCCATT   [436]
T. pfeilii _S                 CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CATCAC CT--CTCATA AGCAGCCATT   [437]
T. claveryi _3                CATAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CT--CACTCA AG--------   [378]
T. claveryi _4                CATAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CT--CACTCA AG--------   [371]
T. arenaria _5                CATAGGGCAT GCCTGTCTGA GCGTCAG--- ----CACCCC CCT-CACTCA AG--------   [369]
T. arenaria _6                CATAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CCT-CACTCA AG--------   [369]
T. boudieri _7                CATTGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CTC-CACTCA AG--------   [383]
T. boudieri _8                CATTGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CTC-TACTCA AG--------   [396]
T. boudieri _9                CATTGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CTC-CACTCA AG--------   [381]
T. boudieri _10               CATTGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CTC-TACTCG AG--------   [377]
T. leptoderma _11             CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CC--CACTCG AG--------   [381]
T. leptoderma _12             CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CC--CACTCG AG--------   [381]
T. olbiensis _13              CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CT--CACTCA AG----TATT   [365]
T. olbiensis _14              CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CT--CACTCA AG--------   [361]
T. pinoyi _15                 CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCT TC--TCCTCA AG--------   [375]
T. nivea _16                  CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCT TC--TACTCA AG--------   [375]
C. echinulatus-12             CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CCC-ATCTCA AG--------   [383]
C. echinulatus-44894          CGTAGGGCAT GCCTGTCTGA GCGTCAG--- ----CTCCCC CCC-ATCTCA AG--------   [383]
M. terfezoides _19            CGTAGGGCAT GCCTGTCTGA GTGTCAG--- ----CTTCCT TC--CTCTCA AACGTCTGTT   [409]
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M. terfezoides _20            CGTAGGGCAT GCCTGTCTGA GTGTCAG--- ----CTTCCT TC--CTCTCA AACGTCTGTT   [409]
Tuber melanosporium           CTTAGGGCAT GCCTGTTCGA GCGTCAC--- ----TACACG CCTTATCACA AA-G------   [356]
C. venosus _24                CTTAGGGCAT GCCTGTTCGA GCGTCACAAA CAAA-CCCCT ----CAC--- --------TT   [374]
C. alveolatus _25             CTTAGGGCAT GCCTGTTCGA GCGTCACTAA CAA--CCTCA T---CAC--- --------TT   [356]


[                                    490        500        510        520        530        540
T. pfeilii-P                  TATTTCTTT- GAGTG----- -GTTCTGTAT TTGAGGACTC -ATTGGATAA GA-AGGTTTT   [488]
T. pfeilii _a                 TATTTCTTT- GAGTG----- -GTTCTGTAT TTGAGGACTC -ATTGGATAA GA-AGGTTTT   [488]
T. pfeilii _b                 TATTTCTTT- GAGTG----- -GTTCTGTAT TTGAGGACTC -ATTGGATAA GA-AGGTTTT   [487]
T. pfeilii _S                 TATTTCTTT- GAGTG----- -GTTCTGTAT TTGAGGACTC -ATTGGATAA GA-AGGTTTT   [488]
T. claveryi _3                TATC-CTTT- C----ATA-- ----AGATAC TTGG------ -ATTGCTTTG GAGAGGTAAC   [419]
T. claveryi _4                TATC-CTTT- A----ATA-- ----AGTTAC TTGG------ -ATTGCTTTG GAGATGTAAC   [412]
T. arenaria _5                TATCCTTTTT CTTTTATAAA ATGTTGATAT TTGG------ -ATTGCTTTG GAGAGGTAAC   [422]
T. arenaria _6                TATCCTTTTT CTTTTATAAA ATGTTGATAT TTGG------ -ATTGCTTTG GAGAGGTAAC   [422]
T. boudieri _7                AATCATTTT- -----ATA-- ----TGATAC TTGG------ -ATTACTTTG GAGATGTAAC   [424]
T. boudieri _8                AATCATTTTT -----ATA-- ----TGATAC TTGG------ -ATTACTTTG GAGATGTAAC   [438]
T. boudieri _9                AATTATTTT- -----ATA-- ----TGATAC TTGG------ -ATTACTTTG GAGATGTAAC   [422]
T. boudieri _10               AATCATTTTT -----ATA-- ----TGATAC TTGG------ -ATTACTTTG GAGATGTAAC   [419]
T. leptoderma _11             TATTTCTTTT A--TG----- ----TTATAC TTGG------ -ATTGCTTTG GAGAGGTAAC   [423]
T. leptoderma _12             TATTTCTTTT A--TG----- ----TTATAC TTGG------ -ATTGCTTTG GAGAGGTAAC   [423]
T. olbiensis _13              TCTTT-A--T G--------- ----TTATAC TTGG------ -ATTGTTTTG AAGACGTAAC   [402]
T. olbiensis _14              TATTTCTTT- A--TG----- ----TTATAC TTGG------ -ATTGTTTTG AAGACGTAAC   [402]
T. pinoyi _15                 TATTCCTTTT ---TG--AAA -------TAC TTGG------ -ATAACTTTG GAGAGGTGAC   [416]
T. nivea _16                  TATTC-TTTT ---GC--AAA -------TAC TTGG------ -ATAACTTTG GAGAGGTGAC   [415]
C. echinulatus-12             CATC-TTTTT ---TG----- ------AAGC TTGG------ -ATTACTTTG GAGAAGTGGT   [421]
C. echinulatus-44894          CATC-TTTTT ---TG----- ------AAGC TTGG------ -ATTACTTTG GAGAAGTGGT   [421]
M. terfezoides _19            TCTCCTTTTT -GGAG----- ---GAAGACT TTGG------ -ATTACCTTG GACAAGCGAT   [453]
M. terfezoides _20            TCTCCTTTTT --GG---A-- --GGAAAACT TTGG------ -ATTACCTTG GACAAGCGAT   [453]
Tuber melanosporium           TTTGTGGTCT T-GGC--A-- --GGAGTGAA TTGCTAG-TC TATT------ -AAAA-TG-T   [399]
C. venosus _24                CTATATA--- --GTG----- GTG--CTGGC GGAAGTGGT- G-CCC-GCGC AGGGGATCGT   [419]
C. alveolatus _25             CCACT----- --GTG----- GTG--TTGGC GGCAGTGGT- T-CCCAGCAC GGAGGATCTT   [400]


[                                    550        560        570        580        590       600] [
T. pfeilii-P                  ACTCCTATGG GTG--AATTC TTCTATCCAG AAAGTTATAG GCAGTACTGG TTAGTTCTTC   [546]
T. pfeilii _a                 ACTCCTATGG GTG--AATCC TTCTATCCAG AAAGTTATAG GCAGTACTGG TTAGTTCTTC   [546]
T. pfeilii _b                 ACTCCTATGG GTG--AATTC TTCTATCCAG AAAGTTATAG GCAGTACTGG TTAGTTCTTC   [545]
T. pfeilii _S                 ACTCCTATGG GTG--AATCC TTCTATCCAG AAAGTTATAG GCAGTACTGG TTAGTTCTTC   [546]
T. claveryi _3                TGTG-TATTA A-----G--T TGCCCTCCAG AAATTTATAG GCAGTA-TGG TT--TGTACT   [468]
T. claveryi _4                TGTG-TATTA A-----G--T TGCCCTCCAG AAATTCATAG GCAGTA-TGG TT--TGTACT   [461]
T. arenaria _5                TTATGTATTA A-----G--T TGCCCTCCAG AAATTCATAG GCAGTA-TGG TT--TGTACT   [472]
T. arenaria _6                TTATGTATTA A-----G--T TGCCCTCCAG AAATTCATAG GCAGTA-TGG TT--TGTACT   [472]
T. boudieri _7                TGTA-TATTA A-----G--T TGCCCTCCAG AAATTCATTG GCAGTA-TGG TT--TGTACT   [473]
T. boudieri _8                TGTA-TATTA A-----G--T TGCCCTCCAG AAATTCATTG GCAGTA-TGG TT--TGTACT   [487]
T. boudieri _9                TGTA-TATTA A-----G--T TGCCCTCCAG AAATTCATTG GCAGTA-TGG TT--TGTACT   [471]
T. boudieri _10               TGTA-TATTA A-----G--T TGCCCTCCAG AAATTCATTG GCAGTA-TGG TT--TGTACT   [468]
T. leptoderma _11             TAG------- ---------T TACCCTTCAG AAATTCATAG GCAGTA-TGG TT--TGCACT   [464]
T. leptoderma _12             TAG------- ---------T TACCCTTCAG AAATTCATAG GCAGTA-TGG TT--TGCACT   [464]
T. olbiensis _13              TAGTTAATAG ---------T TGCCCTCCAG AAATTCATAG GCAGTA-TGG TT--TGCACT   [450]
T. olbiensis _14              TAGT-TAAT- A-----G--T TGCCCTCCAG AAATTCATAG GCAGTA-TGG TT--TGCACT   [450]
T. pinoyi _15                 TTAT-AACAG A-----G--T CGTCCTTCAG AAATTCATAG GCAGTA-TGA TT--TGTACT   [465]
T. nivea _16                  TTAT-AATAA A-----GAGT TGTCCTTCAG AAATTCATAG GCAGTA-TGG TT--TGTTCT   [466]
C. echinulatus-12             TCTA-TAATG A-----G--C CACCCTCTAG AAATTTATAG GCAGTA-TGG TT-AT-ATCT   [470]
C. echinulatus-44894          TCTATAATGA G--------C CACCCTCTAG AAATTTATAG GCAGTA-TGG TT-AT-ATCT   [470]
M. terfezoides _19            GGTTACGTGA T----AA--T GGCTGTCCAG AAAATCATAG GCAGTA-TGG GT-GT-TTTC   [504]
M. terfezoides _20            GGTTACGTGA T----AA--T GGCTGTCCAA AAAATCATAG GCAGTA-TGG GT-GT-TTTC   [504]
Tuber melanosporium           T-CCAGCTGT -----ACACT CTGCTAAAAA TTATGAGAAG G--TTACCAG G-CATGAA-C   [449]
C. venosus _24                CTCCCCC-G- C-GGCCCACC CCACCGAAAT GCATCGGGCG GACAAACCG- -ATCTCAG-C   [473]
C. alveolatus _25             CTCCCCCCGT CTGGC-CACT CCGCCGAAAT AAATCAGGCG GACAAACTG- -ATCTCAG-C   [456]


[                                    610        620        630        640        650       660] [
T. pfeilii-P                  TGTACTGGGC GTAATAATTT ACTTTTATTC TCGTCTAGAA -AAG----GT GAATAG----   [597]
T. pfeilii _a                 TGTACTGGGC GTAATAATTT ACTTTTATTC TCGTCTAGAA -AAG----GT GAATAG----   [597]
T. pfeilii _b                 TGTACTGGGC GTAATAATTT ACTTTTATTC TCGTCTAGAA -AAG----GT GAATAG----   [596]
T. pfeilii _S                 TGTACTGGGC GTAATAATTT ACTTTTATTC TCGTCTAGAA -AAG----GT GAATAG----   [597]
T. claveryi _3                TGAACCAGAC GTAATAAT-- ---AATAAAA TCGTTTGTTT -GAG---TAT GATT-G----   [514]
T. claveryi _4                TGAACCAGAC GTAATAAT-- ---AATAAAA TCGTTTGTTT -GGG---TAT GATT-G----   [507]
T. arenaria _5                TAAACCAGAC GTAATAAT-- ---AATAAAA TCGTTTTGTT TGGGTA-TAT AATTA-----   [521]
T. arenaria _6                TAAACCAGAC GTAATAAT-- ---AATAAAA TCGTTTTGTT TGGGTA-TAT AATTA-----   [521]
T. boudieri _7                TGGACCAGAC GTAATAGT-- ---AATAAAA TCGTTTGTTT -GGG---TAT AATT-G----   [519]
T. boudieri _8                TGGACCAGAC GTAATAGT-- ---AATAAAA TCGTTTGTCT -GGG---TAT AATT-G----   [533]
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T. boudieri _9            TGGACCAGAC GTAATAGT-- ---AATAAAA TCGTTTGTTT -GGG---TAT AATT-G---A    [518]
T. boudieri _10           TGGACCAGAC GTAATAGT-- ---AATAAAA TCGTTTGTCT -GGG---TAT AATT-G----    [514]
T. leptoderma _11         TGAACCAGAC GTAATAAT-- ---AATAAAA TCGTTTGTTT -GGG---TAT AATT------    [509]
T. leptoderma _12         TGAACCAGAC GTAATAAT-- ---AATAAAA TCGTTTGTTT -GGG---TAT AATT------    [509]
T. olbiensis _13          TGAACCAGAC GTAATAAT-- ---AATAAAA TCGTTTGTTT -GGG---TAT GACT------    [495]
T. olbiensis _14          TGAACCAGAC GTAATAAT-- ---AATAAAA TCGTTTGTTT -GGG---TAT GACT------    [495]
T. pinoyi _15             CGAATCAGAC GTAATAAT-- ---AATAAAG TCGTTTATTT -GAA--GTAT AAT-------    [510]
T. nivea _16              TGAATCAGAC GTAATAAT-- ---AATAAAG TCGTTTGTTT -GAG---TAT AATT------    [511]
C. echinulatus-12         -GAACTAGAC GTAATATT-- A--GC-AAAA TCGTCTTTTT -AGG---AAT GATT------    [514]
C. echinulatus-44894      -GAACTAGAC GTAATATT-- A--GC-AAAA TCGTCTTTTT -AGG---AAT GATT-G----    [515]
M. terfezoides _19        CAGACTGAAC GTAATAAG-- A-AAC-AA-- TCAAAAA--- -GGA-CGTTT ATATGG----    [549]
M. terfezoides _20        CAAACTGAAC GTAATAAG-- A-AAC-AA-- TCAAAAAGGA -----CGTTT ATATGG----    [549]
Tuber melanosporium       GACGGACTTT ATAAACGGTT ATAAG-ACC- TGGATCA-GT CACAAGTCTT GTCTGGTCC-    [505]
C. venosus _24            AACTGACTTA GTAAAAT-CA ATCAG--CCA GGAGTTAGG- -TTACGTCTT GCCA---CCC    [525]
C. alveolatus _25         AACTGACTTA GTAAAAT-CA ATCAG--CCA GGAGTGAGG- -TTACGTC-- --CA-G-CCC    [505]


[                                670        680        690        700        710        720
T. pfeilii-P              -------GTG ---CTTGCCT TGAAC-CC-A CAAG------ --TTATGTTA MC--TGGG-T    [634]
T. pfeilii _a..21         -------GTG ---CTTGCCT TGAAC-CC-A CAAG------ --TTATCTTA AC--TGGG-T    [634]
T. pfeilii _b..22         -------GTG ---CTTGCCT TGAAC-CC-A CAAG------ --TTATGTTA AC--TGGG-T    [633]
T. pfeilii _S             -------GTG ---CTTGCCT TGAAC-CC-A CAAG------ --TTATCTTA AC--TGGG-T    [634]
T. claveryi _3            A----TTGT- --ACTTGCCT C-AACTCC-A CCCA------ --TT-TTTAT --TTTGGG-T    [553]
T. claveryi _4            A----TTGT- --ACTTGCCT C-AACTCC-A CCCAT----- --TT-T-TAT -TTTTGGG-T    [547]
T. arenaria _5            A----TTGT- --ACTTGCCT T-AAGCCC-A CCCAT----- --TT-T-TAT --TTTGGG-T    [560]
T. arenaria _6            A----TTGT- --ACTTGCCT T-AAGCCC-A CCCAT----- --TT-T-TAT --TTTGGG-T    [560]
T. boudieri _7            A----TTGT- --ACTTGCCT T-AACCCCCA CCCA------ --TT-TTTAT -CT-TGGG-T    [559]
T. boudieri _8            A----TTGT- --ACTTGCCT TTAACCCCCA CCC------- -AT-TTTTAT -TT-TGGG-T    [574]
T. boudieri _9            A----TTGT- --ACTTGCCT T-AACCCCCA CCCA------ --T-TTTTAT --TTTGGG-T    [558]
T. boudieri _10           A----TTGT- --ACTTGCCT TTAACCCCCA CCC------- -AT-TTTTAT --TTTGGG-T    [555]
T. leptoderma _11         -------GT- --ACTAGCCT T-AACCCC-A CCCAT----- --T-ATTTAA --TTTGGG-T    [546]
T. leptoderma _12         -------GT- --ACTAGCCT T-AACCCC-A CCCAT----- --T-ATTTAA --TTTGGG-T    [546]
T. olbiensis _13          -------GT- --ACTTGCCT T-AACCCC-A CCCAT----- --T-TTTTAC --TTTGGG-T    [532]
T. olbiensis _14          -------GT- --ACTTGCCT T-AACCCC-A CCCAT----- --T-TTTTAC --TTTGGG-T    [532]
T. pinoyi _15             -----T-GTG ---CTTGCCC C-AACT-CAC CCACTTTTTT TTATTTTAT- --TTCGGG-T    [555]
T. nivea _16              -------GTG ---CTTGCCT T-AACT-CAC TCATT----- --T---ATAT CC--TGG--T    [545]
C. echinulatus-12         -------GT- --ACTTGCCT C-AAC-CCAC TCAT------ ----ATTAAA C--TTGGG-T    [549]
C. echinulatus-44894      ------TG-- ---CTTGCCT C-AAC-CCAC TCAT------ ----ATTAAA C--TTGGG-T    [549]
M. terfezoides _19        AGCCTTTGT- --ACTTGCCT TCAAC-CC-A CACA------ --T---TCTT TTTTTGGGTC    [593]
M. terfezoides _20        AGCCTTTGT- --ACTTGCCT TCAAC-CC-A CACA------ --T---TCTT TTTTTGGGTC    [593]
Tuber melanosporium       -----TTACC TTAAGGACCC CCA-T-CCTA ---G------ ----ATGAAC TA--TGGGTT    [543]
C. venosus _24            AGC--TTG-G --ACCCCCAT CCAA---C-- TTGG---TGC G---ACAAAT CA--TGGGTT    [567]
C. alveolatus _25         AGC--TT--- ---CGGACCC CCA-T-CC-- GTTG---CCA C---ACAAAC CATTTG--CT    [545]


[                                730        740        750          ]
T. pfeilii-P              GA-CCTCAGA TCAGGTAGG- ---------- ---------    [652]
T. pfeilii _a             GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTTTAA    [671]
T. pfeilii _b             GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [669]
T. pfeilii _S             GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [670]
T. claveryi _3            GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [589]
T. claveryi _4            GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [583]
T. arenaria _5            GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [596]
T. arenaria _6            GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [596]
T. boudieri _7            GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [595]
T. boudieri _8            GA-CCTCAGA TCAGTTAGGG ATACCCGCCT GAACTT-AA    [611]
T. boudieri _9            GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [594]
T. boudieri _10           GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [591]
T. leptoderma _11         GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [582]
T. leptoderma _12         GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [582]
T. olbiensis _13          GACCCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [569]
T. olbiensis _14          GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [568]
T. pinoyi _15             GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [591]
T. nivea _16              GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [581]
C. echinulatus-12         GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [585]
C. echinulatus-44894      GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [585]
M. terfezoides _19        GA-CCTCAGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [629]
M. terfezoides _20        GA-CCTCAAA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [629]
Tuber melanosporium       GA-CCTCGAA TCAGGGAGGG ATACCCG-CT GAACTT-AA    [579]
C. venosus _24            GA-CCTCGGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [603]
C. alveolatus _25         GA-CCTCGGA TCAGGTAGGG ATACCCG-CT GAACTT-AA    [581]
```

Species used in the alignment	**Accession number in the GenBank**	**Corresponding numbers on the sequences**
Terfezia claveryi	AF301421	**4**
Terfezia claveryi-3	AF387648	**3**
Terfezia arenaria-1	AF276675	**5**
Terfezia arenaria-2	AF276674	**6**
Terfezia boudieri-1	AF092096	**7**
Terfezia boudieri-2	AF092097	**8**
Terfezia boudieri-3	AF092098	**9**
Terfezia boudieri-42a2	AF301418	**10**
Terfezia leptoderma-1	AF276678	**11**
Terfezia leptoderma-2	AF276679	**12**
Terfezia olbiensis-1	AF387657	**13**
Terfezia olbiensis-2	AF387656	**14**
Tirmania pinoyi	AF276669	**15**
Tirmania nivea	AF276668	**16**
Choiromyces echinulatus-12	AF435829	**17**
Choiromyces echinulatus-44894	AF435825	**18**
Mattirolomyces terfezoides-1	AF276681	**19**
Mattirolomyces terfezoides-2	AF276680	**20**
Terfezia pfeilii-a	AF301420	**21**
Terfezia pfeilii-b	AF301422	**22**
Tuber melanosporium	AF167097	**23**
Choiromyces venosus	AF003910	**24**
Choiromyces alveolatus	AF501258	**25**
Terfezia pfeilii ***P***	Pure culture	
Terfezia pfeilii ***S***	Ascocarp	

Appendix IV: Plant Nutrient Solutions and Staining Materials

Preparation of Long Ashton's five nutrient stock solutions (Hewitt, 1996)

Stock Solution A

Chemical	Quantity (g) in 1L distilled water (100x)
$MgSO_4.7H_2O$	36.900
$MnSO_4$ or $MnSO_4.H_2O$	0.223
$CuSO_4.5H_2O$	0.240
$ZnSO_4.7H_2O$	0.0296
H_3BO_3	0.186
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0035
$CoSO_4.7H_2O$	0.0028
NaCl	0.585

Stock Solution B

Chemical	Quantity (g) in 1L distilled water (100x)
FeEDTA	3.000

Stock Solution C

Chemical	Quantity (g) in 1L distilled water (100x)
$CaCl_2$	50.000

Stock Solution D

Chemical	Quantity (g) in 1L distilled water (100x)
K_2SO_4	21.740

Stock Solution E

Chemical	Quantity (g) in 1L distilled water (100x)
$(NH4)_2SO_4$	105.000

Stock Solution F

Chemical	**Quantity (g) in 1L distilled water (100x)**
$NaH_2PO_4.2H_2O$	2.500

Stock solutions were stored in dark bottles in the fridge

Preparation of 10 L nutrient solution

1. Stock solutions were removed from the fridge and allowed to stand for a while
2. A 200ml volume of distilled was placed inside1L measuring cylinder
3. a 10 ml volume of each stock solution was added (200ml of each stock solution for 20L to the measuring cylinder).

Roots clearing and staining solutions (Smith and Dickson, 1997)

50% ethanol

1000ml ethanol
1000ml distilled water

5% KOH

100g KOH
2L distilled water

Alkaline Peroxide H_2O_2

3ml NH_4OH (Ammonia)
30ml 10% H_2O_2
567ml distilled water, prepared only when required.

0.1M HCl (32% MW36.46)

22.79ml HCl
2L Distilld water

Lactoglycerol trypan blue stain

Lactic acid: Glycerol: Water (13:12:16)

520ml lactic acid
480ml Glycerol
640ml distilled water
082g Trypan blue

Lactoglycerol Destain

Lactic acid: Glycerol: Water (13:12:16)
520ml lactic acid
480ml Glycerol
640ml distilled water

APPENDIX V: Internet References

Mycorrhiza and soil phosphorus levels

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Introduction:

Mycorrhizae are an integral part of most plants in nature (Giazninazzi et al., 1982) and occur on 83% of dicotyledonous and 79% of monocotyledonous plant investigated (Wilcox - 1996). All gymnosperms are reported as being mycorrhizal (Newman & Reddell, 1987). Infection of the root system of the plant by these fungi creates a symbiotic (beneficial) relationship between the plant and fungus.

Upon root infection and colonization, mycorrhizal fungi develop an external mycelium which is a bridge connecting the root with the surrounding soil (Toro et al. 1997). One of the most dramatic effects of infection by mycorrhizal fungi on the host plant is the increase in phosphorus (P) uptake (Koide, 1991) mainly due to the capacity of the mycorrhizal fungi to absorb phosphate from soil and transfer it to the host roots (Asimi, et al. 1980). In addition, mycorrhizal infection results in an increase in the uptake of copper (Lambert, Baker & Cole, 1979; Gildon & Tinker, 1983), zinc (Lambert, Baker & Cole, 1979), nickel (Killham & Firestone, 1983), and chloride and sulphate (Buwalda, Stribley & Tinker, 1983). Mycorrhizae also are known to reduce problems with pathogens which attack the roots of plants (Gianinazzi-Pearson & Gianinazzi, 1983).

Influence of Phosphorus on Mycorrhizae:

The benefits listed above are greatest in P-deficient soils and decrease as soil phosphate levels increase (Schubert & Hayman, 1986).

Very high and very low phosphorus levels may reduce mycorrhizal infection/colonization (Koide, 1991). It is well established that:

- infection by mycorrhizal fungi is significantly reduced at high soil phosphorus levels (Amijee et al., 1989; Koide & Li, 1990)
- the addition of phosphate fertilization results in a delay in infection as well as a decrease in the percentage of infection of roots by mycorrhizae (deMiranda, Harris & Wild, 1989; Asimi et al., 1989)
- an increase in the level of soil phosphate results in a reduction in chlamydospore production by the fungus (Menge, et al. 1978). These spores are involved in root infection and spread of the fungus through the soil profile.

Research by Abbott and Robson (1979) concluded that levels of soil phosphorus greater than that required for plant growth eliminated the development of the arbuscles of vesicular-arbuscular (VA) types of mycorrhizae. Arbuscles are structures produced within the host plant cells by the VA mycorrhizae. These structures are responsible for the transfer of absorbed nutrients from the fungus to the plant. The arbuscles resemble miniature shrub-like trees (arbuscular = shrub in Latin). Mosse (1973) reports adding phosphate results in no arbuscles forming.

What levels of P are critical?

When the soil level of bicarbonate-soluble phosphorus exceeded 140 mg kg^{-1} (140 parts per million) the rate of infection was found to decrease (Amijee et al. 1989). Abbott and Robson (1977 & 1978) found the mycorrhizae *Glomus fasciculatum* ceased to be effective when the soil level of phosphorus reached 133 mg kg^{-1} [133 parts per million (ppm)]. Schubert and Hayman (1986) found mycorrhizae was no longer effective when 100 mg or more of P was added per kilogram of soil (100 ppm). Mycorrhizal infection virtually disappeared with the addition of 1.5 grams or more of mono calcium phosphate per kilogram of soil (Mosse 1973). With small additions of phosphorus fertilizer, entry points and fungal growth on the root surface remained normal but arbuscles were small and fewer in number reducing the effectiveness of the fungus/plant relationship. Other researchers have reported mycorrhizal infections tend to die out in soils containing or given much phosphorus (Baylis, 1967; Mosse, 1967). The development of mycorrhizal relationships were found to be the greatest when soil phosphorus levels were at 50 mg kg^{-1} (50 ppm) (Schubert & Hayman, 1986).

Summary and recommendations:

The benefits of mycorrhizae are greatest when soil phosphorus levels are at or below 50 ppm (50 mg kg^{-1}). Mycorrhizal infection of roots declines above this level with little if any infection occurring above 100 ppm P even when soil is inoculated with a mycorrhizae mix.

Prior to inoculating soil with mycorrhizae, a soil test should be conducted. If phosphorus levels are greater than 50 ppm the addition of mycorrhizae will likely be ineffective.

The level of phosphorus in the plant also has been shown to influence the establishment of VA mycorrhizae with high levels inhibiting colonization by mycorrhizae (Menge, et al. 1978). Foliar applications of phosphorus therefore should be avoided when inoculating soil with mycorrhizae.

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DETERMINATION OF QUALITY PARAMETERS OF BACTERIUM INOCU BASED ON LIQUID FORMULATION ELABORATED WITH STRAINS PRODUC INDOLE ACETIC ACID (IAA)

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ABSTRACT

Inoculums produced with strains of *Bacillus cereus*. *Pseudomonas putida* isolated from soil. tw reference strains *Azotobacter chrococcum* and *Pseudomonas putida* and two commercial biological products were evaluated with the aim of establishing quality parameters. The native strains were chosen from a 106 group using antagonistic tests in vitro. germination on trays an IAA (Indol Acetic Acid) detection using a colorimetric technique with the Van Urk Salkowski reagent. The statistic analysis of these tests showed that there are not significant differences between the strains. except in the IAA production using the strain 2 a presumtive *Pseudomonas* and the reference strain *Pseudomonas putida* where an IAA superproduction was observed wit 4 µg/ml and 7 µg/ml respectively. Inoculums were prepared with the isolated and the reference strains in nutrient broth containing L-tryptophan in a concentration of 0.2 %. the final concentration of the inoculum was 10^7 cfu/ml. The inoculums and the commercial products we evaluated in a Biological Activity Test using lettuce seeds (*Lactuca sativa*): nine treatments we evaluated: T1: Selected *Pseudomonas putida*: T2: *Bacillus cereus*: T3: Selected *Pseudomonas putida* + *Bacillus cereus*: T4: 50% selected strains + 50% chemical fertilizer: T5: Reference strains: T6: Commercial biological product A: T7: Commercial biological product B: T8: Chemical fertilizer: T9: Water. The statistical analysis of the test didn't show statistical differences between treatments: although with the Scheffé's Multiple Comparison it could be established that T1. T5. T7 and T8 obtained higher values for the root length and T3. T4 and T for the stem length. Viability tests were performed to the inoculums and it was determined tha conditions of 6 °C ± 2 of temperature during 45 days allow to preserve their viability. The commercial products were evaluated only at room temperature following advises of the producer.

Key words: Indol Acetic Acid. Biological inoculums. Quality control. ***Pseudomonas sp.*** ***Bacillus sp.***

INTRODUCTION

The capacity for responding to internal or external stimuli is one of the essential properties of all living organisms. In the specific case of plants the response can be associated to external factors such as microorganisms or their metabolites used as biological inoculums which can be defined as formulations or preparations of one or more defined biological entities. that can be find in natural form in the soil or be introduced in it. affecting the plants growth and development; with effects like Nitrogen Fixation. Illness Biocontrol. Solubilization. Improvement of minerals absorption and hormonal effects.

In that sense. the biological inoculums have a close relation with the development of agricultural production. giving also response to problems related with the improvement and preservation of the soil fertility.

Actually the concern is around the production of biological inoculums using biotechnological processes with positive effects on the diminution of the environmental and ecological impact. The production process of the inoculums has been done by fermentation using microorganisms that because of their concentration or their production of secondary metabolic substances contribute to the vegetal growth.

For this purpose is important to know the concept of quality. defined as the group of activities done to guarantee the uniform production of batches of products that satisfy the identity norms. activity. purity and integrity into the established parameters.
The production use and quality control rules of the inoculums are in agreement to the Food and Agriculture Organization of the United Nations (4ª)

The aim of this work is to determine some parameters necessary for the quality of biological inoculums based on the production of prototype formulations. using native rizobacteria of the genus *Pseudomonas* and *Bacillus* compared to reference strains of the genus *Azotobacter chrococcum* and Pseudomonas putida and two commercial products.

MATERIALS AND METHODS

Processing of the sample. For the isolation of native strains. samples of different soils were taken. each one received a microbiological study that included quantification using the count of cfu. isolation in a fresh media as King B agar. Mac Conkey agar and Nutrient agar. and microscopic and macroscopic characterization. (9ª)

Strains selection. Three tests were used: production of IAA in vitro. Antagonism according to Gauze 1965 and seed germination. To determine the IAA production by each microorganism a colorimetric technique was performed using the Van Urk Salkowski reagent (1 ml of 0.5 M $FeCl_3$ and 50 ml of 35 %$HClO_4$ in water): the microorganisms were grown in nutrient broth containing 0.2% L-tryptophan. incubated at a 30 °C ± 2 of temperature during 24 hours. After that time they were centrifugated (3000 rpm): 1 ml of the supernatant was mixed with 2 ml of the reagent. 25 minutes later, the optical density reading was done at 530 nm and the concentration of IAA produced was found using the pattern curve.

Growth Kinetics. Were performed to all selected microorganisms and to the reference strains. Azotobacter chrococcum and Pseudomonas putida, provided from the Agricultural and Environmental Microorganisms Stock of the PUJ: each one was grown in nutrient broth with 0.2 % L-tryptophan, using a 25-ml. 24 hours incubated pre-inoculum. This pre-inoculum was taken to a 500-ml final volume measuring the optical density of the culture each 2 hours during 24 hours at 540 nm.

Biochemical characterization. The BBL Crystal microidentification was used with some additional biochemical tests (3), (13).

Formulation production. The selected strains were grown in nutrient broth with 0.2 % L-tryptophan using continuos fermentation.

Viability tests. These were performed to all selected strains individually and mixed, to the mix of reference strains and to the commercial products A and B, used in Colombia, during 45 days.

Room temperature (18 °C ± 2) and refrigeration temperature (6 °C ± 2) were evaluating excepting for the commercial products A and B, which were only evaluated at room temperature.

Biological Activity Tests. Using bioassay in semicontrolled conditions and using as test strain *Lactuca sativa* (seeds), provided from the Seeds National Laboratory ICA, the inoculations were performed and included 9 treatments: (T1: Selected *Pseudomonas putida*; T2: *Bacillus cereus*; T3: Selected *Pseudomonas putida* + *Bacillus cereus*; T4: 50% selected strains + 50% chemical fertilizer (triple 15); T5: Reference strains; T6: Commercial biological product A; T7: Commercial biological product B; T8: Chemical fertilizer; T9: Water.); applying the evaluation method of Bashan Y. 1997 (1), variables like root and stem length (in cm), germination percentage, fresh weight of the plant (in mg) and dry weight of the plant (in mg).were evaluated.

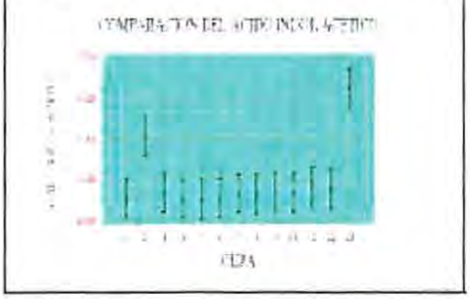
Experimental design and statistical analysis. Included a variance analysis based on a completely random model with sub-sampling, comparing the treatments by the Scheffé's Test.

RESULTS AND DISCUSSION

From the 106 isolated strains, presumptive of the genus *Pseudomonas sp* and *Bacillus sp*, 18 were discarded for presenting characteristics of Enterobacteriaceae on Mac Conkey agar and 35 for being morphologically identical.

To the other 53 strains, an IAA detection test was performed. 11 were selected for producing higher concentrations than 1.32 μg/ml. The strain 2 a presumptive *Pseudomonas sp* and the reference strain *Pseudomonas putida* presented significant differences for IAA superproduction. The other strains produced concentrations that can stimulate the elongation of the root without inhibiting it. (Graphic 1).

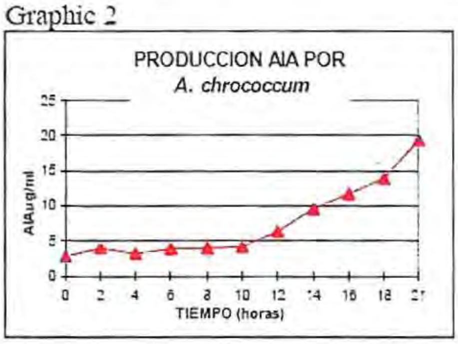
Graphic 1. Comparison of the production of IAA by strain.



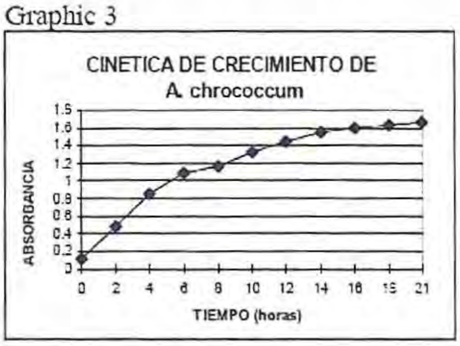
The 11 strains finally selected were confronted to find out their possible antagonic relations, giving negative results with inhibition zones smaller than 0.2 mm. In the germination tests on trays no significant differences were obtained between the strains, and the Scheffé's Multiple Comparison indicated that the higher values between the *Bacillus sp* strains were obtained with the strain 1 (*Bacillus cereus*) and the higher values between the Pseudomonas sp strains were obtained with the strain 9 (*Pseudomonas putida*). These two strains were selected to be producec and evaluated in the Biological Activity Test by bioassay.

In the growth kinetics of the bacteria a directly proportional relation between IAA production and the beginning of the stationary phase of their growth was observed, as an example see graphics 2 and 3 for the *Azotobacter chrococcum* case.

Graphic 2



Graphic 3



The viability test indicated that the microorganisms are better preserved at refrigeration temperatures than at room temperatures. In the formulation with *B. Cereus* a drop in its viability is observed when it was incubated at refrigeration temperature (6 °C ± 2) because of the spores production, which was not evidenced in the culture media unless the product was submitted to high temperatures with the aim of activating the spores. . and the formulation using a mix of bacteria because of the prevailing of the genus *Pseudomonas sp*. maybe for having a short lag phase and a quick growing rate (7), (14).

In the Biological Activity Test using the Bashan's method (1) it could be determined that there are not significant differences between treatments because of the short lasting of the test; however, in the Scheffé's Multiple Comparison higher values could be observed in some treatments about some parameters, like the root length of plants inoculated with the mix of reference strains, where a 23% over the control value was obtained. This difference could be attributed to the synergism between species. The stem length was higher in the inoculation with selected *Pseudomonas putida* and *Bacillus cereus*, and with the mix of reference strains, and this is attributed to asymbiotic free nitrogen fixation and hormones, aminoacids and vitamins production by the microorganisms like *B. cereus* and *Azotobacter chrococcum* (2). In the fresh and dry weight of the plants and in the germination percentage, homogeneous groups were observed in all treatments.

CONCLUSIONS

The initial parameter for the quality determination of inoculum formulations of agricultural use, is the isolation and identification of the guaranteed microorganisms.

The use of the technique for the detection of IAA using the Van Urk Salkowski reagent is an important option for the obtaining of qualitative and semi-quantitative results that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculums.

The nutrient broth with 0.2 L-tryptophan is a good media for the growth of the bacteria and for the IAA production; although its production depends on the microorganisms kinetics stabilization.

The strains of *Pseudomonas sp* evaluated in this study produce higher concentrations than 4.5 µg of IAA/ml, and due to that characteristic they could be used as reference strains for the use of the colorimetric method in future studies

The germination test on trays and the Biological Activity tests using the Bashan's method (1) with *Lactuca sativa* seeds, are not quality parameters that allow to evaluate the agricultural efficiency of the biological inoculums studied in this research, but they could be used to determine the presence of fitotoxic substances that inhibit the germination or affects the plants growth.

The viability evaluation of the inoculums is a very important parameter. The products containing *B. cereus* can be preserved at both temperatures (room and refrigeration) due to its capacity of forming spores.

For a future study is important to increase the time of the Biological Activity Test, so relevant differences during the plant growth can be observed. It's also suggested quantifying the strains capacity for Nitrogen Fixation.

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