Host relations of *Kalaharituber pfeilii* (Henn.) Trappe & Kagan-Zur

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ABSTRACT

Kalaharituber pfeilii (Henn.) Trappe & Kagan-Zur commonly known as the "Kalahari truffle" is a desert truffle species identified from the Kalahari region of southern Africa. Two other species, *Eremiomyces echinulatus* (Trappe & Marasas) Trappe & Kagan-Zur and *Mattirolomyces austroafricanus* (Trappe & Marasas) Trappe & Kovacs are also known to occur in other parts of southern Africa. Truffles are hypogeous fruiting bodies of Ascomycetes, important to humans for their nutritional value and medicinal characteristics. These truffles are known as desert truffles as they prefer to occur under arid or semi-arid conditions characteristic of deserts. Truffle development depends on the presence of a mycorrhizal host, associated microorganisms as well as soil and climatic characteristics. It has been suggested that *K. pfeilii* has a suspected broad plant host range which includes herbaceous to woody trees and shrubs. However, these relationships have not been verified. Indigenous people of the Kalahari believe that truffles are found under grasses. In the Kalahari, truffle fruiting bodies are often found entangled in *Stipagrostis ciliata* (Desf.) De Winter var. *capensis* (Trin. & Rupr.) De Winter roots. *S. ciliata*, also known as the tall bushman-grass, is the most common grass found in the Kalahari.

The objective of this study was to provide conclusive evidence that *S. ciliata* var. *capensis* is a host of the Kalahari truffle. Truffle fruiting bodies and grass roots from where the truffles were found were collected from Upington, South Africa. The fruiting bodies were identified by observing their morphological characteristics using the 'Keys of Truffle genera'. All observed physical properties were similar to those of *K. pfeilii* and further identification was done using molecular techniques. DNA was extracted from the fruiting bodies, mycelial cultures, rhizosheaths and from the *S. ciliata* var. *capensis* grass roots, which were then amplified using the specific *K. pfeilii* specific primers TPF3 and TPR1 and sequenced. The obtained sequence results confirmed that the collected fruiting bodies were those of the *K. pfeilii* and the molecular techniques also confirmed that *K. pfeilii* DNA was present in the *S. ciliata* var. *capensis* rhizosheath and root cells. Microscopy showed an ectendomycorrhizal association between *K. pfeilii* and *S. ciliata* var. *capensis*. Mycorrhizal resynthesis experiments were conducted to establish this mycorrhizal association *in-vitro*. They were unsuccessful because of the structure of the grass and the availability of contaminants.

Truffle and rhizosheath sections were grown on modified Fontana medium. Isolates were confirmed molecularly to be that of *K. pfeilii*. Contaminating bacteria were selected and identified using molecular techniques. Five species were identified, *Phyllobacterium myrsinacearum, Stenotrophomas maltophilia, Leucobacter aluvunii, Staphylococcus cohnii,* and *Pantoea dispersa*. These were further tested for mycorrhizal helper bacterial properties. The bacteria inhibited mycelia growth and only *P. myrsinacearum* produced positive results for Indole Acetic Acid production and none of the isolates solubilised phosphate.

A survey was conducted to investigate the distribution pattern and host range of *K. pfeilii*. Results indicated that *K. pfeilii* occurs from April to May in the Kalahari region and different vegetation was reported in the areas where it has occurred. Data from the South African Weather Service indicated an average maximum temperature of 37°C and irregular rainfall patterns ranging from 15 to 40 mm in yearly averages in Upington where samples for this study were collected. Soil pH values were between 5.6 and 5.9 which is within the range of *K. pfeilii* optimum growth which is 5.5 to 6.5.

Molecular sequences from truffle fruiting bodies, truffle cultures, rhizosheath cultures and rhizosheath root material conclusively proved that *K. pfeilii* was associated with the host plant *S. ciliata* var. *capensis*.

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LIST OF ABBREVIATIONS

°C - Degrees Celsius

µ - Micron (10^6)

BLAST -Basic Local Alignment Tool

bp - Base pairs

BSA - Bovine serum albumin

cm - Centimeters

DNA - Deoxyribonucleic acid

dNTP -Deoxyribose nucleotide triphosphate

e - Exponential

ECM - Ectomycorrhizal

Fig - Figure

FTN - Fontana

g - Gram

GPS - Global Positioning System

IAA - Indole Acetic Acid

ITS - Internal transcribed spacer

l - Litre

M - Mole

MHB - Mycorrhizal helper bacteria

min - Minute

mm - Millimeter

NA - Nutrient agar

NCBI - National Centre for Biotechnology Information

nm - Nanometer

OD - Optical density

pH - Potential hydrogen

PSM - Phosphate solubilising media

S.A. - South Africa

t - Ton

DEDICATION

Dedicated to my father, for all the sacrifices he has made for my family throughout the years.

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CHAPTER 1: LITERATURE

1.1 INTRODUCTION

Fungi are ubiquitous micro- or macroscopic, eukaryotic organisms with unique cellular and behaviour patterns compared to other organisms (Deacon, 2006). They can reproduce sexually or asexually. Asexual reproduction result in the production of spores which germinate to form filamentous structures known as hyphae. The hyphae can be septate or aseptate and can branch out to form a structural network called the mycelium. The mycelium is in direct contact with the surrounding environment and contributes in most biological and chemical reactions taking place in the environment. Various fungal species occur throughout the world but only approximately 5% has been identified and most occur in the soil. Fungi are classified according to their molecular structures and reproductive structures meaning a fungus would be classified differently if reproducing sexually than when reproducing asexually. They are divided into four groups which are Chytridiomycetes, Zygomycetes, Basidiomycetes and Ascomycetes (Smith and Read, 2008).

Ascomycetes are commonly referred to as “sac fungi” because they produce saclike structures called ascus during sexual reproduction (Black, 2008). They produce fruiting bodies which include some edible fungi such as morels and truffles (Smith and Read, 2008). Fungal cell walls are composed of chitin which serves for protection and growth. The main growth factors are temperature, pH, water and nutrient availability. Fungi lack chlorophyll and therefore cannot photosynthesize hence they obtain carbon and nutrients essential for growth from other sources in the environment by absorption. They also have the ability to secrete enzymes such as proteinases, lipases and cellulases which assist in the metabolism of organic matter for nutrient acquisition. The nutrients are absorbed into the cells by diffusion and active transport (Black, 2008).

To obtain essential nutrients and carbon, some fungi form symbiotic relationships with other organisms occurring in their surroundings. A symbiosis is a close, often long term association between different species. Ancestral terrestrial plants could photosynthesise effectively but

lacked developed root systems and therefore had difficulties in acquiring water and mineral nutrients. They formed a mutualistic symbiosis with fungi, which is an association between two species where both participants benefit (Smith and Read, 2008). It is believed that the fungi were attracted to the plants by traces of sugar and amino acids which are released by the plants as root exudates (Kendrick, 1992). Mycorrhizas and lichens are the most important examples of the mutualistic plant-fungal associations (Deacon, 2006).

1.2 MYCORRHIZAS

Mycorrhizas, named from ancient Greek words, "mykos" meaning fungus and "rhizos" meaning roots by Frank in 1885 after he discovered that fungal hyphae invaded plant roots but the roots were still healthy. Mycorrhizal relationships occur in different ways and are responsible for various activities which contribute in plant growth and soil community compositions. This fungus-root relationship occurs in 86% of all plant species found worldwide (Brundrett, 2009). Factors which can influence the occurrence and effectiveness of mycorrhizal associations include plant root properties, climatic factors, soil characteristics and host-fungus compatibility (Smith and Read, 2008).

Developmental stages of the mycorrhizal association include germination of fungal spores into mycelia which recognises the host plant roots. The plant releases chemical signals such as flavonoids, strigolactones and other surface signals which are recognised by receptor proteins on the plasma membrane of the fungus, this signals the fungus to enter a symbiotic mode. The mycelia then colonises the root forming plant fungal interfaces and the hyphae fully develops and spread into the soil (Smith et al., 2003). Once the association has been established, the plant gains use of the fungal mycelia. The fungal hyphae grow further from the plant root to distant surfaces to search for nutrients in places where the root hairs cannot reach. In return the plant supplies the fungus with carbohydrates from photosynthesis to support its growth (Smith and Read, 2008).

Mycorrhizal plants have been observed to have higher levels of phosphorus, nitrogen, calcium, magnesium and iron compared to non-mycorrhizal plants (Bohrer et al., 2003; Smith and Read, 2008; Titus et al., 2002). This is because mycorrhizal associations are responsible for changes in main plant processes such as the rate of nutrient absorption and the allocation of carbon to

various parts of the plant and the fungi. The rate of nutrient uptake is increased by various mechanisms such as increasing the surface area and by altering mycorrhizospheric chemistry. The fungal hyphae provide a large surface area beyond the root nutrient depletion zones and can access smaller soil pores which plant roots cannot penetrate. The exchange of nutrients and carbon between the mycorrhizal symbionts may not be simultaneous and the dependence of each symbiont on the other depends on the environment where the association occurs (Smith and Read, 2008).

A study by Morte et al. (2000), demonstrated that mycorrhizal plants have a higher survival rate compared to non-mycorrhizal plants under drought conditions especially in arid and semi-arid areas. This was attributed to an increase in transpiration, photosynthesis and stomatal conductance in the mycorrhizal plants than the non-mycorrhizal plants. Mycorrhizas also protect the plant from heavy metals by minimizing translocation of metals into aerial parts of the plant. Mycorrhizal plants therefore have high tolerance to harsh conditions such as drought, salinity, acidity and heavy metals and are healthier and resistant to fungal infections (West, 1997; Titus et al., 2002). They also contribute in the development of soil structure as the hyphae assist in capturing soil particles preventing loss of nutrients and other essential components (Brundrett, 2009).

1.3 MYCORRHIZAL HELPER BACTERIA

The mycorrhizal association was initially considered as an association between host plants and fungi. But the presence and activity of bacteria in the rhizosphere and their contribution towards the mycorrhizal association was first investigated by Bowen and Theodorou (1979). They observed that certain bacterial isolates promoted and others inhibited the colonization of *Pinus radiata* roots by *Rhizopogon luteolus.*

Microorganisms are attracted to the rhizosphere (the zone in the soil which is influenced by plant roots) by root exudates which contain essential compounds which are required for growth. The knowledge of soil microbial diversity is limited by the inability to study all microorganisms. Approximately 1% of the soil bacterial population can be cultured by standard laboratory practices. It is not known whether this 1% provides a good enough representation of the soil bacterial population. Soil is heterogeneous therefore studying certain parts of it may not be a

proper indication of its microbial diversity (Kirk et al., 2004). About 4000 different bacterial genomic units are found in 1 g of soil. These microorganisms are vital for the continuing cycling of nutrients and sustaining the terrestrial ecosystems (Madigan and Martinko, 2006). Soil microorganisms associated with plant roots have an important influence on plant nutrition, growth promotion, and disease interactions (Assigbetse, 2005). The taxonomic diversity of soil microorganisms is influenced by both plant roots that can locally affect the chemistry of the rhizosphere through composition and amount of root exudates (Frey-Klett et al., 2007) and by the presence of symbiotic organisms that colonize plant roots such as ectomycorrhizal fungi (Burke et al., 2006).

The mycorrhizosphere which is a zone influenced by mycorrhizal plant and fungi and associated bacteria (Linderman, 1988) provides various essential components for soil bacteria that directly or indirectly affect root ecology (Smith et al., 2003). Bacteria found in the mycorrhizosphere are important in the development of the mycorrhizal association as they may contribute in mycorrhizal activities such as nitrogen fixation and nutrient mobilization to ensure availability of nutrients to their hosts and to one another (Bofante and Anca, 2009).

Bacteria that were observed to promote the mycorrhizal association were termed mycorrhizal helper bacteria (MHB) by Garbaye (1994). There are large populations of MHB that can increase mycorrhizal benefits by different mechanisms which include coordinating nutrient availability and uptake, producing plant hormones, helping mycorrhizal fungi to protect plant roots from pathogens and increasing mycorrhizal colonisation (Azcon-Aguilar and Berea, 1992; Frey-Klett et al., 2007).

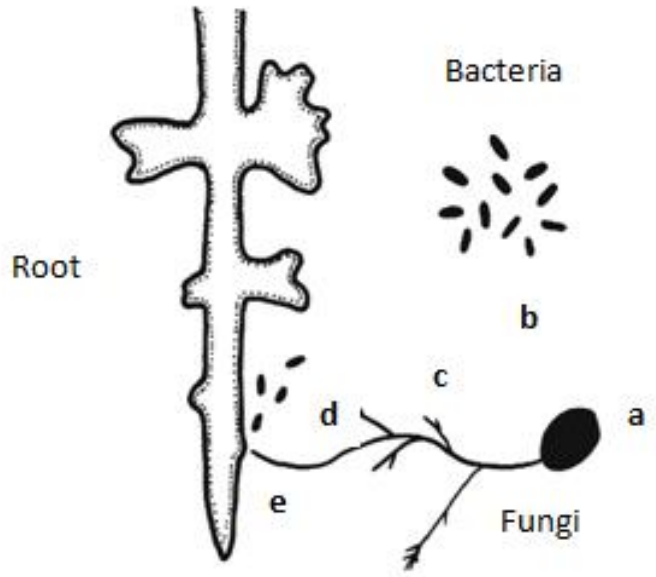

Figure 5.1 Mechanisms of mycorrhizal helper bacteria as proposed by Garbaye (1994) modified from Tarkka and Frey-Klett, (2008). Suggested mechanisms include (a) stimulation of spore germination, (b) release of metabolites that affect soil properties, (c) stimulation of hyphal extension, (d) enhancing host fungal recognition, (e) assisting in root penetration.

Several studies have been conducted to investigate and identify the role of bacteria in the mycorrhizal symbiosis. In 1994, Garbaye proposed various alternative mechanisms by which bacteria can facilitate formation of mycorrhizas (Fig 1.1). He suggested that MHB release exudates which are essential for fungal spore germination and growth of fungal hyphae from germinating spores or other sources such as previously colonised roots. An increase in arbuscular mycorrhizal fungal (*Glomus clarum)* spore germination was observed when direct contact between the fungi and bacteria occurred (Xavier and Germida, 2003). Another study by Will and Sylvia (1990) showed an increase in spore germination of *Glomus deserticola* when *Unicola paniculata* roots were inoculated with *Klebsiella pneumonia*.

MHB can also alter the physic-chemical properties of the soil in order to facilitate the mycorrhizal process (Garbaye, 1994). They improve the soil properties through the production of certain compounds such as siderophores. They are also involved in the detoxification of antagonistic substances produced by both fungi and bacteria which serve as protection against pathogens and competitors (Tarkka and Frey-Klett, 2008). *Amanita muscaria* inhibited the

production of antibiotic compounds released by *Streptomyces spp*. through the release of organic acids (Riedlinger et al., 2006).

Another mechanism of MHB is facilitating the development of external fungal hyphae that spread extensively into the soil environment by producing metabolites that promote mycelial growth such as malic and citric acids (Duponnois and Garbaye, 1990). An increase in mycelia mass would increase the chances of contact between plant root and fungus therefore increasing the mycorrhization rate (Brule et al., 2001).

MHB facilitate the recognition of the plant roots as potential partners by the fungi and *vice versa* (Frey-Klett and Garbaye, 2005). Host plant roots release signals for mycorrhization to fungus and the fungus then extends its mycelial growth towards the root infection site. MHB assist in the enhancement of these mycorrhizal stimulatory signals (Xie et al., 1995). They increase root permeability by producing enzymes such as endoglucanase and xylanase which soften the plant root cell walls for easy penetration by the fungal hyphae (Duponnois, 2006). They also stimulate the root to prepare for mycorrhization through the production of indole acetic acid (IAA) which results in short root formation therefore increasing the contact area between root and soil, increasing surface area for mycorhization to occur (Smith and Read, 2008).

The interaction between soil bacteria and truffles is not well studied but may contribute substantially, through any or all of the above mentioned mechanisms, to the establishment of the relationship between truffles and their host plants.

1.4. CLASSIFICATION OF MYCORRHIZAS

Mycorrhizal associations are divided into various categories depending on the fungal partner (mycobiont), host plant (phytobiont) and the structural changes that occur during the development of the association (Peterson and Farquhar, 1994). The mycorrhizal association has evolved in different directions which have resulted in morphological changes and changes in root structure, providing the best growth conditions to different plant groups. The fungus may colonise the roots of a host plant either intracellularly or extracellularly forming endomycorrhizas or ectomycorrhizas (ECM), respectively. Another group called the Ectendomycorrhizas also exist, they were named because they show both ectomycorrhizal and

endomycorrhizal characteristics. These are mycorrhiza with ECM characteristics but also have intracellular hyphal penetration in the host epidermal and cortical cells (Smith and Read, 2008).

1.4.1 Endomycorrhizas

Endomycorrhizas are the most commonly found mycorrhizal association usually characterized by the internal penetration of the fungal hyphae into host plant cortical cells. This association normally occurs between fungal species of the Glomeromycota and plant species belonging to the Bryophyta, Pteridophyta, Gymnospermae and Angiospermae. Endomycorrhizal association are divided into arbuscular, arbutoid, monotropoid and ericoid mycorrhizas. The arbuscular and ericoid are the two main common types of endomycorrhizas (Imhof, 2009).

Arbuscular mycorrhizas use enzyme activity and hydrostatic force to penetrate the plant root epidermal cells. The hyphae then further penetrate into the cortical cells and forms branchlike structures called the arbuscules. The arbuscules push the plasma membrane aside therefore increasing the contact area between the fungal hyphae and plant root cell components. A large contact area allows better nutrient and carbon exchange between the fungus and host. The arbuscules can be a coiled or multibranched hyphal mass inside the host cells (Imhof, 2009). Some arbuscular mycorrhizas form vesicles which are hyphal swellings with numerous nuclei and lipid bodies that serve as storage compartments. Arbuscular mycorrhizas also produce extraradical hyphae which emerge from the root surface to further parts of the soil thus increasing the nutrient absorption area (Smith and Read, 2008).

Ericoid mycorrhizas are a highly specific symbiotic association between plant species in the Ericaceae, Epacridaceae and Empetraceae families and a diverse group of soil fungi. The first fungal species which was identified to form an endomycorrhizal relationship with ericaceous species was the *Hymenoscyphus* (*Pezizella*) *ericae*. Fungal genera such as *Oidiodendron*, *Myxotrichium* and *Gymnasella* have also been discovered to form mycorrhizal relationships with ericoid plants. Ericoid plants may co-occur with Arbuscular and Ectomycorrhizal hosts. Ericoid mycorrhization is characterised by the invasion of cortical cells of the fine hair roots by fungal hyphae forming hyphal coils. A vascular bundle and a layer of cortical cells make up the fine roots of ericoid plants. The association allows the plants to grow in harsh conditions with low nutrient availability (Smith and Read, 2008).

1.4.2 Ectomycorrhizas

Ectomycorrhizal associations are identified by the presence of a sheath or mantle of fungal hyphae that is wrapped around the root. The sheath serves as protection against plant pathogens and prevents leakage of substrates into the soil as it is wrapped all around the root and everything going in or out of the root must first pass through the sheath isolating it from the soil environment. Hyphal structures also found penetrating around the epidermal and cortical cells are called the 'Hartig net'. The 'Hartig net' serves as a contact area between plant and fungus where nutrient and carbon exchange can take place. There is no intracellular penetration of the root cells by fungal hyphae. Extraradical mycelia are produced which can extend a few millimetres from the root or can be widespread occupying a large soil surface area (Taylor and Alexander, 2005).

The number of plant and fungal species involved in ECM symbioses is estimated to be 6 000 for fungal species (Hawksworth, 2001) and 20 000 - 25 000 for plant species (Brundrett, 2009). Ectomycorrhizal symbionts have been discovered to have low specificity, meaning they can form associations with any available mycobiont or phytobiont. As a result host plants can be colonised by more than one mycobiont. The degree of ECM colonisation varies according to the plant host species and in some cases not all the structural ECM characteristics are present (Smith and Read, 2008).

Basidiomycetes and Ascomycetes are the fungi that usually form ectomycorrhizal relationships with mainly plant species of Gymnospermae and Angiospermae. Ectomycorrhizal associations occur with species under the families such as Pinaceae, Fagaceae, Betulaceae, Myrtaceae and Nothofagaceae to mention a few (Tedersoo et al., 2010). The most researched ECM relationship is between *Tuber melanosporum* Vittad and trees such as *Quercus pubescens* or *Corylus avellana*. Tedersoo et al. (2006) suggested that all members of the Pezizaceae family form ectomycorrhizal associations for the development of their hypogeous fruiting bodies. However some Pezizales occur in areas where no ECM hosts have been identified. Truffle species such as *Eremiomyces echinulatus* and *Kalaharituber pfeilii* have been suggested to form ECM

associations with plants such as *Acacia spp* and *Citrullus vulgaris*, but no known ECM plants have been found in the geographic locations where they occur (Trappe et al., 2008).

1.4.3 Ectendomycorrhizas

Ectendomycorrhizas were given their name as they have both the characteristics of an ECM association such as the 'Hartig net' but may not possess the sheath and they develop intracellular hyphal penetration a characteristic of Endomycorrhizas. The intracellular penetration can be from the 'Hartig net' hyphae or sheath hyphae. Ectendomycorrhizal formation induces the growth of short roots which become covered by highly branched hyphae. The sheath develops behind the apex, between root hairs and further spreads covering the entire root. The 'Hartig net' penetrates between the epidermal and cortical cells. Intracellular penetration occur a few cells away from those where the first 'Hartig net' formation occurred. Once inside a cell, the hyphae branch repeatedly. The ectendomycorrhizal association can remain for at least a year, and there is no evidence of hyphal degeneration or lysis (Smith and Read, 2008).

As with ECM, ectendomycorrhiza occur between Basidiomycete or Ascomycete fungal species and plant species belonging to Gymnospermae or Angiospermae (Smith and Read, 2008). Known ectendomycorrhizal fungi are septate Ascomycetes within the order Pezizales and genus *Wilcoxina* (Danielson, 1982; Egger, 1996) and are associated with phytobionts belonging to the *Pinus* and *Larix* genera (Mikola, 1965; Laiho, 1965; Yu et al., 2001). Truffle species such as *Terfezia* have also been reported to form ectendomycorrhizal associations with their hosts (Adeleke, 2007; Kagan-Zur et al., 1999; Bratrek et al., 1996).

Unlike members of endomycorrhiza, both ecto- and ectendomycorrhizas reproduce sexually and some produce macroscopic fruiting bodies which can be either epigeous (occur above ground) or hypogeous (occur underground) (Tedersoo et al., 2010). The epigeous fruiting bodies include the more recognised mushrooms and the most recognised of the hypogeous fruiting bodies are known as truffles (Hall et al., 2007).

1.5 TRUFFLES

Truffles are known as nutritious seasonal hypogeous fruiting bodies of Ascomycete fungi. They are produced by different Pezizalean genera as reproductive structures filled with spores (O'Donell, 1997; Percudani et al., 1999; Norman and Egger, 1999). Approximately 70 different species have been identified, of which 32 occur in Europe (Karwa et al., 2011). The most appreciated truffles in the world belong to the *Tuber* and *Terfezia* genera, often termed forest and desert truffles respectively, which is mostly reflected in their distribution. These two genera are considered to be of great economic importance because of their potential as food sources (Hall et al., 2003). The most highly recognised are the French Perigold black truffle (*Tuber melanosporum* Vittad.) and the Piemont truffle (*Tuber magnatum* Pico) from Europe are usually purchased at high prices and are in high demand (Hall et al., 2007).

Truffle species of the same genera are known to have similar ecological characteristics and a wide range of host plant species. They occur at different geographic locations (forests and deserts) but most prefer calcareous soils (Salterelli et al., 2008) which are neutral or alkaline (Martin et al., 2010). A few desert truffle species such as *Terfezia arenaria, Terfezia leptoderma* and *Kalaharituber pfeilii* have been observed to occur at a slightly acidic pH (Khabar et al., 2001; Taylor et al., 1995). The formation of truffles is dependent on several factors such as amount and timing of rainfall, soil characteristics, climatic conditions and most importantly the presence of mycorrhizal hosts (Dighton, 2009).

1.5.1 Importance of truffles

Truffles are collected for their distinctive flavour, nutritional value and health benefits (Talou et al., 1990). Several genera including *Tuber, Terfezia* and *Kalaharituber* that produce edible fruiting bodies which include some of the world's most expensive foods and they have a worldwide market (Hall et al., 2003). Their organoleptic properties are responsible for their taste and high commercial value (Salterelli et al., 2008). An analysis of the chemical composition of desert truffles indicated that the dry matter comprised of 20-27% protein, 3-7% fat, 7-13% crude fibre, 60% carbohydrates and 2-5% ascorbic acid (Kagan-Zur et al., 2008). They also contain

water soluble polysaccharides which are used as a natural functional ingredient to increase viscosity and titratable acidity, and improve flavor in the food industry (Miao et al., 2011).

Although they add flavour to staple foods they are also valuable foods which can be enjoyed alone (Al-Laith, 2009). Truffles are normally eaten raw, boiled, roasted or covered in hot ashes (Trappe et al., 2008). In good seasons truffles are dried and ground into powder to supplement the regular diet. Even though the unique aroma of the truffles cannot be preserved by drying, the nutritious flavour is added to a mixture of flatbread which is then baked and eaten with honey. Traditionally desert truffles are cooked simply so as not to mask their delicate aroma (Shavit and Volk, 2007).

Truffles have been used as traditional medicine for centuries without any harmful effects to humans (Al-Rahmah, 2001) and no known toxic compounds have been detected (Ahmed-Ashour et al., 1981). In ancient times truffles were dried and their powder was used for stomach illnesses and open cuts, the juices were used for eye infections (Shavit and Volk, 2007). Truffles have been appreciated in modern medicine as they have shown to be an unlimited source of therapeutic compounds with antiflammatory, antimutagenic, anticarcinogenic (Hanna et al., 1989), antioxidant (Murcia et al., 2002) and antimicrobial (Janakat et al., 2004, 2005) properties. A study by Fratianni et al. (2007) indicated that extracts of the truffle species, *Tuber aestivum*, was observed to contain antimutagenic compounds. *Terfezia claveryi* produced a compound that effectively inhibited the *in vitro* growth of *Staphylococcus aureus* by 66.4% (Shavit and Volk, 2007). A patent was registered which describes the use of truffles (*Terfezia claveryi* Chatin, *Terfezia boudieri* Chatin, *Terfezia arenaria* Trappe, *Terfezia leptoderma* Tul, *Tirmania nivea* (Desf) Trappe and *Tirmania pinoyi* (Maire) Malencon) extracts to treat and prevent senile cataracts in humans and animals (Morgan, A., Patent no US20120039927).

The commercial demand for truffles is growing across the world and successful cultivation can help meet the demand. The most expensive is *T. melanosporum* which can cost up to $3000 per kilogram (Trappe and Claridge, 2010). Truffles are the main source of income for most inhabitants where they are found naturally especially poverty stricken areas (Mandeel and Al-Laith, 2007) such as the Kalahari region of southern Africa.

1.5.2 Factors that affect truffle growth

Truffle development in nature is irregular and ambiguous and depends on several conditions such as soil properties and nutrient availability, climatic characteristics and the type of truffle species (Hall et al., 2003). The occurrence of truffles in a certain area depends on the truffle's specific growth requirements. *Tuber spp* usually occur in forests and *Terfezia, Tirmania* and *Kalaharitube*r species occur in desert as they prefer arid to semi-arid environments (Hall et al., 2007).

Desert truffles are mainly found when rain conditions are favourable with high temperature averages (Trappe et al., 2008). It has been shown that plenty of rain in the beginning of the rainy season is necessary to ensure a good truffle harvest (Kagan-Zur et al., 1999). Water availability is very limited in deserts because of scarce rainfall and sporadic precipitation (Holzapfel, 2008). Although the genera *Terfezia* and *Tirmania* are primarily ectomycorrhizal, they are highly adaptable. Some species like *Terfezia arenaria, Terfezia claveryi* and *Tirmania pinoyi* form endomycorrhizal associations in phosphate poor soils and ectomycorrhizal associations in phosphate rich soils (Fortas and Chevalier, 1992).

In order for truffles to complete their life cycle, several main factors should be present in addition to the abiotic factors, namely the mycorrhizal host plant, the fungus and the associated soil microorganisms (Giamaro et al., 2005). All these occur in the mycorrhizosphere, which is the zone influenced by both the plant root and the mycorrhizal fungi (Linderman, 1988). Like all mycorrhizas, truffles depend on the host plant which provides the fungi with the carbohydrates it needs for growth and development. Bacteria are of importance in truffle development because after fruiting body development the truffle becomes independent of its host plant and depends on bacteria for nutrients because of their ability to modify nutrient availability during the truffle biological cycle (Murat et al., 2008).

1.5.3 Distribution of truffles

Truffles display similarities in their ecological characteristics but their geographic distributions differ (Salterelli et al., 2008). Truffle distribution is mainly determined by the factors that influence truffle growth. Most *Tuber* species (forest truffles) are collected in France, Italy, Spain,

Europe, Asia and certain parts of North America. *Tuber melanosporum* is usually found during winter (November to March) in France, Italy and Spain forming mycorrhizal associations with trees of *Quercus sp*. as well as other hosts. *Tuber magnatum* and *Tuber aestivum* are found in Spain but they occur during summer (May to September) (Hall et al., 2007).

The geographical distribution of desert truffles is limited to arid and semi-arid lands, mostly in countries around the Mediterranean Sea such as Spain, Portugal, Italy, France, Hungary, Turkey, Morocco, Tunisia, Algeria, Egypt, Israel, the Arabian Peninsula, Iran, Iraq, Syria, Saudi Arabia, Kuwait, Iraq and Iran (Al-Laith, 2009) and certain parts of southern Africa (Marasas and Trappe ,1973), North America (Trappe and Sundberg, 1977) Japan and China (Wang, 2011). The regions where desert truffles grow have an annual rainfall ranging from 50 to 380 mm. The truffle season produces good yields if rainfall ranges from 70 to 120 mm in North African countries, and from 100 to 350 mm in countries of southern Europe. The distribution of this rainfall is as important as the quantity; that is, rain is necessary no later than the beginning of December in North African and Middle Eastern countries and no later than the beginning of October in the countries of south Europe (Morte et al., 2008).

1.5.4 Locating fruiting bodies

Locating fruiting bodies is not easy as all truffles are hypogeous. Traditionally, truffles were located by observing the appearance of cracks in the soil which result as the truffles pushes towards the surface (Taylor et al., 1995). This technique proved not to be very effective in areas where soil disturbances occurred due to animal and human activities. Therefore in most countries, animals such as pigs and dogs were trained and used to hunt the truffles. Several studies have been done on certain *Tuber* species to investigate the ability of animals to detect the presence of truffles under the ground (Talou et al., 1995; March et al., 2006). They observed that because of their acute sense of smell these animals could recognise certain volatile compounds produced by the truffles which also contribute to the aroma of the truffles. These animals were found to be able to selectively detect the presence of the compound dimethyl sulphide which is present in the truffles attracting them towards their location (March et al., 2006).

1.5.5 Preservation of truffles

Truffles are seasonal products appreciated and exported worldwide. The most valued truffles such as *Tuber melanosporum* and *Tuber magnatum* are harvested in Europe and are exported to different countries all over the world (Hall et al., 2007). Truffle shelf life is usually shortened due to the presence of certain bacteria and other fungi which are normally found on the peridium and gleba of the truffles. These microorganisms feed on the truffles as they are highly nutritious (Rivera et al., 2010). Effective storage methods are essential to preserve them for longer and maintain them free of contamination. Different methods have been studied which include storage at various temperatures, irradiation and sterilization by high pressure (Salterelli et al., 2008; Adamo, 2004; Murcia et al., 2003). The used techniques should be able to maintain the truffles's nutritional composition such as protein, carbohydrate and fatty acid contents and should also be able to decrease the microbial population.

Storage at low temperatures has been used as low temperatures can slow down microbial growth. Salterelli et al. (2008) studied the effects of different storage treatments on *T. magnatum, T. borchii, T. melanosporum* and *T. aestivum*. Three different treatments were used which included storage at 4°C, exposure to liquid nitrogen before keeping at -20°C and autoclaving then storing at 4°C . Changes in protein and carbohydrate contents were monitored. Storage at 4°C was considered the better option as it best preserved the biochemical characteristics.

Several studies indicate that irradiation could be an effective method for truffle preservation but close analysis on the changes in chemical compounds such phenols and antioxidant compounds which are essential for preservation of products, need to be further studied (Adamo, 2004; Cullerê et al., 2010). According to Adamo, (2004) softening and browning of the truffles occurs at high doses (3-10 kGy) but the microbial load is decreased at low doses (1.5-2.0 kGy). Other preservation methods include drying at low temperatures with good air circulation (Trappe and Castellano), canning (Murcia et al., 2003) and storage in silica gel (van der Westhuizen and Eicker, 1994). Treatment with the correct irradiation dose, proper packaging and storage at low temperature could successfully preserve the truffles for longer. The type of preservation used depends on the intended use of the truffles. For human consumption, methods that retain the moisture of the truffle are preferred and dried samples are often used as flavour additives, baking and for medicinal purposes (Shavit and Volk, 2007).

1.5.6 Cultivation

The decline in truffle harvest over the last century (Fig 1.2) has lead researchers to seek alternative methods for truffle production. The decrease in harvest is due to a number of factors such as overharvesting, deforestation, loss of host plants due to pests and diseases, global warming, acid rain and most importantly, water availability (Hall et al., 2003). Ethnomycological studies have suggested that the cultivation of truffles could contribute to improving the rural economic development in the areas where they can be cultivated (Cano, 2003; Honrubia et al., 2003, 2007; Mandeel and Al-Laith, 2007).

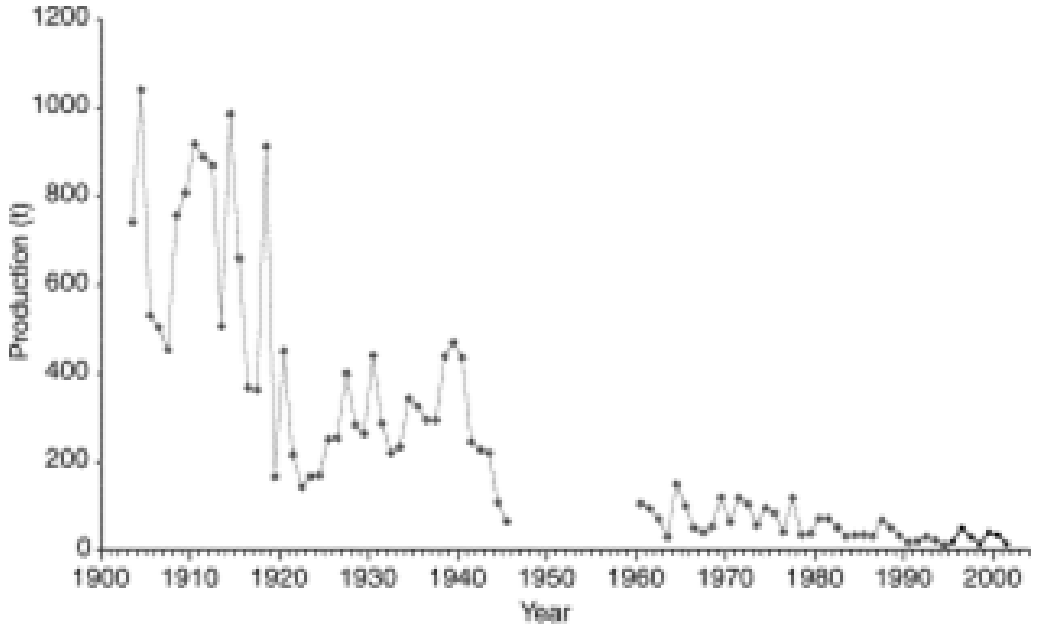

Figure 1.6 *Tuber melanosporum* and *Tuber brumale* production (t) over the last century (Hall et al., 2003).

Interest in truffle cultivation began in the mid 1800's, which led to the discovery of ectomycorrhizas in 1885. The first cultivation experiment was established in France for *Tuber melanosporum* species by Talon. He discovered that if seedlings grown under *T. melanosporum* producing oaks were used to establish new truffières, fruiting bodies would be visible from 5 to 20 years. The method had a few challenges which included infection by pathogens and presence of competing mycorrhizal fungi (Hall et al., 2003). Talon's technique was widely used for years until 1970's when more effective methods were introduced which included inoculation of host

plants with *T. melanosporum* spores or growing the plant with other mycorrhizal plantlets (Kagan-Zur et al., 2002). The successful cultivation of *T. melanosporum* resulted in several cultivation experiments to be conducted throughout the world. These experiments were based on field plantations of mycorrhizal plants. Many truffières have been created in Italy, Spain, China, Finland, Australia, North America and New Zealand (Hall et al., 2007; Morte et al., 2008).

For some time only the *Tuber* species could be cultivated until biotechnological techniques were recently developed which assisted in the successful cultivation of the *Terfezia* species (Morte et al., 2008). Methods of cultivation included inoculation of host plants with spore suspensions (Hall et al., 2002) and slurries from fruiting bodies or pure cultures (Sisti et al., 1998). Even with these methods, only a few species have been successfully cultivated (Hall et al., 2003). Slama et al. (2010) successfully cultivated *T. boudieri* Chatin with the mycorrhizal host plant *Helianthemum sessiliflorum* Desf. Pers. *H. sessiliflorum* seeds were inoculated with a *T. boudieri* fruitbody slurry added on soil and vermiculite mix. The plants were maintained in a greenhouse and fruiting bodies were observed to occur after three years.

There are several factors which need to be considered for successful cultivation of truffles such as site selection which is determined by soil texture and nutrient composition, choice of host plant species which should be best adapted to the selected site and most importantly the conditions should be favourable for truffle production. The absence of any of these required factors can reduce and sometimes hinder their production and growth (Hall et al., 2003). The main challenges in truffle cultivation are infection by other microorganisms and lack of knowledge of each truffle's specific growth requirements. Fertilizer application has been introduced in truffle cultivation but its effects have not yet been confirmed. A study by Suz et al. (2010) concluded that the application of fertilizers has no particular significance in truffle development but some changes were observed in the development of roots of host plants in comparison to the control. Previous studies indicate that fertilizer application can decrease the level of mycorrhization as the plant might become less invested in its fungal partner as it will be able to obtain nutrients from the soil. Additionally, the fertilizer concentrations required by the plant may not be favourable for truffle growth and development (Dupre et al., 1982).

1.6 DESERT TRUFFLES OF SOUTHERN AFRICA

The term 'desert truffles' comprises species of different hypogeous Ascomycetes genera, such as *Terfezia, Balsamia, Delastreopsis, Delastria, Leucangium, Mattirolomyces, Phaeangium, Picoa, Tirmania* and some *Tuber* species. A new desert truffle genus, *Kalaharituber* was introduced to replace some of the truffles occurring under Pezizales which were previously classified under the *Terfezia* genus (Ferdman et al., 2005). Their name matches the nature of their distribution, which are typically areas with arid and semiarid conditions (Honrubia et al., 1992). Development of desert truffle fruiting bodies depends on the amount and distribution of rainfall (Morte et al., 2008, 2009). Desert truffles such as *Eremiomyces*, *Kalaharituber*, *Terfezia* and *Tirmania* are appreciated in the Middle East, North Africa and in the Kalahari region of southern Africa.

The Kalahari Desert is a large semi-arid savannah area in southern Africa. It covers mostly Botswana and parts of Namibia and South Africa (Fig 1.3). It is characterised by low and poorly distributed amounts of rainfall, high temperatures ranging from 20 - 45°C in summer, low relative humidity, extreme temperature fluctuation and frequent droughts. It usually receives about 76-190 mm rainfall per year. In the rainy season grasses grow throughout the Kalahari (Taylor et al., 1995). The Bushmen's grasses (*Stipagrostis sp.*) are the dominant grasses found. The sand is mostly red and its vegetation includes thorn trees, shrubs and grasses. The vegetation can survive throughout the dry season as it usually rains in summer. The *Acacia erioloba* (Camel thorn tree), *Boscia lehmanniana* (Sheperd tree), *Citrullus lanatus* (Tsamma) and *Rhizogum trichotomum* (Drie doring) include some of the plants found in the Kalahari (van Oudtshoorn, 1992). The soil texture is more than 90% sand. The soil is nutrient poor with a slightly acidic to neutral pH (Wang et al., 2007). The soil is low in organic matter and minerals such as N, P and K. Plant species found in the Kalahari need to have deep root structures for water absorption and stability.

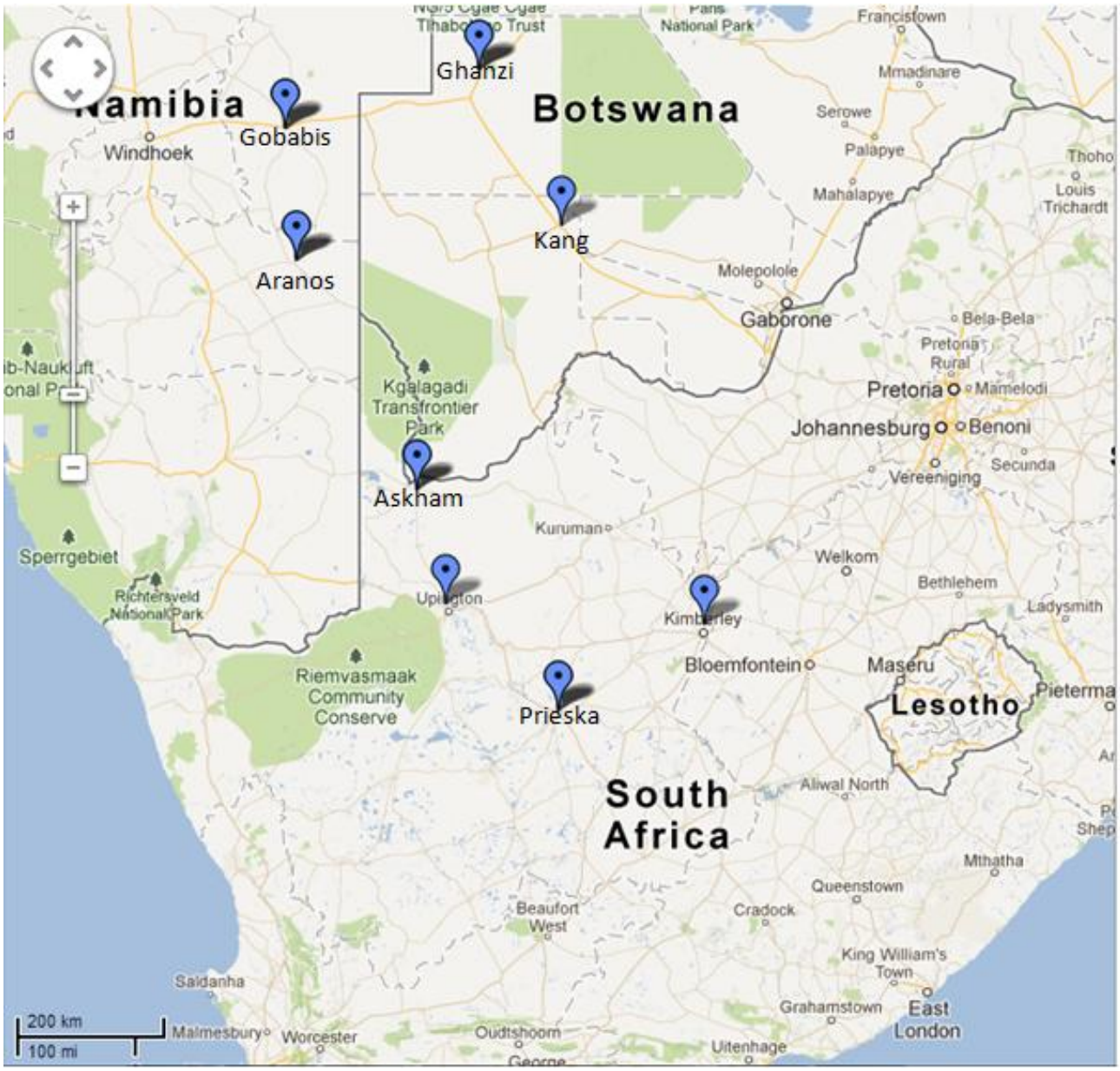

Figure 1.7 Kalahari region of southern Africa adapted from www.googlemaps.co.za. The ballooned areas indicate places where occurrence of truffles has been reported in recent years.

Trappe et al. (1979) concluded that three genera of desert truffles occur in the Kalahari with each represented by one species namely, *Choiromyces echinulatus* Marasas & Trappe, *Terfezia pfeilii* and *Terfezia austroafricanus* all belonging to the Ascomycota. Phylogenetic studies have showed that neither *Terfezia pfeilii* nor *Choiromyces echinulatus* belonged to the *Terfezia* genus

(Ferdman et al., 2005). Two new genera and species were introduced. *Terfezia pfeilii* was reclassified and is now called *Kalaharituber pfeilii* and was placed under the new *Kalaharituber* genus. *Choiromyces echinulatus* was also changed to *Eremiomyces echinulatus* under the genus *Eremiomyces. Terfezia austroafricanus* was also changed to *Mattirolomyces austroafricanus* (Ferdman et al., 2005; Trappe et al., 2008). The Terfeziaceae family under which the *Terfezia* and *Tirmania* were traditionally placed under was abolished and these genera were subsequently placed under the Pezizaceae (Norman and Egger, 1999; Percudani et al., 1999; Ferdman et al., 2005).

A common feature for all desert truffles is the presence of large, inflated, thin walled cells in the peridium and gleba (Alsheik, 1994; Trappe, 1990). *Terfezia* species have spherical and ornamented spores while *Tirmania* species have smooth spores and amyloid asci. The fruiting bodies are round, tan to brown and look like small, sandy potatoes. The truffles produced by both genera have similar morphology and are not easy to distinguish. A simple iodine test can separate them. A drop of iodine on the cut flesh of *Terfezia* fruitbody will not change or turn yellow to orange. Cut fruiting bodies of *Tirmania* will turn blue- green to black. There is a close genetic relationship between species of *Tirmania* and *Terfezia* and they may have risen from a single evolutionary lineage of fungi that adapted to the heat and drought by growing fruiting bodies underground (Trappe et al., 2008)).

The fruiting bodies of *Kalaharituber, Eremiomyces* and *Mattirolomyces* genera are almost similar but they can also be distinguished using a simple iodine test. The *Kalaharituber* and *Eremiomyces* species will not change or will turn yellow to orange and the fruiting bodies of *Mattirolomyces* species will turn blue green to black (Shavit and Volk, 2007).

1.6.1 *Kalaharituber pfeilii* (Henn.) Trappe & Kagan-Zur

Kalaharituber pfeilii, also known as the Kalahari truffle, was described from Namibia by Hennings (1897). The name *Kalaharituber* means "truffle of the Kalahari" and "*pfeilii*" which was given in honour of Count Pfeil, who was known as the original collector of the species (Ferdman et al., 2005). Previously it has been misidentified as *Terfezia boudieri* Chatin, *Terfezia cleveryi* Chatin and *Terfezia pinoyi* Maire. It was also identified as *Terfezia pfeilii* until

molecular evidence proved otherwise and it was assigned to a new genus, *Kalaharituber* (Ferdman et al., 2005).

Very little information is known about the biology and ecology of *K. pfeilii* (Taylor et al., 1995). The fruiting bodies are subglobose, brown and look like sandy potatoes (Shavit and Volk, 2007). It has smooth, subglobose ascomata. The peridium is approximately 1 mm thick with a yellowish to dark brown colour and the gleba is solid, marbled with sterile white veins. It is distributed in the Kalahari Desert region of southern Africa (Ferdman et al., 2005). It is regarded as one of the main staple foods of the Kalahari inhabitants. A mature Kalahari truffle can grow up to 12 cm in diameter and weigh approximately 200 g but in seasons of good rain they can weigh between 400 – 500 g. *K. pfeilii* occurs in the Kalahari from April to May after late summer rains (van der Westhuizen and Eicker, 1994).

1.6.2 Life cycle of *Kalaharituber pfeilii*

Truffle fruiting bodies germinate from spores which are enclosed in the peridium and gleba (Trappe and Weber, 2001). The spores are dispersed by various methods such as by humans, animals and the wind. The ripening fruiting bodies release a distinguishable aroma which grows stronger as they ripen. A variety of animals (humans included) are attracted by the aroma and they enthusiastically collect and consume the truffles and later disperse the spores to new areas, usually in their faeces (Shavit and Volk, 2007). Animal species found in the Kalahari such as baboons (*Papio ursinus*) and hyenas (*Hyaena brunnea*) have been spotted digging up and feeding on the Kalahari truffles (Trappe et al., 2008).

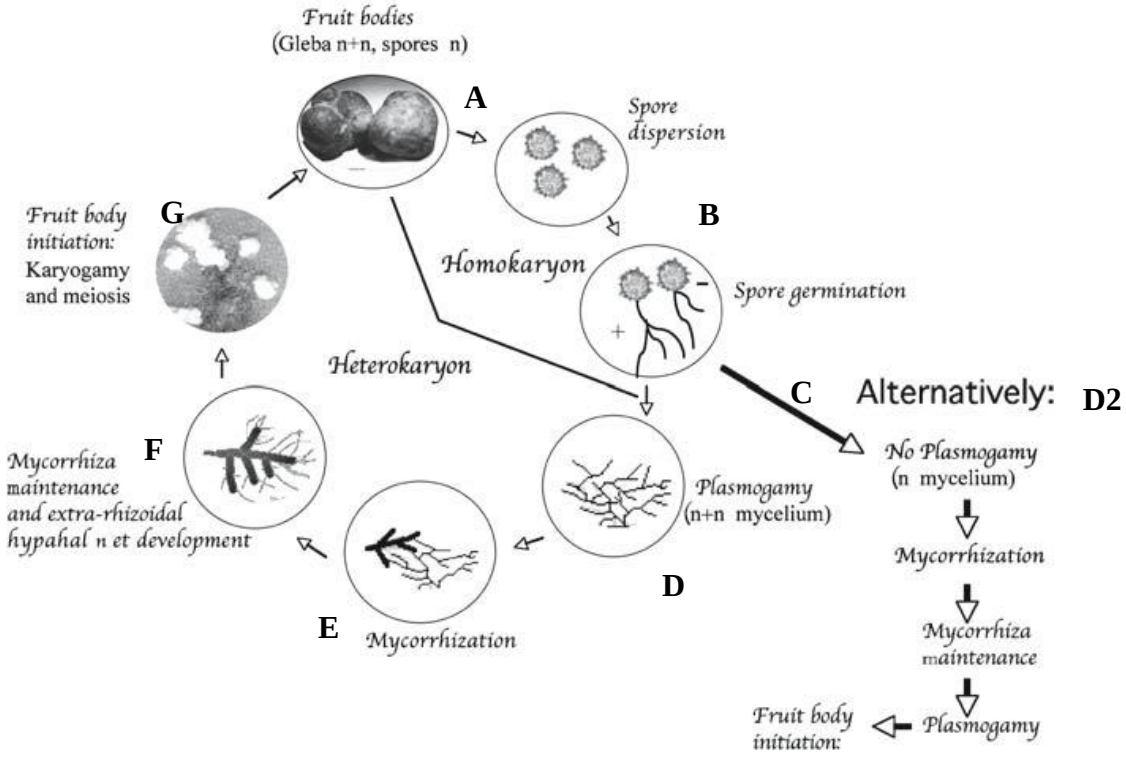

Figure 1.8 The proposed life cycle of desert truffles indicating the reproductive, vegetative and the symbiotic phases (Adapted from Roth-Bejerano et al., 2004; Kagan-Zur et al., 2008): A- truffle dries up and spores are released, B- spores are dispersed either by wind or animals, C- spore germinates into homokaryotic mycelium, D- heterokaryotic mycelium after plasmogamy, E- mycorrhizal initiation resulting from inoculation of host by fungal hyphae, F- hyphal and mycorrhizal development, G- karyogamy and meiosis taking place within the truffle fruiting bodies. D2- sometimes plasmogamy occurs later in the life cycle and mycorrhization is initiated by the homokaryotic hyphae (Roth-Bejerano et al., 2004; Kagan –Zur et al., 2008).

Another form of dispersal is by the wind. On maturation fruiting bodies produce a heap which becomes visible on the surface of the sandy soil (Trappe et al., 2008). The heap dries out producing cracks on the soil surface (Mshigeni, 2001). The wind will enlarge the cracks and the truffles will be exposed to air (Fig 1.4A). The truffles become dry and their thin walled cells also dry out and collapse exposing spores within the truffles which are carried away by the winds (Trappe et al., 2008). The spores remain dormant until rainfall occurs (Trappe and Claridge, 2010). Truffles contain thin walled cells which absorb large quantities of water which results in swelling and therefore providing the moisture needed for spore formation (Trappe et al., 2008).

The spores then germinate and produce a primary homokaryotic mycelium (Fig 1.4B). The mycelium then germinates and inoculates the host either as a primary homokaryotic mycelium or as a secondary heterokaryotic mycelium after undergoing plasmogamy (Fig 1.4D). It is not clear when plasmogamy occurs when inoculated by the primary homokaryotic mycelium. Once the heterokaryotic mycelia inoculate the host (Fig 1.4F), fruiting bodies are formed. Karyogamy and meiosis are presumed to take place within the fruiting bodies (Fig 1.4G) (Roth- Bejerano et al., 2004; Kagan –Zur et al., 2008).

1.6.3 Host plants of *Kalaharituber pfeilii*

Desert truffles have a wide host range. Plants associated with the Kalahari truffle include trees, shrubs and grasses (Taylor et al., 1995). It has also been found on fields cultivated with various food plants (Trappe et al., 2008). Plant species suspected of forming mycorrhizal associations with *K. pfeilii* include *Acacia erioloba* E.Meyer, *Cynodon dactylon* L., *Schmiditia pappophoroides* Steud., *Sorghum bicolour* L., *Citrus vulgaris* Schrad. and *Pennisetum typhoides* (Taylor et al., 1995; Kagan-Zur et al., 1999).

Citrus vulgaris was confirmed a host but it could not be the only host as *K. pfeilii* can be also found in areas where *C. vulgaris* does not grow (Kagan-Zur et al., 1999). Taylor et al. (1995) stated that since there is no sole host plant for *K. pfeilii,* any plants with roots associated with the truffle should be considered a possible host. This is not entirely correct as studies by Kagan-Zur et al., (1999) and Adeleke, (2007) demonstrate that not all plants found in the truffle vicinity are hosts and only *C. vulgaris,* confirmed using PCR and RFLP (Kagan-Zur et al., 1999) and *A. erioloba* (Adeleke, 2007) are possible host so far.

Stipagrostis ciliata var. *capensis* is a perennial grass normally found in the desert areas of Israel and the Kalahari region of southern Africa. It is known to be also annual depending on water availability. It was described in 1965 by Prof. de Winter. *S. ciliata* var. *capensis* previously known as *Aristida ciliata* Desf is an angiosperm from the Gramineae family and is also known as the Tall bushmens grass. It occurs in arid and semi-arid areas such as Spain, Egypt, Tunisia, Botswana, Namibia, South Africa, Kuwait and Saudi Arabia. *S. ciliata* var. *capensis* are found forming circles of clumps known as “fairy rings”, with the older parts of the grass situated in the

centre of the clumps. It is believed that this circular arrangement is due to vegetative multiplication that occurs. *S. ciliata* var. *capensis* possess highly branched bases with stems that can grow up to 1m high (van Oudtshoorn, 1992). They are usually unbranched and yellowish in colour with 2-3 nodes covered with white hairs (van Rooyen, 2001). The leaves are greyish green with leaf sheaths (De Winter, 1965). Leaf sheaths are also dense with long spreading hairs on the tips where the seeds are found (Danin and Orshan, 1994). Flowering occurs from spring to early summer which is usually August to October (Russell et al., 1990). Seed germination can occur at any time of the year but only under favourable conditions such as moisture availability (van Rooyen, 2001).

Desert soils are approximately 95% sand which causes difficulties for plants to remain stable, especially in conditions such as wind and runoff. As a result certain desert plants form the sandy rhizosheath which helps to stabilise them. The occurrence of the rhizosheath was first reported by Volkens in 1887. He found it on grass roots and later Prince (1911) demonstrated its presence on various perennial grasses occurring in North African deserts. Othman et al. (2004) discovered a sand encrusted rhizosheath covering the roots of some of the plant species found in the Sinai desert. *Stipagrostis sp* was one of the species found. The rhizosheath is described as a crust of sand covering the roots and is known to be created from microbial and root excretions (Othman et al., 2004). Moreno-Espindolaa et al. (2007) observed that root hairs and fungal hyphae also play a role in the rhizosheath formation. Various microorganisms which include certain bacterial species such as *Bacillus circulans, Paenibacillus macerans, Enterobacter agglomerans*, *Agrobacterium radiobacter* and *Pseudomonas luteola* were found to thrive on the rhizosheath.

The rhizosheath serves as a protective barrier protecting the plant roots from pathogens and herbivores. It has been observed to play an important role in nutrient uptake and water absorption and retention. The rhizosheath helps maintain the moisture in soils as it closes the air gaps between root and soil particles which are caused by shrinkage of roots (North and Nobel, 1997). Rhizosheaths of plants occurring in dry areas are thicker than those in wetter areas (Watt et al., 1994). For efficient absorption of water and nutrients, plant roots need to be in close contact with the soil particles. Good soil contact is even more difficult to achieve in sandy soils because of their loose structure which results in excessive pores, making it difficult to maintain the moisture (North and Nobel, 1997). The rhizosheath contains mucigel with helps during water retention

(Young, 1995). Nitrogen fixing bacteria have been found to thrive in the rhizosheath (Wullstein et al., 1979; Bergmann et al., 2009).

MOTIVATION

Truffles are collected for their unique flavour, nutritional value and medicinal properties. The demand for truffles has been growing rapidly and even *Kalaharituber pfeilii* has a market in certain parts of the world (Shavit and Volk, 2007). Truffle harvest decreases annually due to the fact that certain growth factors are not always favourable (Hall et al., 2003). The *in vitro* production of the *Kalaharituber pfeilii* has not been successful because its host plants have not been successfully identified. The Kalahari truffle has been associated with trees, shrubs and grasses normally found in its vicinity (Taylor et al., 1995). Plant species such as *Acacia erioloba* E.Meyer, *Cynodon dactylon* L., *Schmiditia pappophoroides* Steud., *Sorghum bicolour* L., *Citrus vulgaris* Schrad. and *Pennisetum typhoides* have been suspected of forming mycorrhizal association with *Kalaharituber pfeilii* but none have been confirmed as the mycorrhizal host (Taylor et al., 1995; Kagan-Zur et al., 1999). The grass species *Stipagrostis ciliata* var. *capensis* is also known to occur in most areas where *Kalaharituber pfeilii* grows but its potential as a possible host has not been previously explored and will therefore be the main focus in this study. Identification of the host species could lead to the first ever commercial production of the Kalahari truffle. Success of its commercial production could lead to it being enjoyed all over the world like other truffles such as *Tuber melanosporium* (Perigold Black Truffle) and *Tuber magnatum* (Oregon White Truffle) which could boost the economy of rural communities of southern Africa.

HYPOTHESIS

Stipagrostis ciliata (Desf.) De Winter var. *capensis* (Trin. & Rupr.) De Winter is the mycorrhizal host plant of *Kalaharituber pfeilii* (Henn) Trappe & Kagan- *Zur*.

Bacteria occurring in the rhizosphere contribute towards the establishment of the mycorrhizal association.

AIM

The aims of this project were to prove that *Stipagrostis ciliata* var. *capensis* is a phytobiont of *Kalaharituber pfeilii* and to identify associated mycorrhizal helper bacteria and establish their role in the relationship.

OBJECTIVES

In order to address the aims of this study, the objectives were to:

- **Collect samples**

Truffle, root and soil samples were collected from selected areas of the Northern Cape.

- **Identify fruting bodies to confirm that they were *Kalaharituber pfeilii***

i. Morphologically identify the collected fruiting bodies.

ii. Molecularly identify the collected fruiting bodies to confirm the morphological identification.

iii. Isolate and maintain pure cultures of *Kalaharituber pfeilii* for use in other experiments throughout the study.

iv. Design *Mattirolomyces austroafricanus* and *Eremiomyces echinulatus* primers, confirm their specificity and use them on collected fruting bodies to confirm and/or eliminate the presence of other truffle species in the collected samples.

- **Investigate that *Stipagrostis ciliata* var. *capensis* is a phytombiont of *Kalaharituber pfeilii***

i. Isolate the mycorrhizal fungi associated with the rhizosheath of *Stipagrostis ciliata* var. *capensis*.

ii. Identify fungi found on roots, rhizosheath and isolated rhizosheath cultures using molecular techniques to confirm any association between *Stipagrostis ciliata* var. *capensis* roots/rhizosheath and *Kalaharituber pfeilii*.

iii. Microscopically examine the type of mycorrhizal association found in roots as *Kalaharituber pfeilii* forms an ectendomycorrhizal association therefore the presence of other mycorrhizal associations will eliminate that particular plant as a host.

iv. Germinate collected *Stipagrostis ciliata* var. *capensis* seeds.and initiate *in-vitro* mycorrhizal re-synthesis experiments to establish mycorrhizal association between *Kalaharituber pfeilii* and *Stipagrostis ciliata* var. *capensis.*

- **Determine the role of associated bacteria**

i. Isolate and identify bacteria found in the truffles and the rhizosheath.

ii. Investigate the role of the associated bacteria by determining their Indole Acetic Acid and phosphate solubilising effects.

- **Conduct a survey**

i. Conduct a survey on *Kalaharituber pfeilii* occurrence and distribution on local farms.

CHAPTER 2: MATERIALS AND METHODS

2.1 Collection of samples

Truffle fruiting bodies, soil, seeds and roots of *Stipagrostis ciliata* var. *capensis* were collected in April 2010 and May 2011 from two sites of the Haakdoring Duin Farm in Upington, Northern Cape, South Africa, (S27 ° 39.261′, E21° 03.964′ (Site 1) and S27° 37.318′, E20° 59.214′ (Site 2). Collected samples were placed in paper bags and stored in cooler boxes for transportation.

2.2 Morphological identification of the collected fruiting bodies

On arrival in the laboratory, fruiting bodies were processed immediately following the methods for morphological and molecular identification described below and some were preserved at 4°C until further use. The fruiting bodies were identified morphologically by observing physical characteristics such as colour and texture of gleba, appearance of veins, shape and colour of peridium following the 'Key to Kalahari truffles' described by Trappe and Castellano (2007).

2.3 Molecular identification the collected fruiting bodies

Further confirmatory identification was done using molecular analysis because morphology can be misleading especially for closely related species (Ferdman et al., 2005).

2.3.1 DNA extraction

Fruiting bodies were rinsed in running water to remove dirt and sand. They were surface sterilised with 50% ethanol, broken open avoiding contamination and explants from inside the truffles were used for DNA extraction. DNA extraction was conducted using the Zymo Research Fungal/Bacterial Miniprep Kit (Catalog no.D6005) according to manufacturer's instructions. About 200 mg of tissue obtained from inside the truffle was placed in a ZR bashing bead lysis tube followed by the addition of 750 µl of the lysis solution. The tubes were vortexed to disrupt and lyse the cells. Tubes were microcentrifuged at 10 000 x g for 1 min. The supernatant was centrifuged through a spin filter in a collection tube at 7 000 x g for 1 min to remove any solid particles and 1 200 µl of DNA binding buffer was added to the filtrate. The mixture was filtered through a spin column to provide a solid support where DNA can bind and the flow through was discarded. A volume of 200 µl of DNA pre-wash buffer was added to the column and

centrifuged at 10 000 x g for 1 min. The column was washed by adding 500 µl of the DNA wash buffer. The pre-wash and wash steps were performed to recover the DNA by precipitation. DNA was eluted with 100 µl of elution buffer and stored at -20°C.

2.3.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was prepared using the KAPA*Taq* ReadyMix DNA Polymerase solution (Catalog no.KK1006) with a final volume of 50 µl. The solution consisted of 2x ReadyMix containing 0.05 U μl^{-1} Kapa*Taq* DNA Polymerase, Reaction Buffer with Mg^{2+} and 0.4 mM each of dNTP. Each PCR reaction consisted of 25 µl ready mix, 2 µl each of forward and reverse primers, 3 µl of 3 mg ml^{-1} bovine serum albumin (BSA) to overcome the effect of PCR inhibitors when processing the samples (Paolocci et al., 1999; Iotti and Zambonelli, 2006), 3 µl DNA template and 15 µl sterile distilled water. The amplification was performed using *K. pfeilii* specific primers TPF3 - 5′ CAA TTG TAG CAA GTG AGC CCT CTG GT 3′ and TPR1 - 5′ ACC TTC TTA TCC AAT GAG TCC 3′ (Adeleke, 2007) synthesised by Intergrated DNA Technologies, Cape Town. The PCR was conducted using the Applied Biosystems 2720 Thermal Cycler with the following cycling parameters : 1 cycle denaturing at 94°C; 35 cycles of 1 minute denaturing at 94°C, 30 seconds annealing at 51°C, 2 minutes elongation at 72°C; 5 minutes final elongation at 72°C.

2.3.3 PCR product clean up

Purification of PCR products was performed to remove excess nucleotides and primers. The PCR products were purified using Promega Wizard SV Gel and PCR clean-up System (Catalog no. A9281/2/5), according to manufacturer's instructions. A membrane binding solution equivalent to 3 times the volume of DNA was added to the DNA. The mixture was transferred to a SV minicolumn in a collection tube and incubated at 25°C for 1 minute then centrifuged for a minute at 16 000 x g. The minicolumn was transferred to a clean collection tube, washed by adding 700 µl of membrane wash solution and centrifuged at 16 000 x g for 1 minute. This wash step was repeated with 500 µl membrane wash solution and centrifuged for 5 minutes. Flow through was discarded and the minicolumn was centrifuged for 1 minute with centrifuge lid open to allow evaporation of residual ethanol. DNA was eluted with 50 µl nuclease free water and stored at -20°C.

PCR products including a positive control (*K. pfeilii* DNA confirmed by Adeleke, 2007) and negative control (autoclaved distilled water) were separated by electrophoresis in 1% agarose gel stained with 0.5 μgml^{-1} ethidium bromide along with a 100 bp Promega molecular marker (Catalog no. 29278810) at 100 Volts for 1 hour and later submitted to the Rhodes University, Sequencing Facility, Grahamstown and Inqaba Biotechnical Industries, Pretoria for sequencing. The sequences were then submitted to NCBI-BLAST online (http://blast.ncbi.nlm.nih.gov/Blast.) for identification (Altschul et al., 1997).

2.4 Isolation and maintaining pure cultures of *Kalaharituber pfeilii*

Fruiting bodies were used fresh and some were preserved by refrigeration at 4°C (Salterelli, et al., 2008). The fruiting bodies were rinsed in running water and surface sterilised with 50% ethanol. After sterilisation they were split open avoiding contamination of the inside layers. Thin inner pieces of the fresh fruiting bodies were removed using sterilised forceps and inoculated onto modified Fontana (FTN) medium (Appendix A) with pH 7.2 and incubated at 32°C which are the optimum pH and temperature for *K. pfeilii* mycelia growth (Adeleke, 2007).

Contaminating bacteria occurring on the *K. pfeilii* cultured plates were subcultured on Nutrient Agar (NA) (Appendix A) for use in the investigation of the role of Mycorrhizal Helper Bacteria (MHB). Fungal mycelia were subcultured by transferring plugs of inoculum to new FTN plates (Adeleke, 2007) in order to maintain pure cultures. Pure fungal mycelial cultures were observed microscopically using the tape mount method and staining with lactoglycerol Trypan Blue (Appendix B) (Smith and Dickson, 1997). The tape mounts were made to confirm the presence of septa and lack of asexual spores.

2.5 Isolation of mycorrhizal fungi associated with rhizosheath

The collected *Stipagrostis ciliata* var. *capensis* roots were gently shaken in sterile distilled water to remove dirt and sand particles. Small pieces of the rhizosheath were removed from the roots using sterile forceps and placed onto modified FTN medium (Adeleke, 2007). Plates were incubated at 32°C. Growth was observed after 48 hours of incubation. Contaminating bacteria were subcultured on NA and maintained for MHB studies. Growth of fungal mycelia was

continually monitored and was subcultured to obtain pure cultures which were then observed microscopically using the tape mount method.

2.6 Identification of isolated truffle and rhizosheath cultures using molecular techniques

Pure fungal cultures obtained from truffle and rhizosheath samples were scraped off the surface of FTN medium and DNA was extracted using the Zymo Research Fungal/Bacterial Miniprep Kit (Catalog no.D6005) according to the instruction manual. PCR was performed to amplify DNA using the *K. pfeilii* primer pair TPR3 and TPF1 (Adeleke, 2007). The PCR products were purified, separated by electrophoresis alongside the negative and positive controls and sent for sequencing as described in section 2.3.

2.7 Microscopic examination of the type of mycorrhizal association found in roots

Root samples were collected underneath and around the vicinity where truffles were located. The collected root samples belonged to *Stipagrostis ciliata* var. *capensis, Stipagrostis obtusa* and *Schimidtia kalihariensis.* The roots were individually rinsed under running water through a sieve to remove excess sand and dirt and soaked in distilled water. Roots were cut and subjected to staining for microscopy as described by Smith and Dickson (1997).

Roots were left in 5% KOH solution (Appendix B) overnight to clear their cells. The KOH was discarded using a sieve to prevent loss of roots. They were rinsed with distilled water and left in the water overnight. Roots were then bleached with alkaline H_2O_2 (Appendix B) for 30 min, rinsed and left overnight in distilled water. After bleaching the roots were acidified by soaking in 0.1M HCl (Appendix B) overnight. The acid was discarded and without rinsing, the roots were stained with 0.5% Trypan Blue in lactoglycerol (Appendix B) overnight. The stain was poured off and a lactoglycerol destain (Appendix B) was added also without rinsing the roots. The roots were left in destain overnight before microscopic examination (Smith and Dickson, 1997).

After staining pieces of the roots were placed onto microscopic slides and covered with cover slips. The slides were observed using the Digital compound light microscope (Olympus BX50) with camera (Olympus DP72) to investigate the type of mycorrhizal relationship present.

2.8 Molecular identification of fungi associated with roots

DNA was extracted from sections of the sand encrusted rhizosheath roots using the UltraClean Plant DNA Isolation Kit (Catalog no. 13000-50) according to manufacturer's instructions. Plant root tissue was cut into small pieces and added to 2 ml bead solution tubes followed by the addition of 60 µl of solution P1. Tubes were vortexed, placed in a water bath at 65°C for 10 minutes and vortexed again horizontally at maximum speed for 10 minutes. After the homogenisation and lysis steps, tubes were centrifuged at 10 000 x g for 30 seconds to pellet plant tissue and unwanted cell contents. The supernatant was transferred into a new micro-centrifuge tube. An aliquot of 250 µl of solution P2 was added to the tube to precipitate unwanted proteins. After mixing, the tubes were incubated at 4°C for 5 minutes and centrifuged at 10 000 x g for 1 minute to pellet the proteins. A volume of 500 µl of supernatant was thoroughly mixed with 1000 µl of solution P3. A volume of 650 µl of the mixture was loaded onto a spin filter and centrifuged for 30 seconds at 10 000 x g to bind the DNA to the spin filter. This step was repeated with remaining sample. The spin filter was transferred to a new centrifuge tube, 300 µl of solution P4 was added and centrifuged for 30 seconds at 10 000 x g to remove residual salts and to clean the DNA. Spin filter was re-centrifuged for 1 minute to remove residues of solution P4. The spin filter was placed into a new collection tube and 50 µl of solution P5 was added to elute DNA by unbinding it from the spin filter. Tube was centrifuged for 30 seconds and spin filter was discarded. DNA was ready for use and stored at -20°C.

Amplification of DNA was done using the nested PCR method. The PCR reaction was prepared using the KAPA*Taq* ReadyMix DNA Polymerase (Catalog no. KK1006). The ready mix consisted of 2x ReadyMix containing 0.05 U μl^{-1} Kapa*Taq* DNA Polymerase, Reaction Buffer with Mg^{2+} and 0.4 mM each dNTP. Each reaction consisted of 25 µl ready mix, 2 µl each of forward and reverse primers, 3 µl of 3 $mgml^{-1}$ bovine serum albumin (BSA) to overcome the effect of PCR inhibitors when processing the samples (Paolocci et al., 1999; Iotti and Zambonelli, 2006), 3 µl DNA template and 15 µl sterile distilled water. The first step was performed using the fungal universal primers ITS1F- 5′ CTT GGT CAT TTA GAG GAA GTA A 3′ and ITS4- 5′ TCC TCC GCT TAT TAG TAT GC 3′ (White et al., 1990) under the following parameters: 1 cycle denaturing at 94°C; 35 cycles of 1 minute denaturing at 94°C, 30 seconds annealing at 47°C, 2 minutes elongation at 72°C; 5 minutes final elongation at 72°C.

PCR products were separated by electrophoresis in 1% agarose gel stained with 0.5 µg ml^{-1} ethidium bromide which was run at 100 Volts for 1 hour.

The PCR products were cleaned up using the Promega Wizard SV Gel and PCR clean up system. The second step of the nested PCR was performed on the cleaned product using the *K. pfeilii* specific primers TPR3 and TPF1 (Adeleke, 2007) with the following parameters: 1 cycle denaturing at 94°C; 35 cycles of 1 minute denaturing at 94°C, 30 seconds annealing at 51°C, 2 minutes elongation at 72°C; 5 minutes final elongation at 72°C. The products were also purified using the Promega Wizard SV Gel and PCR clean up system. The PCR products, positive control (*K. pfeilii* DNA) and negative control (autoclaved distilled water) samples were visualised in a 1% agarose gel stained with 0.5 µg ml^{-1} ethidium bromide along with a 100 bp Promega molecular marker (Catalog no. 29278810). Final PCR products were submitted to Inqaba Biotechnical Industries, Pretoria for sequencing. The sequences were then submitted to NCBI-BLAST online for identification (Altschul et al., 1997).

2.9 Primer design

Primers for *Mattirolomyces austroafricanus* and *Eremiomyces echinulatus* were designed using their sequences which were obtained from NCBI-Genbank online (Altschul et al., 1997). Primers were designed using the Gene Runner software programme (Hastings Software, 1994) taking into consideration primer length, melting temperature and G, C content. The primers were synthesised by Integrated DNA Technologies (IDT), Cape Town. Primers were tested on cultures of *K. pfeilii* to confirm their specificity as no samples of *M. austroafricanus* and *E. echinulatus* were available. PCR products were separated electrophoretically and visualised on an ethidium bromide stained 1% agarose gel to determine the sizes of the product.

2.10.1 Isolation and identification of associated bacteria

Bacteria isolated from truffle isolates and rhizosheath were serially diluted and spread on NA plates. Colonies were discontinuously streaked until pure colonies were obtained. In order to determine purity, colonies were Gram stained as follows: A smear was prepared by mixing a loopful of water with a bacterial colony on a slide and smear was heat fixed. After fixing, the bacteria were stained with crystal violet for 60 seconds. Stain was rinsed off with distilled water and slide was flooded with iodine solution for 30 seconds. Destaining was done by rinsing with

95% ethanol followed by rinsing with distilled water. Safranin which served as a counter stain was added and left for 1 minute (Madigan and Martinko, 2006). Slides were rinsed, air dried and observed under the Nikon YS100 compound light microscope using the oil immersion objective.

2.10.2 Molecular identification of isolated bacteria

Selected bacteria were inoculated into Nutrient Broth (NB) and incubated at 32°C for 24 hours. Bacterial DNA was extracted from pure cultures pelleted from NB using the Zymo Research Fungal/Bacterial Miniprep Kit following manufacturer's instructions as described in section 2.3 and amplified by PCR using the Kapa*Taq* ReadyMix DNA Polymerase with universal primers Fd1 - 5′ AGA GTT TGA TCC CTC AG 3′ and rP2 - 5′ ACG GCT ACC TTG TTA CGA CTT 3′ (Wiesburg et al., 1991) under the following conditions: 1 cycle initial denaturation at 95°C for 90 seconds; 30 cycles of denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds and elongation at 72°C for 60 seconds and 1 cycle final elongation at 72°C for 120 seconds. PCR products were separated by electrophoresis in 1% agarose gel stained with 0.5 μgml^{-1} ethidium bromide which was run at 100 Volts for 1 hour along with a Lambda DNA EcoR+ Hind III Promega molecular marker (Catalog no. 22673502). PCR products were purified using Promega Wizard SV Gel and PCR clean-up System and sent for sequencing to Inqaba Biotechnical Industries, Pretoria. The sequences were then submitted to NCBI- BLAST online (http://blast.ncbi.nlm.nih.gov/Blast.) for identification (Altschul et al., 1997).

2.11 Investigation of the role of associated bacteria

2.11.1 Indole Acetic Acid production test

An indole acetic acid (IAA) test was performed to determine whether the isolated bacterial species produced IAA. Bacterial isolates were inoculated into DEV Tryptophan broth, Catalog no. 1.10694.0500 (Appendix A) and were incubated in the dark for 96 hours at 32°C including a control with the broth only. Three replicates were made for each bacteria and the control. Cultures were centrifuged at 4500 x g for 20 minutes and the supernatant was used to test for the IAA production by adding 300 µl of each sample to 600 µl of Salkowski reagent or Kovacs reagent (Appendix B). After mixing, a colour change to red was recorded as positive for IAA production.

2.11.2 Phosphate solubilisation assay

Bacterial isolates were grown in 1 ml nutrient broth (NB) at 32°C for 72 hours and a control with the NB only. Three replicates were made for each isolate and the control. Samples were centrifuged at 13 000 x g for 5 minutes. The supernatant was removed and the cells were resuspended in 1ml of saline. After vortexing optical density (OD) was measured at 600 nm and the bacterial concentrations were adjusted to the same OD reading of 0.56 by diluting with sterile 0.2 % saline. In order to determine the phosphate solubilising characteristics of the bacteria, four perpendicular wells were made on the phosphate solubilising media (PSM) (Appendix A) and 80 µl of the bacterial sample was added into each well. The development of halos around each well was indication of phosphate solubilisation.

2.11.3 Effect of isolated bacteria on mycelia growth

Bacterial isolates were grown for 72 hours at 32°C in micro-centrifuge tubes containing 1 ml NB. Three replicates were made for each isolate and the control. Samples were centrifuged at 13 000 x g for 5 minutes. The supernatant was removed and the cells were resuspended in 1ml of 0.2 % saline. After vortexing optical density (OD) was measured at 600 nm and the bacterial concentrations were adjusted to the same OD reading of 0.56 by diluting with sterile saline.

The effect of bacterial activity on *K. pfeilii* culture growth was observed by creating 4 perpendicular wells all equidistant from the centre of each FTN plate using a 5 mm core borer (Fig 2.1).

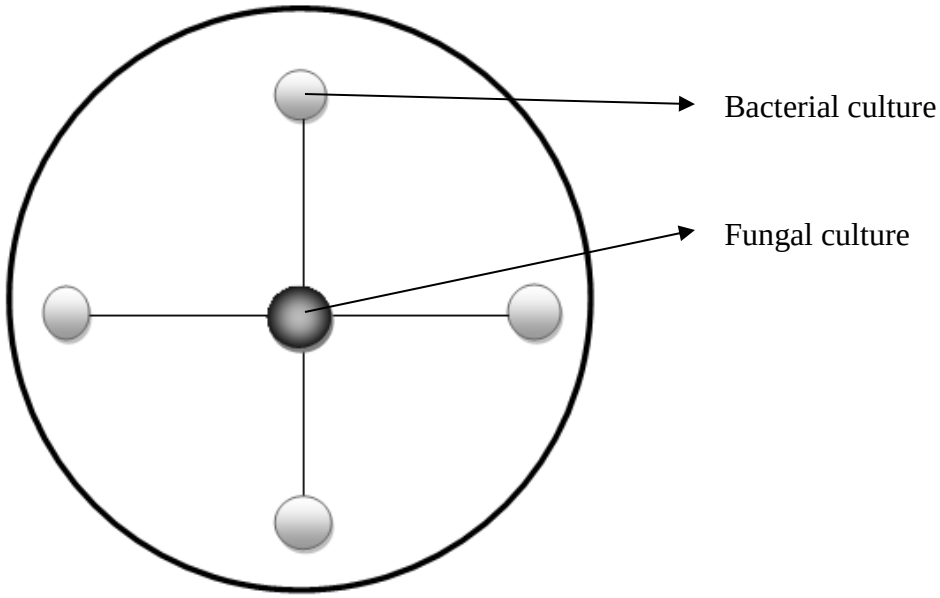

Figure 2. 1 Experimental design of the bacterial effects on Kalaharituber pfeilii experiment: dark circle- Kalaharituber pfeilii culture; light circles- same bacterial species.

After adjusting the concentration, a volume of 60 µl of bacterial sample was loaded in each well with 1 bacterial species per plate, for the control the wells were loaded with autoclaved saline. A plug containing the fungal culture was placed at the centre of each plate and all plates were incubated at 32°C. Three replicates were prepared for each bacterial species and controls. Colony diameter (mm) was measured daily and the effects of the bacteria on truffle culture growth were determined by comparing results with those obtained from the controls.

In order to determine the effect of the bacteria on *K. pfeilii* growth, the obtained results were analysed using the STATISTICA program. Data was analysed using repeated measures analysis of variance and Fischer least significant differences tests.

2.12 Mycorrhizal re-synthesis

Seeds of *Stipagrostis ciliata* var. *capensis* were collected from the vicinity where truffles were found and were kept at room temperature until further use. Collected seeds were surface sterilised by soaking in 3% sodium hypochlorite for 5 minutes, rinsed and soaked overnight in sterile distilled water. The seeds were germinated by placing them in damp sterile cotton wool on petri dishes or by placing on 1.5% water agar.

After germination, seeds were surface sterilised in 70% ethanol for 30 seconds and placed in glass jars containing modified FTN medium with reduced glucose concentration and inoculated with plugs of truffle mycelia culture. The jars were incubated at 25°C and growth was monitored. Contaminated jars were removed. Detection of mycorrhizal formation was observed by root staining as described in section 2.7 (Smith and Dickson, 1997).

2.13 Soil analysis

Soil samples collected beneath and around the truffles were kept at 25°C. A total of 6 composite samples of 200 g were obtained by mixing the soil from sites where the truffles were collected. The samples were sent to the Dohne Agricultural Development Institute, Analytical Services, Stutterheim where their P, K, Ca, Mg, Zn, Mn, Cu, exchangeable acidity, total cations and organic C contents were analysed.

2.14 Rainfall and temperature data collection

Rainfall and temperature (minimum and maximum) data for Upington for the 2009 -2011 period, were requested from the South African Weather Service.

2.15 Truffle distribution survey

After sampling, a survey was conducted in order to determine current distribution and to observe changes related to distribution as well as to assess other truffle species in the southern African region. A letter was published in the Farmers Weekly and Landbou Weekblad magazines where farmers were asked to participate in the survey. The farmers were chosen as the participants of the survey because their farms are mostly located at the areas where truffles have been previously reported to occur and therefore had some knowledge of what to look for. Those that responded were sent a copy of the survey questionnaire (Appendix D). These included farmers from the Kalahari region of South Africa and Namibia. They were asked for their geographic location, the time of the year when they often find the truffles, the type of vegetation around the area where the truffles are found and the description of truffles normally collected. All answered questionnaires were analysed and the descriptions provided were matched with characteristics of known truffle species.

CHAPTER 3: RESULTS

3.1 Collection of samples from selected areas of the Northern Cape

In April 2010, 15 fruiting bodies of *Kalaharituber pfeilii* were collected and 34 were found in May 2011 at Haakdoring Duin Farm, Upington, Northern Cape, South Africa. Fruiting bodies were located by observing cracking in the sand (Fig 3.1a) which usually indicates that there is a truffle protruding underneath (Fig 3.1b). The collected fruiting bodies weighed an average weight of 15 g. It was observed that all the collected fruiting bodies were found beneath or next to *Stipagrostis ciliata* var. *capensis* grass clumps.

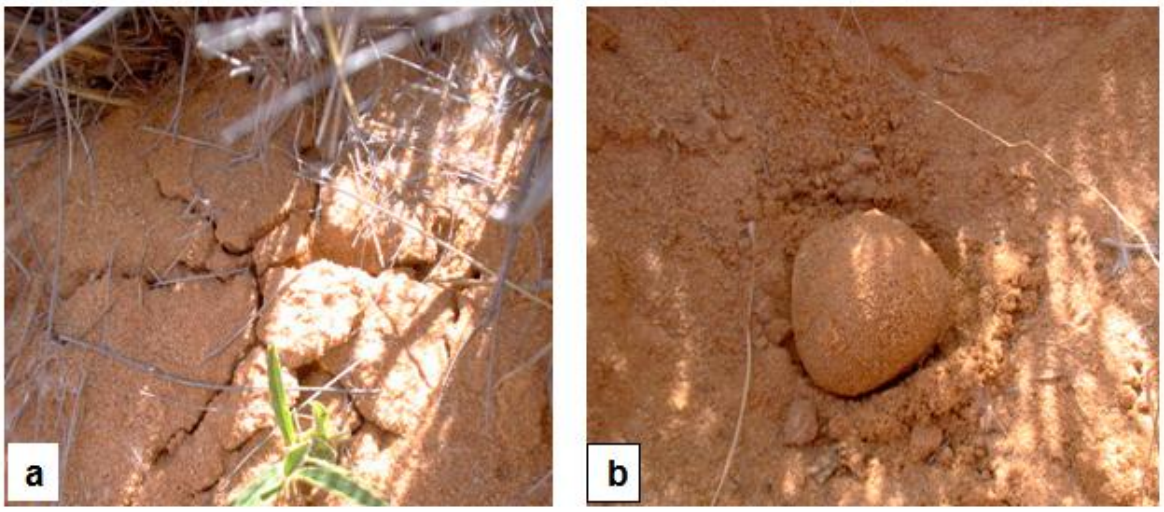

Figure 3.1 (a) Cracks in the sand indicating the presence of the *Kalaharituber pfeilii* fruiting bodies underneath which is formed as the truffle pushes towards the soil surface. (b) *Kalaharituber pfeilii* fruiting body found just beneath the soil surface.

3.2 Morphological identification of the fruiting bodies

Morphological identification was conducted by observing the physical characteristics of the fruiting bodies. The collected truffles had a yellowish to dark brown peridium (Fig 3.2) with a solid gleba marbled with white, yellowish or brown veins and had a distinctive fungoid odour. All these characteristics were consistent with the *K. pfeilii* characteristics as observed by Trappe et al. (2008) and Ferdman et al. (2005). On exposure to air, cut surfaces of fruiting bodies turned yellow in colour.

Figure 3.2 Washed *Kalaharituber pfeilii* fruiting bodies with average weight of 15 g showing typical morphological characteristics.

3.3 Molecular identification the collected fruiting bodies

Amplicons of approximately 400 base pairs were observed (Fig 3.3). The positive control also had a band around the 400 bp region and no bands were observed for the negative control (not shown). Primer dimers which are known to be the by- products of the PCR reaction were also observed around the 100 bp area. The primer dimers were removed after the PCR cleanup process. A total of 13 fruiting body DNA extracts were amplified and sent for sequencing. The sequencing results were submitted to NCBI-BLAST and all submitted sequences had a 100%

identity with *K. pfeilii,* AF301422.1 (Appendix B) and 99% identity with *K. pfeilii,* AF301420.1 (Appendix B). Overall, they had on average 99% coverage (Table 3.1).

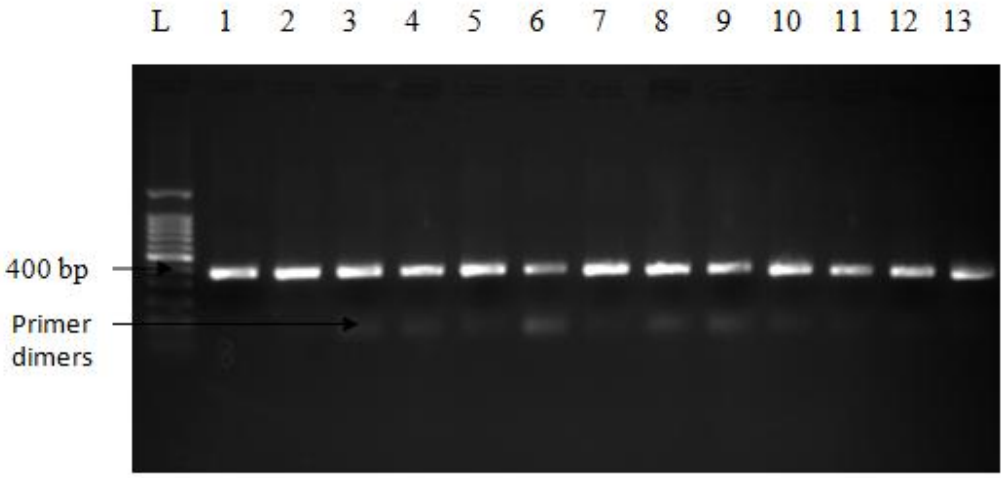

Figure 3.3 Ethidium bromide stained 1% agarose gel showing PCR products of a selection of *Kalaharituber pfeilii* fruiting body samples before purification with lane L: 100 bp DNA marker, lane 1- 13: Truffle samples T1 to T13. Primer dimers were also observed around the 100 bp region. Positive and negative controls not shown.

Table 3.1 NCBI-BLAST results of amplified truffle fruiting bodies indicating the query coverage percentage, E-value and maximum identity percentage. All were 100 or 99 % identical to *Kalaharituber pfeilii*, accession numbers AF301422.1 and AF031420.1.

Sample	**GenBank Accession no.**	**E-value**	**Maximum Identity %**
T1	AF301422.1	1e162	100
	AF301420.1	1e161	99
T2	AF301422.1	2e161	100
	AF301420.1	9e160	99
T3	AF301422.1	1e76	100
	AF301420.1	1e76	100

Sample	**GenBank Accession no.**	**E-value**	**Maximum Identity %**
T4	AF301422.1	2e160	100
	AF301420.1	1e158	99
T5	AF301422.1	2e160	99
	AF301420.1	5e162	100
T6	AF301422.1	8e140	100
	AF301420.1	8e140	100
T7	AF301420.1	7e166	100
	AF301422.1	3e164	99
T8	AF301420.1	2e161	100
	AF301422.1	9e160	99
T9	AF301420.1	5e162	100
	AF301422.1	2e160	99
T10	AF301420.1	2e161	100
	AF301422.1	9e160	99
T11	AF301422.1	7e161	100
	AF301420.1	3e159	99
T12	AF301422.1	7e161	100
	AF301420.1	3e159	99
T13	AF301422.1	5e162	100
	AF301420.1	2e160	99

3.4 Isolation and maintenance of *Kalaharituber pfeilii* cultures

The truffle mycelia started to emerge from 3-4 weeks after incubations in the dark at 32°C. The mycelia from fruiting body tissue appeared cream-white on the modified FTN medium (Fig 3.4a). The maintained cultures were microscopically examined using the tape mount method and observed to be non-sporulating with septate hyphae (Fig 3.4b) which is a characteristic of several Ascomycete fungi. All fast growing, sporulating fungal cultures were discarded.

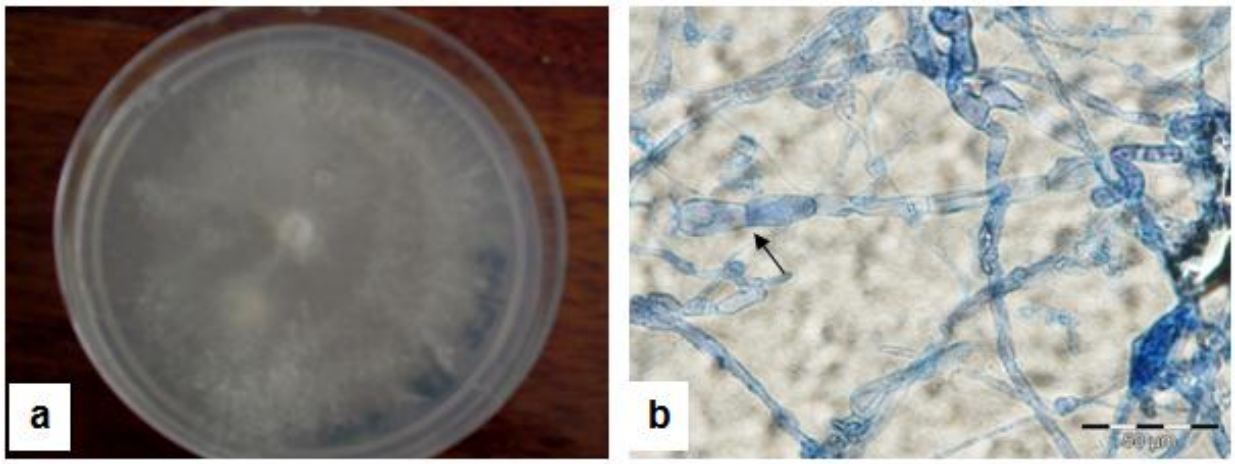

Figure 3.4 (a) *Kalaharituber pfeilii* culture appearing cream-white on FTN medium after 3-4 weeks of incubation at 32°C in the dark. **(b)** Micrograph of the pure mycelia growth in culture showing the septation (arrow) without any spores.

3.5 Isolation of mycorrhizal fungi associated with rhizosheath

The grass species, *Stipagrostis ciliata* var. *capensis* were successfully collected and the roots were observed to be covered with a sand encrusted rhizosheath (Fig 3.5). On removal of the rhizosheath only the vascular cylinder remained. Fungal growth from the rhizosheath cultures varied. Fast growing, sporulating cultures were discarded and creamy white slower growing cultures resembling *K. pfeilii* cultures were maintained (Fig 3.6a). Microscopic observation of the tape mounts confirmed the presence of septation (Fig 3.6b) and lack of sporulation.

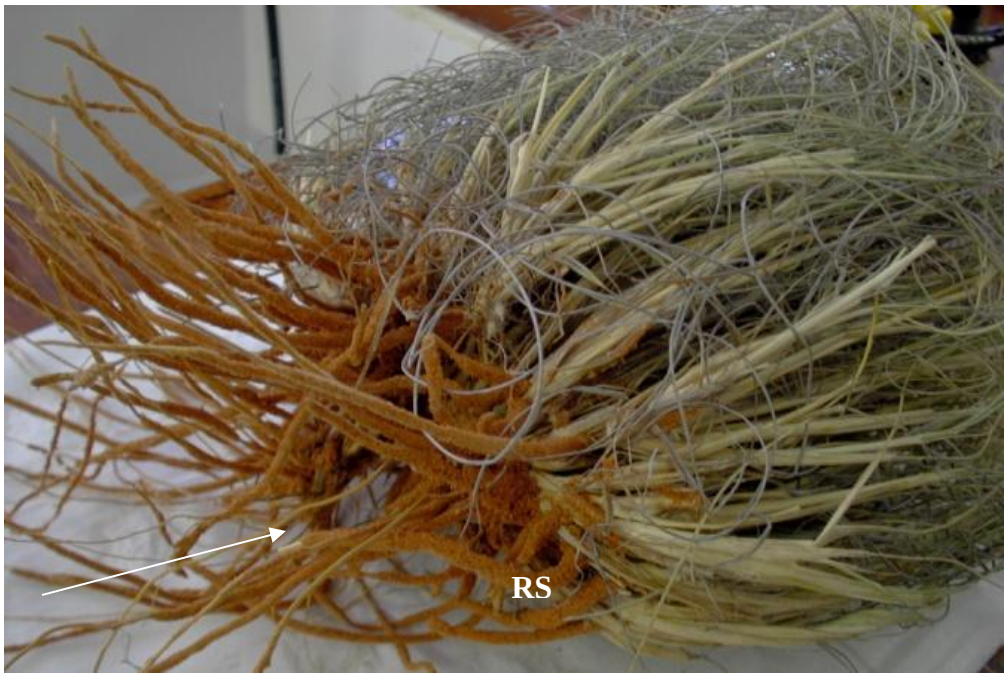

Figure 3.5 *Stipagrostis ciliata* var. *capensis* plant collected in the vicinity where the truffles were found. The roots were covered with a sand rhizosheath (RS) and vascular cylinder (arrow) was left when rhizosheath was removed.

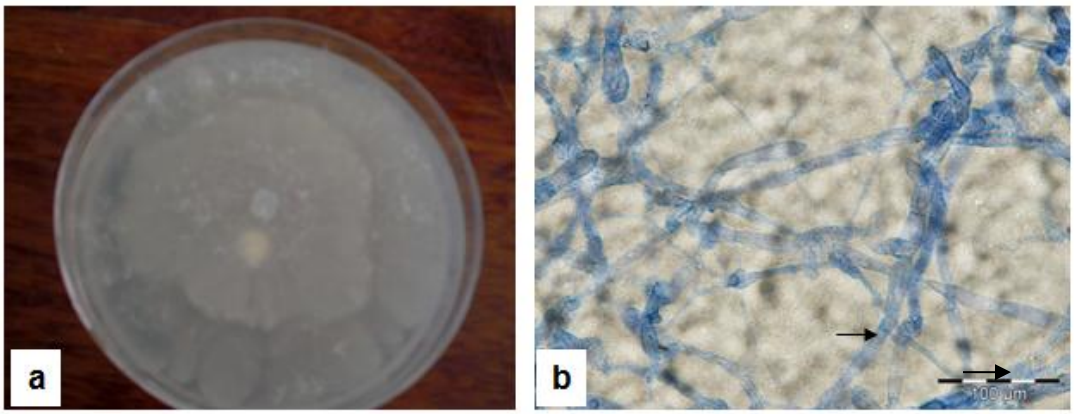

Figure 3.6 (a) Creamy-white growth isolated from the rhizosheath on modified FTN medium. **(b)** Micrograph of the rhizosheath fungal culture showing septation (arrow) and no spores were observed.

3.6 Identification of isolated truffle and rhizosheath cultures using molecular techniques

Fungal DNA from both the truffle and rhizosheath fungal cultures was successfully amplified with *K. pfeilii* specific primer pair TPR1 and TPF3 (Adeleke, 2007) to confirm if they were pure *K. pfeilii* cultures. PCR products from both truffle and rhizosheath cultures produced approximately 400 bp amplicons (Fig 3.7 and Fig 3.8). The positive control also had a band along the 400 bp region and no band was observed for the negative control (not shown). A total of 10 truffle cultures (Fig 3.7) and 9 rhizosheath cultures (Fig 3.8) were amplified. The sequencing results were submitted to NCBI-BLAST and all submitted sequences had a 100% identity with *K. pfeilii,* AF301422.1 (Appendix B) and 99% identity with *K. pfeilii,* AF301420.1 (Appendix B). They had on average 99% coverage for all submitted sequences. The e- value for the truffle cultures was 1e162 and 8e 114 for the rhizosheath cultures (Table 3.2). This confirmed that *K. pfeilii* was isolated from the rhizosheath.

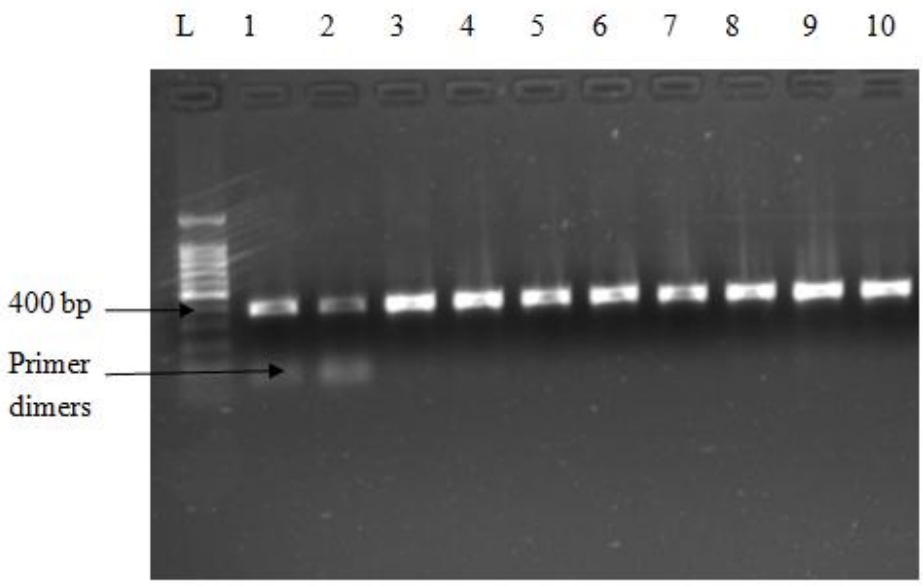

Figure 3.7 Ethidium bromide stained 1% agarose gel showing PCR products of truffle cultures amplified with *Kalaharituber pfeilii* primer pair TPR1 and TPF3. Lane L: 100 bp ladder; Lane 1-10: Truffle cultures 1TC to 10TC.

Table 3.2 NCBI-BLAST results of amplified truffle cultures indicating the query average percentage, E-value and maximum identity percentage. All were 100 or 99% identical to *Kalaharituber pfeilii*, accession numbers AF301422.1 and AF031420.1.

Sample	**GenBank Accession no.**	**E-value**	**Maximum Identity %**
1TC	AF301422.1	1e163	100
	AF301420.1	5e162	99
2TC	AF301422.1	5e165	99
	AF301420.1	4e165	99
3TC	AF301422.1	2e176	100
	AF301420.1	5e174	99
4TC	AF301422.1	9e165	100
	AF301420.1	4e163	99
5TC	AF301420.1	7e161	100
	AF301422.1	3e159	99
6TC	AF301422.1	5e162	100
	AF301420.1	2e160	99
7TC	AF301422.1	9e160	99
	AF301420.1	9e160	99
8TC	AF301422.1	7e166	100
	AF301420.1	3e164	99
9TC	AF301422.1	2e161	100
	AF301420.1	9e160	99
10TC	AF301422.1	5e174	100
	AF301420.1	1e171	99

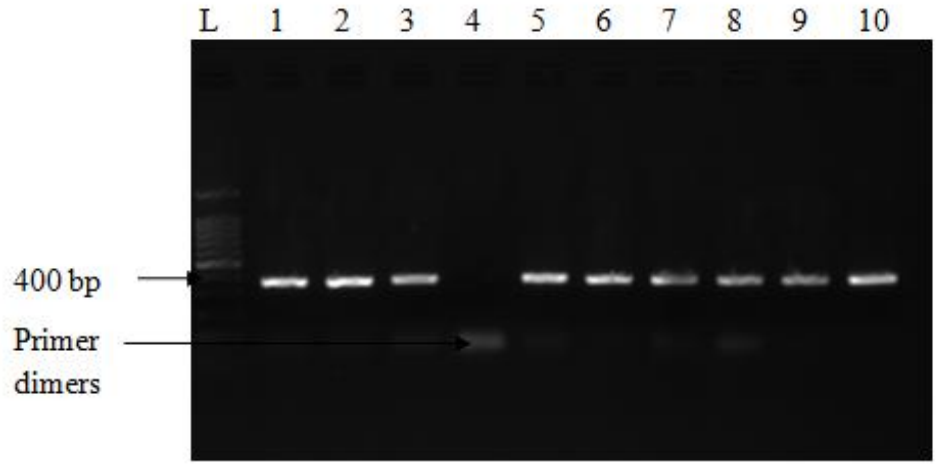

Figure 3.8 Ethidium bromide stained 1% agarose gel showing PCR products of rhizosheath cultures amplified with *Kalaharituber pfeilii* primer pair TPR1 and TPF3. Lane L: 100 bp ladder; Lane 1-10: Rhizosheath cultures HDD1 to HDD10. No truffle DNA was amplified at lane 4.

Table 3.3 NCBI-BLAST results of amplified rhizosheath cultures indicating the query average percentage, E-value and maximum identity percentage.

Sample	**GenBank Accession no.**	**E-value**	**Maximum Identity %**
HDD1	AF301422.1	8e116	99
	AF301420.1	8e116	99
HDD2	AF301422.1	2e124	100
	AF301420.1	6e122	99
HDD3	AF301422.1	8e136	99
	AF301420.1	8e136	99
HDD5	AF301422.1	1e135	99
	AF301420.1	1e135	99
HDD6	AF301422.1	3e144	99
	AF301420.1	1e142	99
HDD7	AF301422.1	2e160	99

Sample	GenBank Accession no.	E-value	Maximum Identity %
	AF301420.1	1e158	99
HDD8	AF301422.1	3e159	99
	AF301420.1	1e157	99
HDD9	AF301422.1	3e150	99
	AF301420.1	1e148	98
HDD10	AF301420.1	2e157	98
	AF301422.1	7e156	97

The sequences had an average 99% identity with *Kalaharituber pfeilii,* AF301422.1 (Appendix B) and *Kalaharituber pfeilii,* AF301420.1 (Appendix B). The culture of the unsuccessful amplification HDD4 was discarded as it was regarded as not *K. pfeilii* and was therefore not sequenced.

3.7 Microscopic examination of the type of mycorrhizal association found in roots

Microscopic observations were difficult due to the sand encrustation which could not removed by washing. This limited the focussing ability during microscopy. *S. ciliata* var. *capensis* roots appeared to have an ectendomycorrhizal association as fungal hyphae were seen penetrating the epidermal and cortical cells with loose hyphae inside some of the cells (Fig 3.9). The roots of *Schidtia kalihariensis* (Suurgrass) and *Stipagrostis obtusa* (Small bushmens grass) possessed an endomycorrhizal association as vesicles were observed within the cells (Fig 3.10).

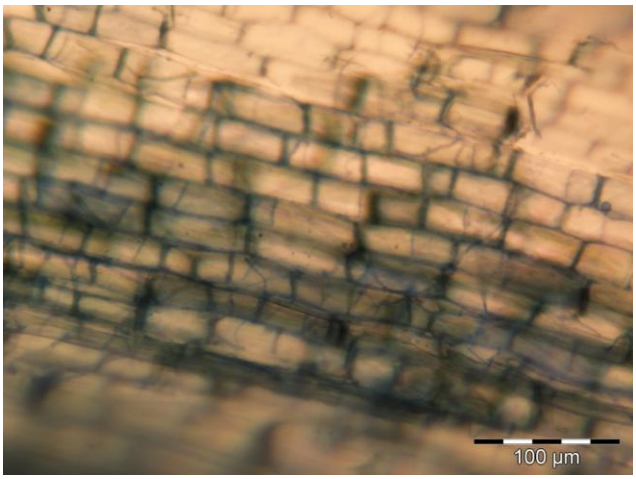

Figure 3.9 *Stipagrostis ciliata* var. *capensis* root with fungal hyphae penetrating between the epidermal and cortical cells with loosely arranged hyphae inside some of the cells.

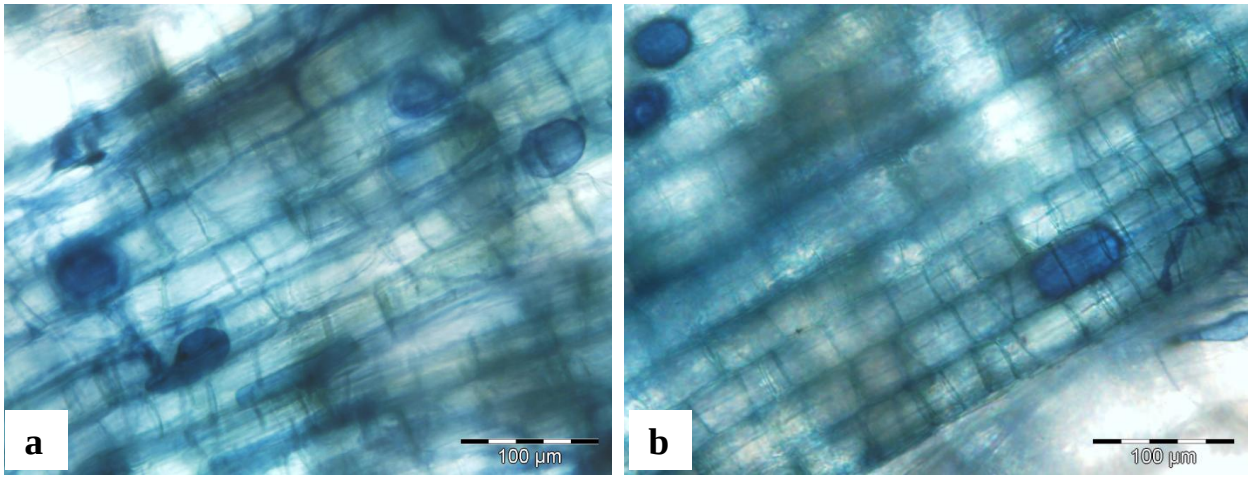

Figure 3.10 Root of (a) *Schidtia kalihariensis* and (b) *Stipagrostis obtusa* forming an endomycorrhizal association indicated by the presence of vesicles within the root cells.

3.8 Molecular identification of fungi associated with roots

Nested PCR was performed on *S. ciliata* var. *capensis* roots covered with rhizosheath to identify the fungi present in the roots. Nested PCR was performed to amplify the DNA increasing the presence of the *K. pfeilii* DNA, to ensure successful with the truffle primers (Adeleke, 2007). The root DNA was amplified with universal primer pair ITS1 and ITS4 produced approximately 620 bp amplicons (Fig 3.11). After cleaning the product was successfully amplified with TPR1 and TPF3 where approximately 400 bp amplicons were produced (Fig 3.12). The unpurified products had primer dimers at the 100 bp region of the gel which are formed as by products of the PCR reaction (Fig 3.12).

After the second nested PCR step successfully amplified products were sent for sequencing. The sequencing results were submitted to NCBI-BLAST (Table 3.4) and all submitted sequences had a 99% identity with *K. pfeilii*, AF301422.1 (Appendix B) and 98% identity with *K. pfeilii*, AF301420.1 (Appendix B). They had an average 99% coverage and 2e160 e-value for all submitted sequences. This confirmed that *K. pfeilii* was present in the *S. ciliata* var. *capensis* roots. Fig 3.13 below illustrates that the collected fruiting bodies, grass roots, rhizosheath culture and truffle culture were all successfully amplified with *K. pfeilii* primer pair TPR1 and TPF3. This strongly suggests that there is a mycorrhizal association between *K. pfeilii* and *S. ciliata* var. *capensis.*

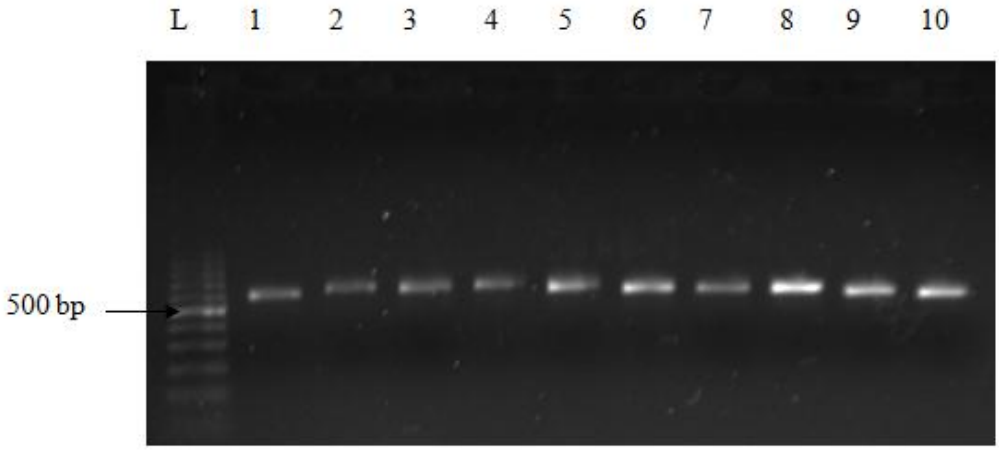

Figure 3.11 Ethidium bromide stained 1% agarose gel showing nested PCR products of *Stipagrostis ciliata* roots DNA after amplification with universal fungal primer pair ITS1 and ITS4. Lane L: 100 bp DNA marker, Lane 1-10: Roots DNA samples R1 to R10.

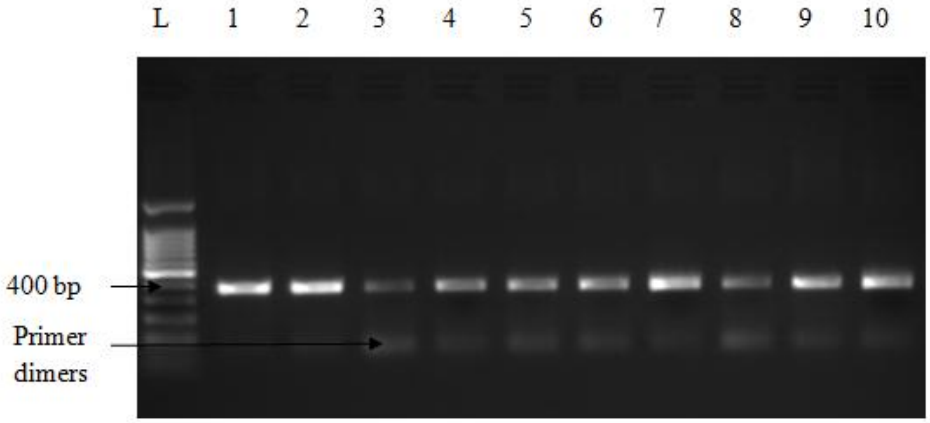

Figure 3.12 Ethidium bromide stained 1% agarose gel showing nested PCR products after the second amplification with *Kalaharituber pfeilii* specific primer pair TPR1 and TPF3. Lane L: 100 bp DNA marker, Lane 1- 10: Root DNA samples R1 to R10. Primer dimers were also observed around the 100 bp region.

Table 3.4 NCBI-BLAST results from amplified root material indicating the query average percentage, E-value and maximum identity percentage.

Sample	GenBank Accession no.	E-value	Maximum Identity %
R1	AF301422.1	1e133	99
	AF301420.1	1e133	99
R2	AF301422.1	3e123	99
	AF301420.1	3e123	99
R3	AF301420.1	2e165	100
	AF301422.1	1e163	99
R4	AF301422.1	2e160	100
	AF301420.1	1e158	99
R5	AF301422.1	6e110	99
	AF301420.1	6e110	99
R6	AF301420.1	9e160	99
	AF301422.1	4e158	99
R7	AF301422.1	1e163	100
	AF301420.1	5e162	99
R8	AF301422.1	2e167	100
	AF301420.1	7e166	99
R9	AF301420.1	5e174	100
	AF301422.1	1e171	99
R10	AF301420.1	2e167	100
	AF301422.1	5e165	99

The sequences had a 100% or 99% identity with either *Kalaharituber pfeilii,* AF301422.1 (Appendix B) or *Kalaharituber pfeilii,* AF301420.1 (Appendix B).

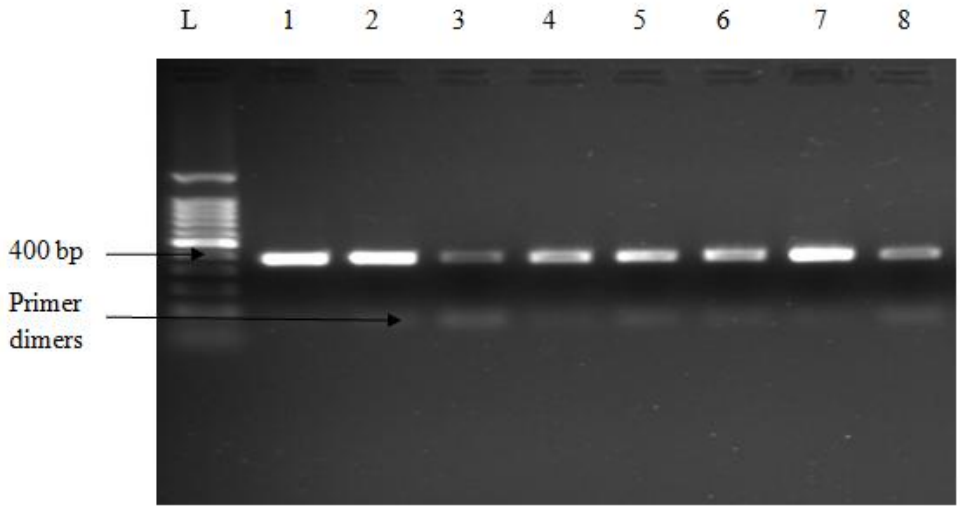

Figure 3.13 Ethidium bromide stained 1% agarose gel showing PCR products after amplification with *Kalaharituber pfeilii* primer pair TPR1 and TPF3. Lane L: 100 bp molecular marker, Lane 1-2: *Kalaharituber pfeilii* fruiting bodies, Lane 3-4: *Stipagrostis ciliata* var. *capensis* roots, Lane 5-6: Rhizosheath culture, Lane 7-8: Truffle culture. Primer dimers were also observed around the 100 bp region.

3.9 Primer design

In order to confirm the presence of *Mattirolomyces austroafricanus* and *Eremiomyces echinulatus*, primers were designed and used to amplify the collected *K. pfeilii* fruiting bodies as no *M. austroafricanus* or *E. echinulatus* fruiting bodies were collected. For *M. austroafricanus* the sequence 5′ GAA AGA ACC ACA GGG ATT G 3′ was used to design both forward and reverse primers. The sequence 5′ CCAGAGAGTTTGCCCGTGGTAA 3′ was used to design the primer pair for *E. echinulatus. M. austroafricanus* designed primers produced bands of about 600bp and *E. echinulatus* primers produced bands of about 700bp. These results rendered the primer design unsuccessful as no bands were expected but the primers amplified the fruiting bodies (Fig 3.14) which were already confirmed as *K. pfeilii* and the primers were therefore regarded as not specific.

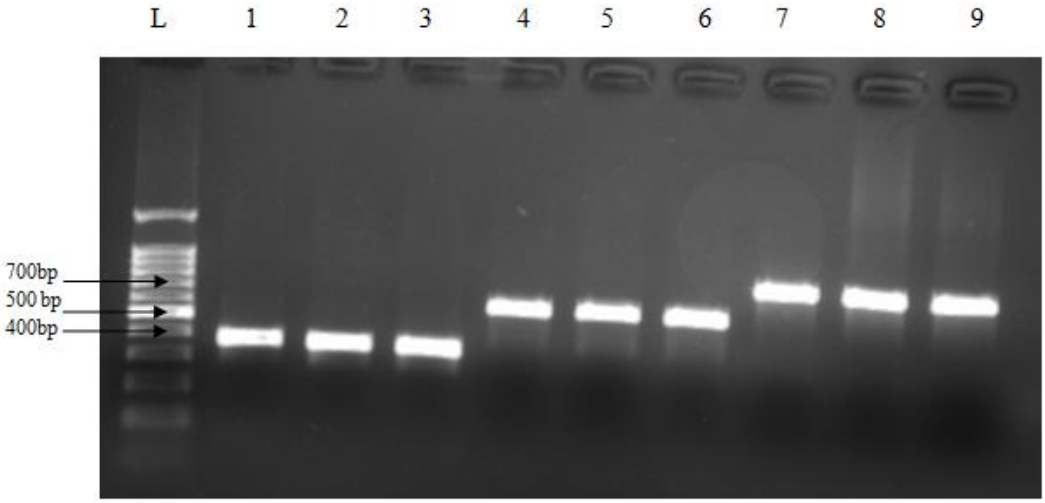

Figure 3.14 Agarose gel showing fruiting body PCR products after amplification with *Kalaharituber pfeilii*, *Mattirolomyces austroafricanus* and *Eremiomyces echinulatus* primers. Lane L: 100bp molecular marker, Lane 1-3: Fruiting bodies amplified with *Kalaharituber pfeilii* primers, Lane 4-6: Fruiting bodies amplified with *Mattirolomyces austroafricanus* primers, Lane 7-9: Fruiting bodies amplified with *Eremiomyces echinulatus* primers.

3.10 Isolation and identification of associated bacteria

Bacterial colonies obtained from the truffle fruiting body cultures and rhizosheath cultures were isolated and grouped according to morphology, growth rate and colour. Six different colonies with distinct characteristics were selected. All colonies were Gram stained as the first identification step (Table 3.6). Amplicons of about 1500 bp were observed (Fig 3.15).

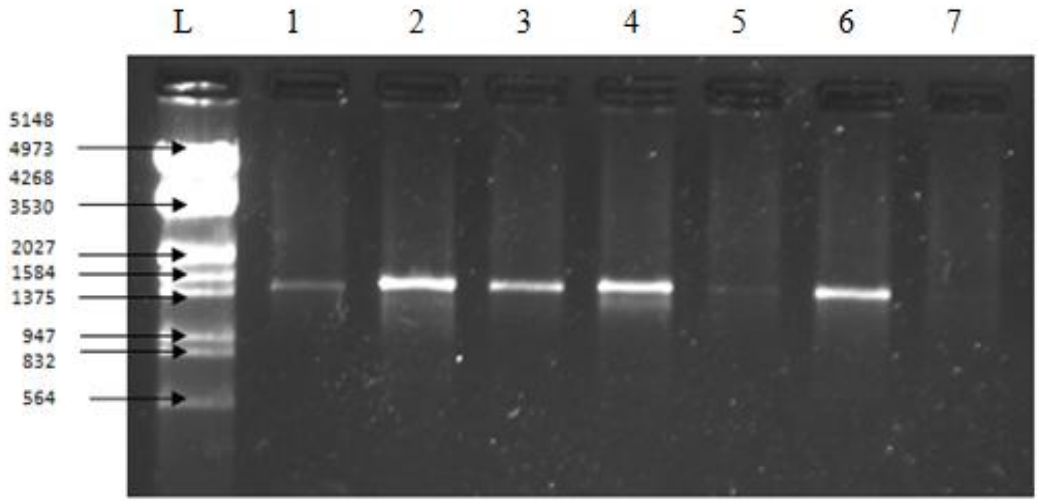

Figure 3.15 Ethidium bromide stained 1% agarose gel showing PCR products of isolated bacteria amplified with bacterial universal primer pair Fd1 and rP2. Lane L: Lambda molecular marker, Lane 1: Isolate TBA, Lane 2: TBB, Lane 3: TBC, Lane 4: TBD, Lane 5: TBE, Lane 6: TBF, Lane 7: Negative control.

Table 3.5 NCBI-BLAST results of submitted bacterial sequences showing the identical species with percentage identity, query coverage and E-value.

Sample	GenBank Accession no.	Species name	E-value	Maximum Identity %
TBA	JN863503.1	*Leucobacter sp.*	1e154	95
TBB	HM584272.1	*Stenotrophomona*	0.0	100

Sample	GenBank Accession no.	Species name	E-value	Maximum Identity %
		maltophilia		
TBC	AB681132.1	*Phyllobacterium myrsinacearum*	1e163	98
TBD	JN128237.1	*Staphylococcus cohnii*	0.0	99
TBE	JN120941.1	*Dietzia sp.*	0.0	96
TBF	JN391535.1	*Pantoea dispersa*	0.005	99

3.11 Investigation of the role and effect of associated bacteria

Only one isolate produced IAA positive results using Salkowski reagent (Fig 3.18) but none had any positive results using Kovacs reagent. All the isolated bacterial isolates were observed to produce inhibitory effects towards the growth of *K. pfeilii* cultures (Fig 3.17).

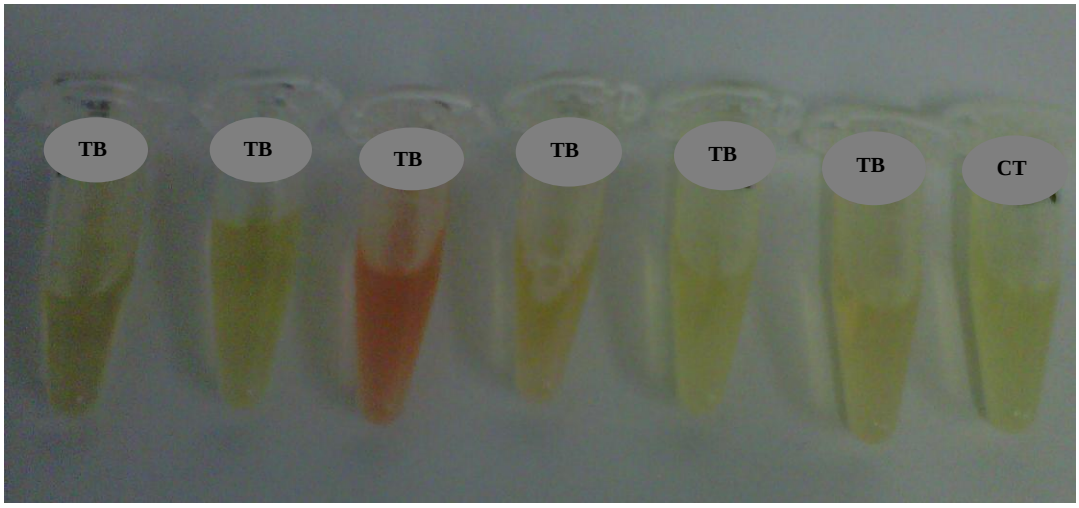

Figure 3.16 Bacterial isolates cultured in Tryptophan broth with Salkowski reagent to investigate their role in indole acetic acid production. All the samples were negative except for sample TBC.

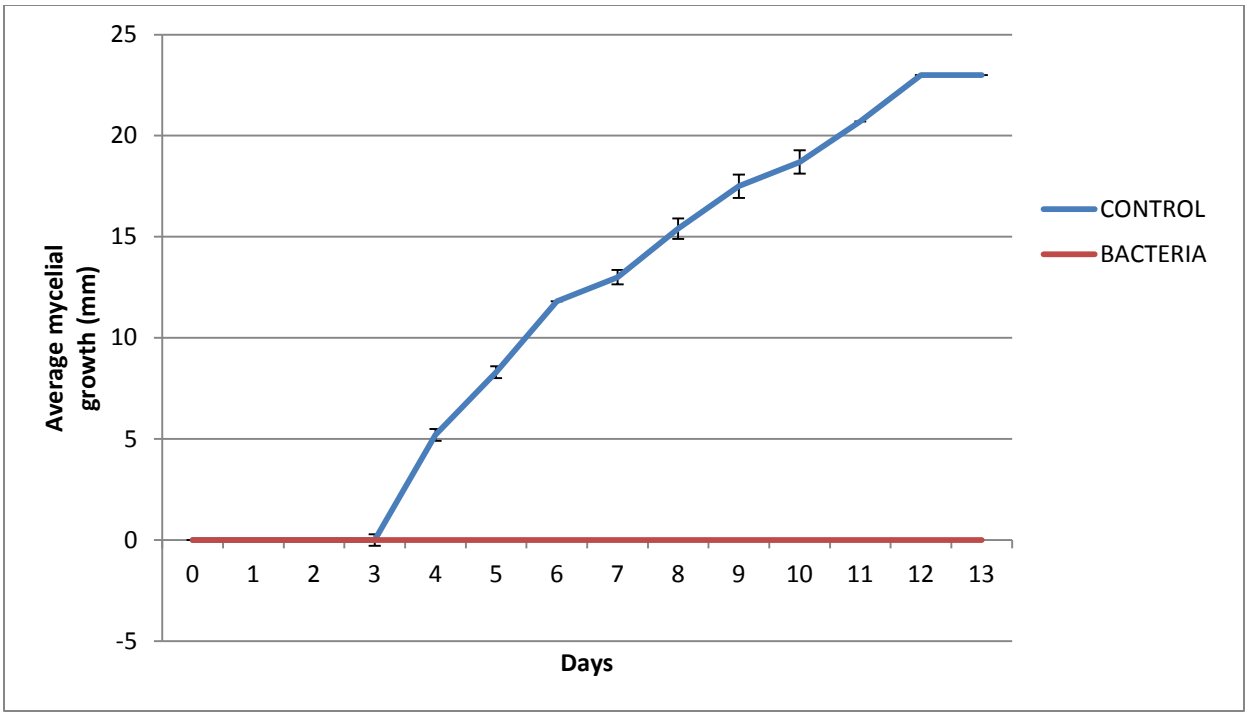

Figure 3.17 Effect of bacterial isolates TBA, TBB, TBC, TBD, TBE and TBF on *Kalaharituber pfeilii* mycelia growth in FTN media.

Table 3.6 Characteristics of bacteria isolated from the truffle fruiting bodies and the rhizosheath

Sample	**Gram reaction**	**Indole test**	**Phosphate solubilisation**	**Appearance on nutrient agar plate**
TBA	+ve bacilli	-	-	Cream
TBB	-ve bacilli	-	-	Cream
TBC	-ve bacilli	+	-	Cream
TBD	+ve cocci	-	-	Creamish yellow
TBE	-ve rod	-	-	Orange
TBF	-ve rod	-	-	Translucent

Sample	Gram reaction	Indole test	Phosphate solubilisation	Appearance on nutrient agar plate
Control		-	-	-

Isolates TBA and TBD were Gram positive, the majority of the samples were Gram negative. Only isolate TBC was positive for the indole acetic acid production test when using the Salkowski reagent. None of the samples were able to solubilise phosphate.

3.12. Collection and germination of seeds.

Stipagrostis ciliata var. *capensis* is the dominating grass species in the Kalahari (Fig 3.17). The seeds were collected from the flowering structures of *S. ciliata* var. *capensis*. After sterilisation the seeds were successfully germinated on 1.5% water agar and sterile damp cotton wool.

Figure 3.18 *Stipagrostis ciliata* var. *capensis* grass with flowering structures containing seeds occurring in the Kalahari region where fruiting bodies were collected.

3.13. Initiation of mycorrhizal re-synthesis experiments

Sterile healthy seedlings were used for the mycorrhizal resynthesis experiments. The petri dish method was unsuccessful because of contamination and also the structure of the seedlings after germination and their growth pattern made it difficult to work with them in axenic culture. Due to time constraints this experiment could not be repeated.

3.14 Soil analysis

Table 3.7 Soil analysis results from 6 composite soil samples collected in the areas where truffle fruiting bodies were found. Site 1 was collected in 2010 and site 2 was collected in 2011.

Sample	Average (site 1)	Average (site 2)
P (mg l^{-1})	2.33	2.33
K (mg l^{-1})	154.33	147.33
Ca (mg l^{-1})	478.33	453.33
Mg (mg l^{-1})	127.00	98.00
Exch.acidity (c mol l^{-1})	0.08	0.10
Total cations (c mol l^{-1})	3.91	3.54
Acid sat. %	2.33	3.00
pH (KCl)	5.87	5.57
Zn (mg l^{-1})	0.00	0.00
Mn (mg $l^{-1)}$	0.00	0.00
Cu (mg l^{-1})	0.00	0.00
Organic C %	0.12	0.14

The P, K and Ca concentrations did not indicate any significant differences between the soil samples. The soil pH was slightly acidic and no Zn, Mn or Cu were detected.

3.15 Rainfall and temperature data

Temperature and rainfall averages data was obtained for Upington for the years 2009-2011. Average monthly data is presented in Table 3.8, 3.9 and 3.10.

Table 3.8 Average monthly maximum temperatures (°C) for Upington for the years 2009 to 2011.

	2009	**2010**	**2011**
JAN	37.4	35	36
FEB	34.4	36.4	34.3
MAR	32.8	36	34.4
APR	30.8	30.4	29.1
MAY	24.6	26.4	24.9
JUN	20.8	22.2	20.6
JUL	20.6	24.3	20.9
AUG	25.3	26	25.1
SEP	27.8	29.8	29.6
OCT	31.2	30.8	31
NOV	33	33.6	32.7
DEC	36.2	35.9	35.9

The Maximum temperatures are experienced from December till March, with January being the hottest month. The year 2009 had the highest average compared to the other years.

Table 3.9 Average monthly minimum temperatures (°C) for Upington for the years 2009 to 2011.

	2009	2010	2011
JAN	19.3	19.8	20.8
FEB	21.1	20	19.4
MAR	18.1	19.2	18.3
APR	15.4	14.1	11.6
MAY	8.7	9.1	7.3
JUN	5.7	3.1	1.8
JUL	4.4	6	1.7
AUG	6.9	6.2	4.5
SEP	8.4	10.8	6.8
OCT	14.1	12.4	10.9
NOV	16.3	16.1	12
DEC	18.2	18.4	16.4

The minimum temperature values increase from December and start to decrease after March each year. The lowest value recorded over this period was 1.7°C in July 2011.

Table 3.10 Monthly rainfalls (mm) for Upington for the years 2009 to 2011.

	2009	2010	2011
JAN	7.8	85	73
FEB	49.6	26.3	144.4
MAR	56.4	23	135.9
APR	20.7	41.4	17.5
MAY	3.7	0	36.2
JUN	3.6	0	29.2
JUL	1.1	0	0.4
AUG	0	0	3.8
SEP	0	1.5	0

OCT	26.3	0.4	0
NOV	4.2	42.6	5
DEC	33.4	56.1	34.4
Annual rainfall	206.8	276.3	479.8

The period from July to September had the lowest rainfall in all the years. The highest rainfall was recorded from December till March. The year 2011 had the highest rainfall average.

3.16 Survey

Although 50 questionnaires were sent out to different farmers only 8 responded. The results are presented in Table 3.7.

Table 3.11 Survey results indicating location and period when truffles are found, dominant vegetation and truffle species normally found.

Farm name	Address/GPS coordinates	Period when truffles are found	Dominant vegetation	Type of truffles found
Farm Ootmoed	Aranos, Namibia, 24S19E45	March, April, May	*Stipagrostis sp.*	*K. pfeilii*
Loch Maree	S 27° 06. 978 E 20° 29.882, SA	May	*Stipagrostis sp.*	*K. pfeilii*
Weltevrede	Askham, SA	April	*Stipagrostis sp, Rhigozum trichotomum*	*K. pfeilii*
Farm 3E12	Hartswater, Northern Cape, SA	April, May	*Stipagrostis amabilis*	*K. pfeilii*
Van Druten	Upington, Northern Cape, SA	April, May	*Stipagrostis ciliata, Stipagrostis*	*K. pfeilii, M. austroafricanus*

Farm name	Address/GPS coordinates	Period when truffles are found	Dominant vegetation	Type of truffles found
			obtuse	
Plaas Uitsig	S28. 05972° E21. 02575°, SA	May	*Stipagrostis ciliata, Stipagrostis obtuse*	*K. pfeilii*
Gottesgabe no. 159	Gobabis, Namibia	April, May	*Aristida, Stipagrostis uniplumis, Acacia meridionalis*	*K. pfeilii, M. austroafricanus, E. echinulatus*
Askham	S26 58′51.2 E 20 46′53.9	May	*Stipagrostis sp., Rhigozum trichotomum*	*K. pfeilii, E. echinulatus*

According to received responses, most truffles are found in Namibia and the Upington region of South Africa. They are usually found in April and May. *Stipagrostis sp*. is the dominant vegetation in areas where the truffles are found and *Kalaharituber pfeilii* is the most common truffle species found. However, these results are not conclusive as only 8 out of 50 farmers responded.

4. DISCUSSION

4.1 Collection of *Kalaharituber pfeilii*

The truffles were located by observing cracks in the sand (Fig 3.1a) which are a result of the truffle protruding underneath the soil pushing towards the soil surface (Mshigeni, 2001). Locating truffles using this method is labour intensive and time consuming as not all cracks in the soil are caused by the truffles and is therefore used in low scale collections. However for commercial purposes animals such as pigs and dogs have been used for the location of forest truffles. These animals were observed to have the ability to detect an aromatic compound called dimethyl sulphide which helps them locate the truffles from underneath the ground. The truffle aromas are not detected in immature fruiting bodies but intensify as the truffle matures which help prevent the harvesting of immature fruiting bodies when animals are used (Trappe and Claridge, 2010). The use of animals as an alternative for the location of desert truffles including *Kalaharituber pfeilii* has not been explored and it is unknown whether they produce the compound dimethyl sulphide. Signs of animal disturbances at the collection sites do suggest that some volatile compounds are released and detected by the animals. The use of trained animals to locate truffles would increase the natural harvest, as currently truffle collection is a time consuming task. This approach may be unsustainable, resulting in reduced populations. Decline in forest truffle harvests has been attributed to overharvesting, loss of habitat and urban development (Hall et al., 2003).

Truffles fruit throughout the year depending on the species. The Kalahari truffles are often found from April to June depending on when the rain occurs. Collection trips at Spitskop Nature Reserve, Upington in April 2010/2011 yielded no truffles. This was unexpected as truffles have been successfully harvested in the same area in April in previous years (Adeleke, 2007; J. Dames pers. comm.). Due to the lack of truffles in Spitskop, Haakdoringduin farm also in Upington was chosen as the second site and fruiting bodies were successfully collected in May 2011.. All collected fruiting bodies were *K. pfeilii* and no other species were found.

A decrease in harvest has been noted over the years (Adeleke, 2007; J. Dames pers. comm.). This indicated a possible change in distribution patterns. According to the survey conducted

(Table 3.11), truffles have been found in Askham, Hartwater and Upington which are all in the Northern Cape province, South Africa. Responses from Namibia included the areas of Aranos and Gobabis. It was also observed that the fruiting bodies are seldom found in the same location as previous collections, this supports observations made in this study. This could be due to the release of exudates by truffles which can alter the characteristics of the soil (Walker et al., 2003) and land-use practices such as over grazing. Samples were only collected from a limited area and more in depth distribution studies are required. No evidence was found to suggest that either *E. echinulatus* or *M. austroafricanus* were collected. It has been noted that *E. echinulatus* appears later in season and is not regarded as edible (van der Westhuizen and Eicker, 1994). Survey results indicate that *E. echinulatus* has been found from Askham, Upington (South Africa) and Gobabis (Namibia). *M. austroafricanus* was originally described from Kimberly (Marasas and Trappe, 1973), situated on the fringes of the Kalahari but the survey shows that it has been found to occur in Upington and Gobabis. The Kalahari is a vast area of about 900 000 km^2 stretching from the Northern Cape in South Africa, through Botswana and parts of Namibia, Angola and Zambia (Fig 1.3) and a more detailed distribution survey would be valuable in monitoring any changes to their occurrence.

The amount and weight of truffles collected in this study (Fig 3.2) was considerably less than collected in previous years (Adeleke, 2007; J. Dames pers. comm.). These differences could have been due to certain environmental changes such as amount and timing of rainfall as water availability is the main contributing factor in truffle growth (Leisner, 1967). During the dry winter months spores of the Kalahari truffle become dormant and require moisture for germination (Trappe and Claridge, 2010). Rainfall is thought to trigger germination and subsequent hyphal growth and would be required for fruiting body formation.

According to Awamah and Alsheik (1979) desert truffle species of *Terfezia* and *Tirmania* are known to fruit in years with a minimum rainfall of 180 mm distributed from October to March. Total rainfall recorded from October to March 2009/2010 in Upington was 198.2 mm with 452.4 mm being recorded for the same period in 2010/2011 (Table 3.10). Fruiting bodies collected later in 2011 were smaller than those collected in 2010, this indicated that truffles were in the early stages of development possibly due to the heavy rainfall in February and March 2011 (a total of 280.3 mm). Average temperatures during these periods did not differ greatly being

17.9^{o}C minimum (Table 3.9) and 34.6^{o}C maximum (Table 3.8) for the period October to March 2009/2010 and 17.7^{o}C and 34.2^{o}C respectively for the same period in 2010/2011. No studies have been published about the amount of rainfall required for adequate fruiting of the Kalahari truffles (Trappe et al., 2008). However the timing of rainfall is thought to be important. In North African and Middle Eastern countries rain is essential until only the beginning of December (Morte et al., 2008). Although the Haakdoring Duin site is approximately 150 km North of Upington, rainfall data was not available for this area, so there may been some differences from the rainfall experienced in Upington. A long term study on truffle formation in relation to these climatic factors would greatly enhance our understanding of the Kalahari truffle.

4.2 Identification of *Kalaharituber pfeilii*

4.2.1 Morphological Identification

Morphological identification using The Key to Kalahari Truffles by Trappe and Castellano, (2007) confirmed that the collected fruiting bodies were *K. pfeilii*. The collected fruiting bodies were observed to have a yellowish to cream peridium, gleba with marbled white veins (Fig 3.2) and had a fungoid odour. These were the same characteristics which were observed to be characteristic of *K. pfeilii* by Ferdman et al., (2005) and those described by Adeleke (2007) and van der Westhizen and Eicker (1994). The collected truffles were firm but the texture became softer as they gradually lose their freshness.

Each truffle species is known to have distinctive aromatic compounds which develop stronger as the spores mature (Trappe and Claridge, 2010). Truffles never share the same aroma even when they have similar physical features (March et al., 2006; Cullere et al., 2010). The aroma is due to approximately 36 volatile compounds, the most important include methane and dimethyl sulphide which normally aid in the location of truffles. The geographic location of the truffles contributes to their aroma. Zeppa (2004) demonstrated that the host plant also contribute to the aroma of its truffle. He demonstrated that the compound aromadendrene was detected in *T. borchii* mycelium growing with host plant *Tilia platyphyllos* but was absent in *T. borchii* free living mycelia. *K pfeilii* has a fungoid aroma compared to the "wet forest" aroma of *T. melanosporum* and the "cheesy garlic" aroma of *T. magnatum.* Aroma profiles have been established for the correct identification of truffles in *Tuber* genera (Luard, 2006).

Truffle species are believed to have evolved over many years resulting in loss of certain characteristic features (Ferdman et al., 2005). According to Hall et al. (2007), truffles do not possess a stalk or gills like most mushrooms, this also was also observed on the collected truffles. Taylor et al. (1995) contradicts these findings as the truffles he collected contained a stalk, this is more of a small protuberance but often becomes dislodged when harvested and may be related to maturity of the truffle. Truffles collected for this study did show stalk-like structures but it could not be confirmed whether the stalk was starting to develop or was dislodged during harvesting as previously mentioned.

K. pfeilii is an Ascomycete and like several other Ascomycete fungi it does not produce asexual sporulating structures in culture (Fig 3.4a) and has septate hyphae (Fig 3.4b). These characteristics were used to initially identify truffle cultures and eliminate contaminating fungi. *K. pfeilii* mycelia colonies emerged from explants after 3 to 4 weeks but took 3-4 days when subculturing after incubation in the dark as *K. pfeilii* fruits underground, at 32°C. Temperature is an important factor in *in-vitro* growth and *K. pfeilii* was shown to grow best at 32°C (Adeleke, 2007). Subcultured colonies grew faster than the initial inoculums, this could be because the mycelia in the colonies are more adapted to utilise the nutrients of the media than the initial truffle inoculums. The ability of the subcultured colonies to grow faster in media is essential in mycorrhizal resynthesis experiments and further cultivation studies of *K. pfeilii*. Mycelial culture viability was observed to decrease after several subsequent subculturing attempts. This was also observed by Kagan-Zur et al., (2008) and therefore it is essential to investigate various methods for maintaining cultures.

4.2.2 Molecular Identification

In this study molecular techniques which include PCR and DNA sequencing were used to identify the collected fruiting bodies. DNA extraction kit was used instead if the CTAB method because it is simple, does not contain organic solvents such as chloroform and obtained DNA is more pure and clean. . Addition of BSA (Bovine Serum Albumin) in all PCR reactions assisted to overcome PCR inhibitors (Adeleke, 2007). Amplification with specific primer pair TPR1 and TPF3 allowed for the specific identification of *K. pfeilii* from all the samples. Adeleke (2007) designed *K. pfeilii* specific primer pair TPR1 and TPF3 which was tested against a selection of

fungi isolated from truffle fruiting bodies. He conducted a nested PCR using first the ITS universal primers which produced a band for all fungi tested. The second round of PCR using primer pair TPR1 and TPF3 amplified only *K. pfeilii* confirming their identity and successful isolation when subjected to BLAST analysis. Amplification with specific primers shortens the period and cost of molecular identification (Paolocci et al., 1998). Specific primers have been successfully used to selectively amplify truffle species such as *Tuber spp.* (Amicucci et al., 1998; Mello et al., 1999). .

Specific primers were also designed in this study, with the intention to specifically amplify *Mattirolomyces austroafricanus* and *Eremiomyces echinulatus* which are truffle species also known to be found in the Kalahari region of southern Africa. These species including *K. pfeilii* are closely related and may be mistaken for one another. Having specific primers would assist in the rapid identification of these truffles. The designed primers were not tested on samples of *M. austroafricanus* and *E. echinulatus* as neither were found to occur during sample collection. When tested on *K. pfeilii,* both primers did produce amplicons but of different sizes (Fig 3.14) which would unfortunately not successfully differentiate between the different species. Further primer design would need to be tested. In the absence of fruiting bodies, herbarium samples of these truffles obtainable from the National Mycology Collection can be used.

DNA sequencing is used to determine unknown PCR- amplified sequences using one of the amplification primers. After sequencing Basic Local Alignment Tool (BLAST) programs are used as they are essential for searching identical DNA sequences in DNA databases (Altschul et al., 1997). The fruiting bodies had 100 and 99% similarities with *K. pfeilii* sequences available on the BLAST program (AF301422.1 and AF301420.1) which therefore confirmed that collected fruiting bodies were *K. pfeilii*.

4.3 *Kalaharituber pfeilii* host relations

This study investigated the possible mycorrhizal relationship between *K. pfeilii* and the roots of *Stipagrostis ciliata* var. *capensis, S. obtusa* and *Schmidtia kalihariensis*. These are grass species found in the vicinity where the fruiting bodies were collected and therefore had the potential to be hosts. Grass species are easier to grow in semiarid conditions as they do not require large amounts of water to germinate and grow. The roots of *S. ciliata* var. *capensis, S. obtusa* and *S. kalihariensis* were subjected to staining so that their mycorrhizal associations could be examined. All these grasses have sand encrusted rhizosheaths which when removed exposes the vascular cylinder (Fig 3.5). The rhizosheath which could potentially be held in place by mycorrhizal fungal hyphae emanating from the colonized root cells is known to be essential for nutrient uptake, water absorption and also acts as a barriers against pathogens, dehydration and herbivores (Othman et al., 2004). The presence of a rhizosheath is not sufficient evidence to confirm a relationship between plant and truffle. Despite washing and soaking of roots, sand particles were difficult to remove without dislodging the cortical root cells. This made microscopic examinations difficult due to inadequate focus ability. An ectendomycorrhizal association was observed within the roots of *S. ciliata* var. *capensis* (Fig 3.9), although this could not be confirmed. Adeleke (2007) also suggested that the *K. pfeilii* produced ectendomycorrhizal relations with potential hosts. The other grass species were observed to have endomycorrhizal associations with the presence of vesicles (Fig 3.10) suggesting a relationship with arbuscular mycorrhizal fungi, although arbuscules were not confirmed. These grasses were therefore eliminated as potential hosts because even though truffles can form endomycorrhizal associations none have been observed to produce vesicles (Smith and Read, 2008).

Adeleke, (2007) surveyed several plants species and microscopically observed that most were not hosts, but showed arbuscular mycorrhizal relationships and spores of *Glomus sp*. were also extracted from the soil, confirming the presence of Glomalean fungi. The results including a study by Kagan-Zur et al. (1999) showed the presence of an endomycorrhizal association between *K. pfeilii* and *Citrus vulgaris.* Fungal hyphae were observed within plant cortical root cells. Other suggested hosts have not been positively confirmed. Adeleke, (2007) amplified *K. pfeilii* from roots of *Acacia erioloba* and the grass *Cynodon dactylon*, this was done from a resynthesis trial and the results were not repeatable, so the relationship could not be confirmed.

Direct amplification of rhizosheath material was used to confirm presence of the fungus (Fig 3.13) in the root cells. Sequencing of PCR products (Table 3.3) confirmed and conclusively showed the presence of *K. pfeilii* in the rhizosheath of *S. ciliata*. It would be interesting to determine whether the colonisation structures found in the *S. obtusa* and *S. kalihariensis* were in fact those of the *K. pfeilii* using the same PCR amplification with the specific primers. This was not conducted in this study as truffles are usually not found where these grasses are the more dominant species (J. Dames pers. comm.). *S. kalihariensis* is a known indicator of drought and overgrazing and often replaces areas previously dominated by *S. ciliata* which is an indicator of good veld and grazing conditions (van Oudtshoorn, 1992).

Soil characteristics play a role in the occurrence and distribution of truffles as they can hinder the availability of hosts under specific limiting conditions such as pH (Diez et al., 2002). The amount of nutrients available in the soil also plays a major role in the initiation of the mycorrhizal symbiosis. Studies have shown that the type of mycorrhiza formed depends on the availability or lack of certain nutrients. Fortas and Chevalier (1992) observed that some plants form ectomycorrhizal association when phosphate levels are sufficient and an endomycorrhizal association is formed at low phosphate concentrations. Desert truffles are known to occur in calcareous soils but *K. pfeilii* was observed to occur in slightly acidic soils with pH ranging from 5.5 – 6.5 (Taylor et al., 1995). Soil analysis conducted in this study was also within this range (Table 3.7). Adeleke (2007) collected truffles from the Spitskop Nature Reserve and these soils had a pH of 5.9. However the optimal *in vitro* cultural conditions for *K. pfeilii* was pH 7.0-8.0 which is higher than the pH recorded for the collection sites. Percentage organic carbon and P levels were similar to soil of this study (Adeleke, 2007).

There is interest in the potential to cultivate *K. pfeilii* like the other successfully cultivated truffles. The mycorrhizal resynthesis experiments were conducted to establish a mycorrhizal association between *K. pfeilii* and its host which could be the first step towards the successful cultivation of this truffle. Seeds of *S. ciliata* var. *capensis* were collected for this study because it was observed that it occurred in all the areas where the truffles were collected. *S. ciliata* var. *capensis* produces flowers from August to October and February to June, inflorescence with spikelets (Fig 3.18) where collected in April and May (van Oudsthoorn, 1992). The actual seed is very small and removal from the spikelet can be difficult. Soaking seeds in water proved to be a

more effective way to stimulate germination. Only germinated seedlings were selected for the mycorrhizal resynthesis experiments.

The petri dish method used for mycorrhizal synthesis was unsuccessful because of contamination and the structure of the grass, it was difficult to maintain the roots only in the medium which protruding leaves. Other axenic approaches were also not successful. Because of the extensive contamination and death of the seedlings the roots were not stained to verify the mycorrhizal relationship. The resynthesis methodology proved challenging and did not ultimately prove the relationship between *K. pfeilii* and *S. ciliata* var. *capensis*, the molecular evidence from the collected field samples was convincing enough to state that *S. ciliata* var. *capensis* is a host of the Kalahari truffle. A more natural approach under greenhouse conditions is recommended for future studies. Growing medium can be pasteurised and seeds can be applied directly to the damp surface of the growing medium. It has been observed in the collection area that when conditions are damp the seed awn assist the seed to burrow into the soil. Inoculum of *K. pfeilii* can subsequently be applied. Growing medium could be a combination of sand from the collection site and vermiculite for aeration. Alternatives inoculums types can be tested such as fruiting body slurry that could be used to inoculate the grass seeds (Slama et al., 2010) or pre-germinated seeds (Morte et al., 2008). According to Morte et al. (2008) seedlings should first develop a proper root system which is normally after 5 months before it is inoculated with the fungi for mycorrhizal association to be successful.

4.4 Isolation and identification of associated bacteria

Mycorrhizal associations have been considered as an association between mycorrhizal plants and fungi but the presence and activity of bacteria in the rhizosphere is evidence that bacteria contribute towards the mycorrhizal association. Several studies have been performed to investigate and identify the role of bacteria in the mycorrhizal symbiosis (Fry-Klett et al., 2007; Poole et al., 2001; Tarkka and Frey-Klett, 2008). Bacteria can improve the ability of mycorrhizal fungi to colonise the plant roots. Some bacteria influence the fruitbody formation of certain fungal species (Barbieri et al., 2007). This study investigated the effects of bacteria isolated from the truffle fruiting body and the rhizosheath on *K. pfeilii* mycelia growth. No mycelia growth as observed in the presence of the bacteria. Inhibition of mycelia growth by bacteria is often due to

chitinase which some bacteria produce (Chet et al., 1990). Pacioni (1990) studied the relationship between bacteria and the fruiting bodies of *Tuber sp*. using scanning electron microscopy. He observed bacteria in the hyphae and spores, and they are responsible for interchange of metabolites required for hyphal development.

Bacteria are also important for their phosphate solubilising properties and their ability to produce indole acetic acid. These characteristics were also investigated in this study. Isolate TBA was identified as *Leucobacter sp*. but the sequence was short and additional tests could have confirmed the results. Bacteria in the *Leucobacter* genus are Gram positive, irregular rods which were confirmed by Gram staining. *Leucobacter* has an optimum growth temperature of 30°C but can also grow at higher temperatures such as 37°C. These bacteria are airborne and can be isolated from soil. They belong to the Microbacteriaceae family which possess a characteristic 2,4 diaminobutyric acid in the peptidoglycan. *Leucobacter sp*. are usually found in metal stressed environments as they have developed resistance and detoxification mechanisms against the metals (Morais et al., 2006). Detoxification is one of the characteristics of MHB as it results in the adjustment of the mycorrhizospheric soil properties (Duponnois and Garbaye, 1990).

Isolate TBB was identified as *Stenotrophomas maltophilia* a Gram negative bacilli cells which were confirmed by Gram staining. *S. maltophilia* occurs in water, soil and plants. It is commonly isolated from the rhizosphere of several plant species such as wheat, oat and maize (Swings et al., 1983). It was also isolated by Adeleke (2007) from the fruiting body of *K. pfeilii* but it did not show any significant stimulatory effects on mycelia growth in dual culture. It has been observed to have antagonistic effects although little is known about its antagonistic mechanisms (Berg et al., 1996). It is suggested that the antagonistic effects may result in the production of antibiotics important against pathogenic bacteria, parasitism and competition (Chet et al., 1990). *S. maltophilia* has properties useful in biological control of soil borne plant pathogens. It was successfully used as a biological agent against *Rhizoctonia solani* (Berg et al., 1996). The ability to inhibit pathogenic microorganisms thus eliminating competition for nutrients and parasitism is essential for mycorrhizal association, therefore *S. maltophilia* can be considered as MHB.

Phyllbacterium myrsinacearum (TBC) is gram negative and rod shaped and was one of the isolated bacteria. *Phyllobacterium* species have been shown to have antifungal and antibacterial activities (Lambert et al., 1990) which can assist in the elimination of competing microorganisms

occurring in the mycorrhizosphere. Non pathogenic properties to plants have been observed and are therefore considered a potential candidate for use in plant growth promotion or biological control of soil borne diseases. They are N_2 fixing bacteria associated with root surface of sugarbeet plant and have the capacity to communicate with plant tissues. IAA production was positive suggesting that *P. myrsinacearum* can influence mycorrhizal formation by promoting short root and hair development, although this could not be tested in this study due to the challenges with resynthesising the relationship. Although utilisation of nitrogen was not tested in this study, the reported fixation ability could greatly enhance nitrogen availability to both symbionts.

Isolate TBD was *Staphylococcus cohnii* which is a Gram positive, coagulase negative cocci commonly found as part of the human skin flora but can be a pathogen in human disease (Rupp and Archer, 1994). It has also been isolated as a component of the soil microbial flora with a potential in rhizoremediation (Abhilash et al., 2011). Isolate TBE was identified as *Dietzia sp.* but the sequence was too short (Appendix C) therefore the results were disregarded. The last isolate was identified as *Pantoea dispersa* (TBF) which is a Gram negative rod with an optimum growth temperature of 30°C. *Pantoea* species are usually isolated from plants and the natural environment. It is a detoxifying bacterium known to detoxify albicidin which is important in sugar cane leaf scald disease development and is therefore an effective biocontrol agent against leaf scald disesase in sugar cane. A strain of *P. dispersa* was observed to contain growth promoting properties (Selvakumar et al., 2007).

Leucobacter sp, S. maltophilia, *P. myrsinacearum* and *P. dispersa* could possess certain mycorrhizal helper characteristics which were not explored in this study such as chitinase, protease and cellulose to mention a few. Although all isolates were observed to inhibit growth of *K. pfeilii* in culture they could potentially contribute to mycorrhization through the increase in plant root growth, supply of nitrogen, detoxification and as biological control agents against competing soil fungi.

CONCLUSION

Kalaharituber pfeilii is an indigenous truffle of the Kalahari. The occurrence of two other truffle species, *Mattirolomyces austroafricanus* and *Eremiomyces echinulatus* have been reported. In this study only *K. pfeilii* fruiting bodies were found, both *M. austroafricanus* and *E. echinulatus* were not found in this study or a previous study by Adeleke, (2007). The availability and size of truffles collected indicated that the growth of the fruiting bodies is dependent on the amount and timing of rainfall. According to the literature (Story, 1958; Mshigeni, 2001) and also the results of the conducted survey, *K. pfeilii* occurs from April to May in the Kalahari.

The morphological and molecular identification methods were regarded as being more effective in confirming host relations. . Primer pair TPR1 and TPF3 (Adeleke, 2007) successfully and specifically amplified *K. pfeilii*. This study was dependant on this use of these primers and there is a need to develop specific primers to assist with the identification of *M. austroafricanus* and *E. echinulatus.* Although DNA extraction and PCR kits were used in this study some problems were encountered with amplification. The addition of BSA helped to overcome PCR inhibitors for the successful amplification of the DNA, indicating that modifications to current kits are sometimes required.

Obtaining pure cultures of *K. pfeilii* were essential for investigating between bacterial interactions and ultimately for the mycorrhizal resynthesis experiments. Adeleke (2007) showed that isolates were sensitive to pH and temperature and cultures were therefore incubated at 32°C and the pH of the modified Fontana medium was adjusted to 7.2. *K. pfeilii* does not sporulate in culture and contains septate hyphae. However it was observed that cultures lost viability after successive rounds of subculturing. This could be problematic in future inoculation experiments. *K. pfeilii* occurs in slightly acidic soils with a pH range of 5.5-6.5 unlike other truffle species which prefer high pH calcareous soils.

The presence of the sand encrusted rhizosheath, which effectively replaced the roots caused difficulties during microscopy as the sand grains were entangled interfering with focussing ability. However evidence of an ectendomycorrhizal association was observed between *K. pfeilii* and *Stipagrostis ciliata* var. *capensis.* Molecular analysis through direct DNA extraction and

amplification from the root and using the BLAST program confirmed *S. ciliata* var. *capensis* as a host of *K. pfeilii*. The presence of vesicles confirmed that *Schidtia kalahariensis* and *Stipagrostis obtusa* are endomycorrhizal hosts possibly associating with the arbuscular mycorrhizal fungi. This led to the conclusion that they are not hosts of *K. pfeilii.* Mycorrhizal resynthesis experiments under axenic conditions were unsuccessful because of several factors such as extensive contamination by bacteria and depletion of nutrients in the media.

Bacterial species were successfully isolated in culture from the truffle fruiting bodies and rhizosheath. Four of the isolates were Gram negative and two were Gram positive. Only one, *Phyllbacterium myrsinacearum,* tested positive for indole acetic acid production and none of the isolates able to solubilise phosphate. Molecular identification through sequencing and BLAST programmes confirmed the isolates as *Leucobacter sp, Stenotrophomas maltophilia, Phyllobacterium myrsinacearum, Staphylococcus cohnii,* and *Pantoea dispersa* and the sixth isolate was identified as *Dietzia sp* but this was not confirmed because the sequence was too short.

Phyllobacterium myrsinacearum can be considered as a mycorrhizal helper bacterium because of its IAA producing characteristics. IAA producing isolates improve the formation of plant short roots which results in a larger root surface area hence increasing chances of mycorrhizal colonisation. *Stenotrophomonas maltophilia* which had been previously isolated from *K. pfeilii* fruiting bodies (Adeleke, 2007) could have a certain role in *K. pfeilii* production which is not yet known. All the isolated bacterial species could possess certain mycorrhizal helper characteristics which were not explored in this study and could potentially contribute to mycorrhization through the increase in plant root growth, supply of nitrogen, detoxification and as biological control agents against competing soil microorganisms.

FUTURE RECOMMENDATION

Truffle fruiting bodies tend to lose freshness quicker and means of preservation for laboratory studies needs to be investigated. Distribution studies also need to be conducted as changes in distribution patterns has been observed over two different sampling sites. No fruiting bodies were found in locations where they have been previously harvested from. Although root and truffle exudates could limit production of fruiting bodies in subsequent years, this needs to be investigated further. Mycorrhizal resynthesis experiments require further investigation to optimise the process for successful results as that would be the next step toward successful cultivation of *K. pfeilii*. Inoculation in greenhouse trials may be more successful than axenic systems. Knowledge about the effects of abiotic and biotic factors on truffle growth needs to be explored further in order to understand the relationship between the symbionts.

Bacterial interactions both physical and biochemical and their role in mycorrhizal development could also contribute to furthering our knowledge of the Kalahari truffle life cycle and mycorrhizas in general. Adeleke (2007) did isolate some bacteria that were shown to stimulate fungal growth *in vitro*, although this was not shown in this study there are many different soil bacteria several of which were not isolated due to the selection of faster growing isolates. These isolates should not be ignored. Investigation of the microbial population of the rhizosheath could also reveal many interesting interactions. The non-culturable components of the population also need to be considered in order to get a true reflection of the microbial diversity associated with the mycorrhizal relationship. Further knowledge is required to identify the compounds or mechanism involved in fungal growth promotion and to determine the effects of mycorrhizal helper bacteria on the plants and fungi. In a complex soil environment it is likely that several interactions results in successful mycorrhizal establishment, some bacteria may be able to provide nutrients through nitrogen fixation and phosphate solubilisation while others may produce hormones to stimulate root production. This coupled with any antagonistic ability through production of enzymes, antibiotics or other inhibitory compounds could assist *K. pfeilii* to out compete other soil borne fungi.

Success in the cultivation of the Kalahari truffles would increase the commercial value of these truffles and also make them available to worldwide markets. It would also contribute towards improving the rural communities where they naturally occur.

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APPENDICES

APPENDIX A: Media and reagents

Modified Fontana media 1L (Adeleke, 2007)

15 g	Bacteriological agar
1.65 g	Peptone powder
6.5 g	$C_6H_{12}O_6.H_2O$
0.33 g	KH_2PO_4
150 µl	1 % $MgSO_4.7H_2O$
150 µl	1 % $FeCl_2.4H_2O$
150 µl	1 % $ZnSO_4.7H_20$
150 µl	1 % $MnSO_4.H_2O$
1 650 µl	CaCl

Heat, cool down and adjust to pH 7.2. Autoclave and filter sterilise 0.01g ampicilin, 0.01 g chloramphenicol and 0.33 g BSA.

Phosphate Solubilising Media 1L (Mehta and Nautiyal, 2001)

10 g	Glucose
5 g	$Ca_3 (PO_{4)2}$
5 g	$MgCl_2.6H_2O$
0.25 g	$MgSO_4.7H_2O$
0.2 g	KCl
0.1 g	$(NH4)_2\ SO_4$
15 g	Agar

Salkowski reagent (Glickmann and Dessaux, 1994)

12 g $FeCl_3$ in 7.9 M H_2SO_4

APPENDIX B: Nutrient Solutions and Staining materials (Koske and Gemma, 1989; Smith and Dickson, 1997)

5 % KOH

100 g KOH

2 L Distilled water

Alkaline H_2O_2

3 ml NH_4OH

3 ml 30 % H_2O_2

594 ml Distilled water

0.1M HCl

22.79 HCl

2 L Distilled water

Lactoglycerol Trypan Blue Stain

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

440 ml Lactic acid

406.2 ml Glycerol

541.6 ml Distilled water

0.694 g Trypan blue

Lactoglycerol destain

440 ml Lactic acid

406.2 ml Glycerol

541.6 ml Distilled water

APPENDIX C: Nucleotide sequences

Kalaharituber pfeilii (AF301420.1)

5′GGAGTTTGCCGGTGGGTAGCCCCTTTTATAATCAAAACCTGTCTAATAGAGAAACCTTTTGGAGTTTGCCGGTGGGTAGCCCCT
TTTATAATCAAAACCTGTCTAATAGAGAAACCTTTTTGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAAC
GGATCTCTAGTGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATGA
AGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGCTCTTGCATCGATGAAGAACGCAGTGAATTGCGATAAGTAA
TGTGAATTGCAGAATCTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCATCACCTCTCATAAGCAGCCA
TTTATTTCTTTGAGTGGTTCTGTATTTCTGAGCGTCAGCATCACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATT
TGAGGACTCATTGGTGAGGACTCATTGG 3′

Kalaharituber pfeilii (AF301422.1)

5′GGAGTTTGCCGGTGGGTAGCCCCTTTTATAATCAAAACCTGTCTAATAGAGAAACCTTTTGGAGTTTGCCGGTGGGTAGCCCCT
TTTATAATCAAAACCTGTGTAATAGAGAAACCTTTTTGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAAC
GGATCTCTAGTGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAGGCTCTTGCATCGATGA
AGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTCGCTCTTGCATCGATGAAGAACGCAGTGAATTGCGATAAGTAA
TGTGAATTGCAGAATCTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCATCACCTCTCATAAGCAGCCA
TTTATTTCTTTGAGTGGTTCTGTATTTCTGAGCGTCAGCATCACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATT
TGAGGACTCATTGGTGAGGACTCATTGG 3′

Comparison between the obtained *K. pfeilii* sequences, AF301422.1 and AF301420.1. A conversion from G to C was observed in the 113 position.

```
22.1        GGAGTTTGCCGGTGGGTAGCCCCTTTTATAATCAAAACCTGTCTAATAGAGAAACCTTTT      60
20.1        GGAGTTTGCCGGTGGGTAGCCCCTTTTATAATCAAAACCTGTCTAATAGAGAAACCTTTT      60

22.1        GGAGTTTGCCGGTGGGTAGCCCCTTTTATAATCAAAACCTGTGTAATAGAGAAACCTTTT      120
20.1        GGAGTTTGCCGGTGGGTAGCCCCTTTTATAATCAAAACCTGTCTAATAGAGAAACCTTTT      120

22.1        TGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAG      180
20.1        TGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAG      180

22.1        TGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAG      240
20.1        TGTCTGATATTAAATGAAATAAAATGAAAAAGAATAAAACTTTCAACAACGGATCTCTAG      240

22.1        GCTCTTGCATCGATGAAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTC      300
20.1        GCTCTTGCATCGATGAAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTC      300

22.1        GCTCTTGCATCGATGAAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTC      360
20.1        GCTCTTGCATCGATGAAGAACGCAGTGAATTGCGATAAGTAATGTGAATTGCAGAATCTC      360

22.1        GTGAATCATCGAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTG      420
20.1        GTGAATCATCGAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTG      420

22.1        GTGAATCATCGAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTG      480
20.1        GTGAATCATCGAATCTTTGAACGCACATTGCGCCCTATGGTATTCCGTAGGGCATGCCTG      480

22.1        TCTGAGCGTCAGCATCACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATT      540
20.1        TCTGAGCGTCAGCATCACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATT      540
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```
22.1          TCTGAGCGTCAGCATCACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATT       600
20.1          TCTGAGCGTCAGCATCACCTCTCATAAGCAGCCATTTATTTCTTTGAGTGGTTCTGTATT       600

22.1          TGAGGACTCATTGGTGAGGACTCATTGG                                       628
20.1          TGAGGACTCATTGGTGAGGACTCATTGG                                       628
```

Mattirolomyces austroafricanus (GQ231753.1)

```
5’GAAAGAACCACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAGATTTGAAATCTGGCGTCATTATGGCGTCTG
AGTTGTAATCTGTAGAGGAGTATTCGAGTATGGATTTGGCCTAAGTTCTTTGGAACAGGGCGTCATAGAGGGTGAGAACCCCGTTA
ACGGCCTAGGTCTTATGCTCATGTGAATCTCCTTCAACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTCCAT
CTAAAGCTAAATACTGGCAAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGAACTCTGAACAGAGAGTTAAATAG
TACGTGAAATTGTTGAAAGGGAAGCACTTGCTACCAGACACGCTTGTAGTTGATCAGCCTCCATTCTTGGTGGTGCACTCAACTAT
GGGTGGGCTAGCATCAGTTATACTGGTAGGACAAAGACTGAGGGAATGTAGCTCCTTTCGGGGAGTGTTATAGCCTTCAGTGCAAT
ACTATCTAGTGTGACTGAGGACTGCGCTTCGGCGAGGATGCTGGCATAATGGTCGTAAGTGACCCGTCTTGAAACACGGACCAAGG
AGTCTAACATCTATGCAAGTGTTTGGGTGTTGAAACCCTTACGCGAAATGAAAGTGAACGAAGGTGGGAACCGCAAGGTGCACCAT
CGACCGATCCTGATGTTCTCGGATGGATTTGAGTAGGAGCATAGCTGTTGGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGT
GAAGCCAGAGGAAACTCTGGTGGAGGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTCAAATTTAGGTATAGGGGCGAAAGACTA
ATCGAACCAT 3’
```

Eremiomyces echinulatus (AF435823.1)

```
5’CAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAGATTTGAAATCTGGTG
CCATTTTGGCACTCGAGTTGTAATCTGTAGAGGAGTATTCGAGTGTAGCTTTGGCTTAAGTTCCTTGGAACAGGACGTCATAGAGG
GTGAGAACCCCGTTAACGGCCTTTGTCTTATGCTCATATGAATCTCCTTCAATGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATG
GGTGGTAAATTCCATCTAAAGCTAAATACTGGCAAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGAACTCTGAA
CAGAGAGTCAAATAGTACGTGAAATTGTTGAAAGGGAAGCACTTGTGACCAGACGCACTTGCAGTTGATCAACCTCCATTTTTGGT
GGTGCACTCGGTTGTAGGTGGGTCAGCATCAGTTATAGTGATGGGAAAAAGATTGAGGGAATGTGGCTCTCTCCGGGGAGTGTTAT
AGCCCTTGATGTAATGCTACCAGCTGTAACTGAGGACCGCGCTTCGGTGAGGATGCTGACACAATGGTCGCAAGTGACCCGTCTTG
AAACACGGACCAA 3’
```

Alignment of the the truffle species *Kalaharituber pfeilii* (gi11127911gb), *Eremiomyces echinulatus* (gi17226850gb) and *Mattirolomyces austroafricanus* (gi255966097gb) using MAFFT Alignment Online.

```
gi|11127911|gb| ---ATCATTATTGAGTAAGCTTTA-------------------------------------
gi|17226850|gb| AGGATCATTACTGAAAAAACTATC-------------------------------------
gi|255966097|gb GAAAGAACCACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAGATTTGA
                   *  *..*. *..   .*. *

gi|11127911|gb| --------------------TTGTAGCTTTCTCTC--------TTATCCCTTTGTTTACT
gi|17226850|gb| --------------------TCTTAGTTCTTC-----------ATATCCTATTGTTAACT
gi|255966097|gb AATCTGGCGTCATTATGGCGTCTGAGTTGTAATCTGTAGAGGAGTATTCGAGTATGGATT
                                  *.  **.* *             ***.*   *.*  *.*
```

```
gi|11127911|gb| TTACCCTGTTGCTT----------------------------CCAC-TGGACAGTGTGAG
gi|17226850|gb| T-ACCATGTTGCTT----------------------------CCACGTGGACAGGCTCAC
gi|255966097|gb TGGCCTAAGTTCTTTGGAACAGGGCGTCATAGAGGGTGAGAACCCCGTTAACGGCCTAGG
                * .**  . * ***                            ** * * .**.*  * .

gi|11127911|gb| CTTTGCTGGCA----------GTTGAAGAAGTTCAATTGTAGGCAAGTGAGCCCTCTGG-
gi|17226850|gb| CCT-----------------------------------------GAGTGAGCCCTTTGGT
gi|255966097|gb TCTTATGCTCATGTGAATCTCCTTCAACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAAT
                ..*                                        .*.** . *... ..

gi|11127911|gb| ------------TTTTGGTGCACTCGGTACCATTGCTGGGGAGTTTGCCGGTGGGTAGCC
gi|17226850|gb| ATAGTGTGAATATTTTCATACATT-----------CCAGAGAGTTTGCCCGTGGTAAGAC
gi|255966097|gb GGGTGGTAAATTCCATCTAAAGCTAAATACTGGCAAGAGACCGATAGCGCACAAGTAGAG
                          .. *   . ..*            .*.  * * **  ....  **

gi|11127911|gb| CCTTTTATAATCAAAACCTGTGTAATAGAGAAACCTTTTTGTCTGATATTAAATGAAATA
gi|17226850|gb| CTCC---AAACCAAAACTTGAAGAAAACCAAAAC-----AGTCTGACAAT--------TT
gi|255966097|gb -------TGATCGAAAGATGAAAAGAACTCTGAACAGAGAGTTAAATAGTACGTGAAATT
                       .*.*.***  ** . *. *    .*       **. .*.* *        *

gi|11127911|gb| AAATGAAAAAGAATAAAACTTTCAACAA----------CGGATCTCTAGGCTCTTGCATC
gi|17226850|gb| ATTGCAAATGAAATAAAACTTTCAACAA----------CGGATCTCTAGGCTCTTGCATC
gi|255966097|gb GTTGAAAGGGAAGC---ACTTGCTACCAGACACGCTTGTAGTTGATCAGCCTCCATTCTT
                .    **. ..*..   **** * ** *          ..* *  ..** ***.  . *.

gi|11127911|gb| GATGAAGAACGCA-------GTGAATTGCGATAAGTAATGTGAATTGCAGAA--------
gi|17226850|gb| GATGAAGAACGCA-------GTGAAATGCGATAAGTAATGTGAATTGCAGAA--------
gi|255966097|gb GGTGGTGCACTCAACTATGGGTGGGCTAGCATCAGTTATACTGGTAGGACAAAGACTGAG
                *.**. * ** **       ***.. *.  ** *** **.. ..* * * **

gi|11127911|gb| -------------TCTCGTGAATCATCGAATCTTTGAACGCA--------CATTGCGCCC
gi|17226850|gb| -------------TCTCGTGAATCATCGAATCTTTGAACGCA--------CATTGCGCCC
gi|255966097|gb GGAATGTAGCTCCTTTCGGGGAGTGTTATAGCCTTCAGTGCAATACTATCTAGTGTGACT
                           *.*** *.* ..*.. * *.** *..***        .* **.* *.

gi|11127911|gb| TATG---GTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCATCACCTC-TCATA-----
gi|17226850|gb| TATG---GTATTCCGTAGGGCATGCCTGTCTGAGCGTCAGCTCCCCCCCATCTCA-----
gi|255966097|gb GAGGACTGCGCTTCGGCGAGGATGCTGGCATAATGGTCGTAAGTGACCCGTCTTGAAACA
                 * *   *...*.**  *.* ****. *. *.*  ***.    .  *.* ** ..

gi|11127911|gb| ---------------AGCAGCCATTTATTTCTTTGAGTG----------------------
gi|17226850|gb| ---------------AGCATCTTTTTTGAAGCTTGGATT----------------------
gi|255966097|gb CGGACCAAGGAGTCTAACATCTATGCAAGTGTTTGGGTGTTGAAACCCTTACGCGAAATG
                           *.** *. * .     .***..*


gi|11127911|gb| ------------------------------------------GTTCTGTATTTGAGGAC
```

```
gi|17226850|gb| ------------------------------------------------ACTTTGGAGAAG
gi|255966097|gb AAAGTGAACGAAGGTGGGAACCGCAAGGTGCACCATCGACCGATCCTGATGTTCTCGGAT
                                                                .. **   *.*

gi|11127911|gb| TCATTGGATAAGAAGGTTTTACTCCTATGGGTGAATTCTTCTATCCAGAAAGTTATAGGC
gi|17226850|gb| TGGTTCTATAATGAG----------------------CCACCCTCTAGAAATTTATAGGC
gi|255966097|gb GGATTTGAGTAGGAG----------------------CATAGCTGTTGGGACCCGAAAGAT
                  .**  *  * .**                      .  .  ...*.*  . * **..

gi|11127911|gb| AGTACTGGTTAGTTCTTCTGTACTGGGCGTAATAATTTACTTTTATTCTCGTCTAGAAAA
gi|17226850|gb| AGTA----TGGTTATATCTGAACTAGACGTAATATTAGCAAAATCGTCTTTTTAGGAATG
gi|255966097|gb GGTG-----AACTATGCCTGAATAGGGTGAAGC------------------CAGAGGAAA
                .**.      . * . .*** *. .*..* *..                 .  .*.* .

gi|11127911|gb| GGTGAATAGGTGCTTGCCTTGAACCCACAAGTTATGTTAA--CTGGGTGACCTCAGATCA
gi|17226850|gb| ATT------GTACTTGCCTCAACCC----ACTCATATTAAACTTGGGTGACCTCAGATCA
gi|255966097|gb CTCTGGTGGAGGCTCGCAGCGGTTCTGACGTGCAAATCGA--TCGTCAAATTTAGGTATA
                  .      . .**.**  ... .*    .  .* .*..*  ..*   .*..* .*  .*

gi|11127911|gb| GGTAGGGATACCCGCTGAACTTAA
gi|17226850|gb| GGTAGGGATACCCGCTGAACTTAA
gi|255966097|gb GGGGCGAAAGACTAATCGAACCAT
                ** . *.* . *.. * .* ..*
```

*All species have a similar nucleotide
.Two species have a similar nucleotide
Highlighted parts indicate sequences used to design primers

Phyllobacterium myrsinacearum **(AB681132.1)**

```
5’CGGGTGAGTAACGCGTGGGAATCTACCAACTCTACGGAATAACGCATGGAAACGTGTGCCGGGTGAGTAACGCGTGGGAATCTA
CCCATCTCTACGGAATAACGCATGGAAACGTGTGCTAATACCGTATACGTCCTTCGGGAGAAAGATTTATCGGAGATGGATGAGCC
CGCGTTGGATAATACCGTATACGTCCTTCGGGAGAAAGATTTATCGGAGATGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGG
TAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCA
TAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGSAKTGGGGATGATCAGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCASCCAT
GCCGCGTGAGTGATGAAGGCCCTAGATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGG
GTTGWAAAGCTCTTTCACCGGYGAAGATAATGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATG 3’
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Stenotrophoma maltophilia **(HM584272.1)**

```
5’CGGCAGCACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCCGGCAGCACAGAGGAGCTTGCTCC
TTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACC
GCATACGACCGGAATCTACTTTTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGG
GGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTATACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGAA
TGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGAGCTAGTTG
GCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
```

TTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTTGGACAATGGGCGCAAGCCTGATCC
AGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCCGGCTAATACCTGGTTGGG
ATGACGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCCGGCTAATACCTGGTTGGGATGACGGTACCCAAAGAATAAGCACC
GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGG
TAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTATTTAAGTCCGTTGTGAGCGTTACTC
GGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTTATTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATA
CTGGATGACTAGAATGTGGTAGAGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGATGACTAGAATGTGGTAGAGGG
TAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGGTAGCGGAATTCCTGGTGTAGCAGTGA
AATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGTGGGGAGCA
AACAGGGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATG
TTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGCACGCAGTATC
GAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT
GGAGTATGTGGTTTAATTCGATGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAA
CGCGAAGAACCTTACCTGGCAACGCGAAGAACCTTACCTG 3′

Leucobacter sp. (JN863503.1)

5′GGGATAAGCGCTGGARACGGYGTCTAATACTGGATATGTCCTATCACCGCATGGTGTGTAGGGATAAGCGCTGGAAACGGCGTC
TAATACTGGATATGTCCTATCACCGCATGGTGTGTAGGTGGAAAGAATTTTGGTTCGGGATGGACTCGCGGYCTATCWGCTAGATG
GKGAGGKAATGGTGGAAAGAATTTTGGTTCGGGATGGACTCGCGGCCTATCAGCTAGATGGTGAGGTAATGGCTCACCATGGGCTC
ACCATG 3′

Dietzia sp. (JN120941.1)

5′GAACTCCTGCCGMATGGTGGGGGTGAACTCCTGCCGCATGGTGGGGGT 3′

Staphylococcus cohnii (JN128237.1)

5′CAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTWWCACGTGGGTAACCAGATAAGGAGCTTG
CTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGAATAACTCCGGGAAAC
CGGGGCTAATGCCGGATAACATTTAGAACTACCTATAAGACTGGAATAACTCCGGGAAACCGGGGCTAATGCCGGAT
AACATTTAGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTACCGCAT
GGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGG
CTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGAT
ACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGAYACGGTCCAGACTCCTACGGGAGGCAGCAGTAG
GGATGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG
GCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGATCTTCCGCAATGGGCGAAAGCCTGACGGAG
CAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTAT
GCACGTCTTGACGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTAC
CTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGGTACCTAATCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGT
AGGCGGTTTCTTAAGTCTGATGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTC
TGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGRAAACTTGAGTGCAGAAGTGAAAGCCCACG
GCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTG
AAATGCGCAGAGATATGGAGGAACACCAGTGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATG
GAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACGGCG
AAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACC
GCAAGCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAA

AGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGTTGAAACTCAAAGGAATTGACGGGGACC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGGAAGCAACGCG 3’

***Pantoea dispersa* (JN391535.1)**

5’GAACGGCAGCACAGAAGAGCTTGCTCTTTGGGTGGCGAGTGGCGGACGGSTGAGTAATGTGAACGGCAGCACAGAAGAGCTTGC
TCTTTGGGTGGCGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAAT
ACCGCATAAYCTGGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCRAGACCAAAGT
GGGGGACCTTCGGGCCTCACACCATCGGATGTGCCCAGATGGGAGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCACACCATC
GGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATTAGCTAG
TAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGATATTGCACAATGGGCGCAAGCCTGA
TGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAATAACCTCGC
CGATTGACGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAATAACCTCGCCGATTGACGTTACCCGCAGAAGAAGC
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AACGCGAAGAACCTTACATGCAACGCGAAGAACCTTAC 3’

APPENDIX D: Survey questionnaire

Kalahari Truffle Survey – 2011

Thank you for participating in this survey, your input is greatly appreciated. Once completed please Email (J.dames@ru.ac.za) or Fax: 086 683 9595 this form back to Dr Joanna Dames, Rhodes University.

Name:	
Farm name:	
Location or GPS co-ordinates:	
Contact details: Postal address	
Email:	
Cell and/or Tel:	

1) At what time of the year do you generally expect to find truffles (tick the months)

March	April	May	June	July	Other

2) Describe the vegetation of the area where truffles are collected (you are welcome to email some photographs)

Dominant Vegetation	**Additional comments**

3) There are three types of truffles tick the box/es that describes the truffles that you find.

Scientific name	**Description**	**Tick**
1) *Kalaharituber pfeilii* (previously called *Terfezia pfeilii*)	Truffles surface brown. Flesh white marbled becoming yellowish to brown with white veins.	
2) *Mattirolomyces austroafricanus* (previously called *Terfezia austroafricana*)	Truffles surface pale to orange brown when dried. Flesh pale marbled with veins.	
3) *Eremiomyces echinulatus* (previously called *Choiromyces echinulatus*)	Truffles surface pale cream coloured to brown when dried. Flesh marbled with dark brown veins. Usually regarded as being inedible	

4) Are there any further comments you would like to make regarding the Kalahari truffles, or observations you have made.

Thank you for your time

Yours sincerely

Dr Joanna Dames

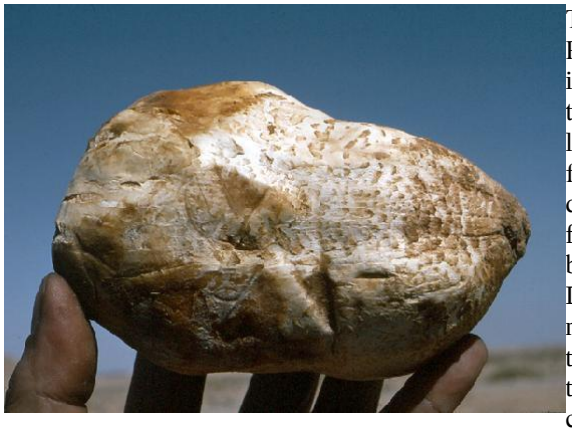
APPENDIX E: Internet refences

Terfezia and *Tirmania*, Desert Truffles (terfez, kama, p/faqa)
Delicacies in the sand or manna from Heaven?

Tom Volk's Fungus of the Month for January 2007 by Elinoar Shavit and Tom Volk

Please click TomVolkFungi.net for the rest of Tom Volk's pages on fungi

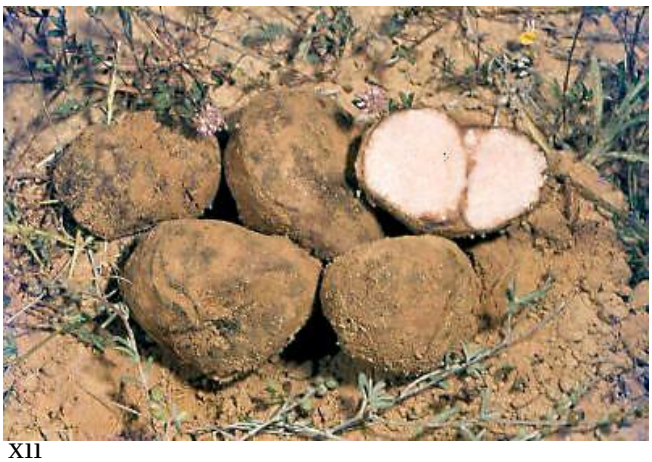
Welcome to the tenth anniversary edition of the Fungus of the Month! Since the first Fungus of the Month in January 1997 was a truffle (*Tuber gibbosum*, the Oregon white truffle), I decided on another truffle for this month's fungus. My good friend Elinoar Shavit, an Israeli native, has written most of this web page on the desert truffle, found in the Middle East and along the southern Mediterranean coast. I hope you will enjoy this tenth anniversary page and continue to enjoy ten years of Fungus of the Month pages. Thanks for visiting!



The famed 19th Century French gastronome and author of "The Physiology of Taste," Brilliant Savarin, once said, "The truffle is not exactly an aphrodisiac, but it tends to make women more tender and men more likeable." Quite an achievement for a little potato of a mushroom. Meanwhile, Robyn Dixon, writing for the LA Times, described truffles as "desiccated donkey dung." Both are passionate descriptions, but not of the same fungus. While Savarin was referring to the famed Périgord black truffle, a 'true truffle' (*Tuber melanosporum*), Robyn Dixon was describing a desert truffle, *Terfezia pfeilii*, or in its new name – *Kalaharituber pfeilii* Trappe. In the marketplace, the rare *Tuber melanosporum* is one of the most expensive of the eagerly sought after perishable items bought for human consumption. In the 'souks' of Damascus (Syria) and Beer-Sheva (Israel), one can get a pound of fresh desert truffles for the price of a good piece of steak. Passion aside, there is much more to desert truffles than their sandy first impression.

Truffles are hypogeous fungi, which means they form their fruitbodies below ground. Ecologically, they are mycorrhizal, forming mutually beneficial associations with the roots of plants. Taxonomically, they are members of the Ascomycota (having a saclike ascus that contains the ascospores). Like most other hypogeous fungi, desert truffles depend on animals to disperse their spores. Toward this end, the ripening fruitbodies omit a distinct aroma, which grows stronger as they mature. The aroma attracts a variety of animals (humans included) who eagerly collect and consume the truffles, and later disperse the spores to new areas, usually in their feces.

'Desert truffle' is a term used to refer to members of the genera *Terfezia* and *Tirmania* in the family Terfeziaceae, order Pezizales, which grow in arid and semi-arid areas of the Mediterranean region, the Arabian Peninsula, and North-Africa. Some have been found in South Africa and China. The photograph to the right was taken by Dr. Nissan Binyamini in the Israeli Negev Desert. Nissan Binyamini wrote the only mushroom guide for amateurs in Hebrew, and headed the Dept. of Botany at the Tel Aviv University for many years. Species of *Terfezia* and *Tirmania* prefer high pH calcareous soils, typical of desert soils.



Although the genera *Terfezia* and *Tirmania* are primarily ectomycorrhizal (forming a sheath around the roots of their host plant), they are highly adaptable. Some species, like *Terfezia arenaria*, *Terfezia claveryi*, and *Tirmania pinoyi*, form endomycorrhizal associations in phosphate-poor soils and ectomycorrhizal associations in phosphate-rich soils. Species of both genera form mycorrhizas on roots mainly of members of the genus *Helianthemum* (family Cistaceae), relatives of the North American rock rose, but can also form relationships with members of other families in the absence of species of *Helianthemum*. These relationships contribute to *Helianthemum's* adaptability to drought conditions and facilitate absorption of nutrients, particularly nitrates. This finding may provide an explanation for folklore shared by Bedouins in the Israeli Negev and truffle hunters in Morocco, claiming that truffles will grow where lightning strikes during thunderstorms. (more on this subject)

It has been shown that plenty of rain in the beginning of the rainy season is necessary to ensure a good truffle crop in spring. Even so, many truffle hunters, including the Bedouins of the Negev, believe that truffles appear suddenly, without seed or root, swollen by early season's rains, and loosened from their sandy bed by the loud rumblings of strong thunderstorms. These beliefs go back thousands of years. In 1st century C.E., Pliny the Elder (Gaius Plinius Secundus), wrote in his *Naturalis Historia*, Book xix, "Among the most wonderful of all things is the fact that anything can spring up and live without a root. These are called truffles (tubera). They are surrounded on all sides by earth, and supported by no fibers. There are two kinds: one is sandy and injures the teeth, the other without any foreign matter. Those of Africa are the most esteemed. Peculiar beliefs are held for they say that they are produced during autumn rains, and thunderstorms especially, and are best for food in the spring. They grow…where there is much sand." The Jewish Talmud (the record of rabbinic discussion of the Jewish law), echoed the same claim. Truffles and mushrooms are usually discussed together in the Babylonian Talmud (compiled and redacted in Iraq in the 5th Century C.E.). The Rabbis considering the issue concluded that truffles and mushrooms do not grow from the soil. Rather, they spontaneously appear in the soil. In one place it is said that, "they emerge as they are in one night, wide and round like rounded cakes".

The most common species of the genus *Terfezia* are *Terfezia arenaria* (*syn. T. leonis*), *T. boudieri*, *T. claveryi*, *T. leptoderma*, and *T. terfezioides* (=*Mattirolomyces terfezioides*) and although just separated - *T. pfeilii* (*syn. Kalaharituber pfeilii*). The most common species of the genus *Tirmania* are *Tirmania nivea* and *T. pinoyi (syn. T. africana)*. *Terfezia spp*. have spherical and ornamented spores, while *Tirmania spp*. have smooth spores and amyloid asci. The fruitbodies are round, tan to brown, and look like small, sandy potatoes. They are a few centimeters across, and weigh about 1-10 oz. The truffles produced by both genera are similar in overall look, and are not easy to tell apart. A simple iodine test can separate them. A drop of iodine on the cut flesh of *Terfezia* fruitbody will not change or turn yellow to orange. Cut fruitbodies of *Tirmania* will turn blue-green to black. The color of their gleba (the flesh of the fruitbodies) depends on the species, and its shade can be white to light cream, cream to yellow, and even pink. At this stage the spores are unripe, and they will ripen once the truffle matures, usually above the ground in the sun. The gleba is then much darker and can even be black. Their unique flavor develops as they reach maturity.

There is a close genetic relationship between species of *Tirmania* and *Terfezia*, and they may have arisen from a single evolutionary lineage of fungi that adapted to the heat and drought by growing its fruitbodies underground. *Terfezia spp*. generally prefer semi-arid habitats, and *Tirmania spp*. are more adapted to arid deserts, but species of both genera often share the same habitat, and even the same *Helianthemum* host, forming their fruitbodies around it. This may show that the initiation of a fruitbody by one species is not inhibited by the initiation of another fruitbody close by. The truffles of both genera grow at the end of the rainy season, in spring, usually between March and June.

Desert truffles are called by a number of different names and by variations of these names. They are called *terfez* or *terfas* in

Morocco, Libya, and Egypt. Since truffles were shipped from North Africa to the Roman Empire for many years, *terfez* may be the source of the scientific name for the genus *Terfezia*. The white *Tirmania nivea* is called *zubaydiya* in Saudi Arabia and Oman, and the Nama people of Namibia call the Kalahari truffle: "*! / nabba"* (the "!" stands for a click of the tongue on the upper palate, combined with an 'N' sound). However, the most prevalent names by which desert truffles are called all over the Arabian peninsula and the Eastern shores of the Mediterranean, are variations on the words *kama* and *faqa*. These are ancient terms which have been used in the region for thousands of years, and still refer to truffles in modern Arabic, Hebrew, and Aramaic. The term *'pagua'* is even mentioned in the Bible.

Even though they are formed underground, the fruitbodies of desert truffles are not very deep and can be visible to the trained eye. They are connected to their rhizomorphs and to their host's roots by a stalk-like formation made of hyphae. It is thin, but some species form a thick 'stem' that can reach a length of 40cm. Local truffle hunters believe that this stem pushes the truffle out of the ground. In his book, "The Bedouins and the Desert", Jibrail Jabbur writes, "If left ungathered, the white truffle [*Tirmania nivea*] grows underground by leaps and bounds until it bursts through and appears on the surface of the ground. When it has reached its maximum size, it sometimes rolls out of the mouth of its little 'volcano' and its skin begins to wrinkle…in the sun". Exposed ripe desert truffles spoil within a few hours, and are often overtaken by insect larvae. Truffle collectors hunt for them in the early hours of the morning. The sun light reflecting off of the dew drops on the sand particles helps detect the cracked bumps in the sand, which are the tell tale signs of a truffle emerging. Because desert truffles grow so close to the surface, there is no need to use pigs or trained dogs to find them. Experienced truffle collectors can detect an emerging truffle from a distance, and often return season after season to the same areas, keeping the locations of their favorite spots a secret.

Where desert truffles grow, people eagerly await their appearance. They are collected for their unique flavor, nutritional value, and medicinal properties. For these reasons, they have always been in demand, making them a lucrative cash crop for local populations. In the past few years, the demand for desert truffles in Europe has been growing rapidly. It occupies a special niche, and benefits from the growing Middle Eastern population in Europe and from the Europeans' traditional passion for truffles. *Terfez* from North Africa reach the European markets each spring, and even the Kalahari truffle has a market in Germany. Irrigation of the native truffle producing areas in times of drought could help secure a more predictable supply of truffles to the growing markets. Efforts have been made to cultivate desert truffles in Turkey, Israel, Saudi-Arabia, Namibia and other places within their growing areas, but so far with only modest success.

Truffles have always been a popular delicacy. *Kamma* were craved by rulers of Mesopotamia. In the excavations of the 4000 year old Amorite palace in Tel-Hariri (eastern Syria), archeologists found remnants of truffles still in their special baskets as well as mentions of them in the palace's inventory lists. The Pharaohs of ancient Egypt cherished them as food… fit for the Pharaohs, and arranged for large quantities to be brought to their palaces. The Roman Emperors regularly imported massive quantities of *terfez* from Libya and Greece. In the Jewish Mishna (first recording of the Jewish oral law, redacted around the 2nd Century C.E.), truffles ('kmehin' in plural) symbolize plenitude and Devine reward, as presented by the 1st Century B.C.E. story of Honi the Circle Drawer.

The Bedouins have always held truffles in high esteem, and consider them a special gift. Their folklore includes stories about the contribution of truffles to Bedouin life. In his travels across the deserts of Arabia, Jibrail Jabbur observed Bedouins collecting their most appreciated truffle, the White Truffle, Terfezia nivea. In "Bedouins and the Desert" he writes, "its skin and pith are both white, its pith is softer, neither as firm nor as round as the brown and black truffle. The Bedouin calls them Shaykh [the leader of a Bedouin tribe] and when the Bedouin girl is collecting truffles she sings to them…I personally saw a white Shaykh… and dug it out with my own hands. It weighed more than 1,400 grams [about 3 lbs] and measured 24 cm [about 10 inches] in diameter. From a distance I thought it was a large rock, since it lay exposed on the surface of the ground, as white truffles generally do when they are fully mature".

The Bedouins of the Negev use truffles for food, as a cash crop and as medicine for a variety of ailments. They dry them and use the flour for stomach ailments, and open cuts. They use the juice of the truffles to treat eye infections. They were recommended by the Prophet Muhammad (in the 7th Century C. E.), especially for problems of the eyes. It is written in the Tirmidhi Hadith (No. 1127), "When some companions of Allah's Messenger (peace be upon him) remarked to him that truffles were the smallpox of the earth, he replied, "Truffles are a kind of Manna, which Allah the Glorious and Exalted sent down upon the people of Israel, and their juice is a remedy for the eyes". The Bedouins in the Negev do not allow truffles anywhere near fermenting milk when preparing *samene* (fermented butter, similar to gee). They say that it will stop the delicate process of fermentation. Antibacterial substances have been isolated from the juice of a variety of desert truffles. In 2004, a Jordanian research team isolated a promising peptide antibiotic from the juice of *Terfezia claveryi*, which effectively inhibited the growth (in vitro) of *Staphylococcus aureus* by 66.4%.

Desert truffles are nutritious, and particularly high in protein. In good seasons, truffles are dried and ground to powder to supplement the regular diet. Even though the unique aroma of the truffles cannot be preserved by drying, the nutritious flour is added to a mixture of flatbread, which is then baked and eaten with honey. In times of famine, people have been known to rely on truffles. Particularly compelling is the story of an Iraqi woman who describes bad times during the 1970's in Baghdad, when her family and neighbors could not get food for months. According to the Iraqi woman, one year truffles were so plentiful that people prepared them in the same way they would normally prepare meat. They ate desert truffles every day for four months, cooking them in every imaginable manner. Yet at the end of this period, she had not tired of them, still finding them nutritious and tasty.

Traditionally, desert truffles are cooked simply, so as not to mask their delicate aroma. The oldest way, which is still very popular today, is to roast them in the embers of the fire. Truffles are also baked, sliced and fried in butter. They are made into fragrant soups, usually with camel's milk. A very popular dish, served at souks and restaurants all over the Middle East, is scrambled eggs with desert truffles, served in a pita-pocket. (recipes with desert truffles)

In a few months, the time will be right to travel to Morocco, Israel, Turkey or Namibia, to collect desert truffles. But before you pack your bag, consider this: Truffle hunters in Namibia carry a special 'snake stick' to ward off poisonous adders when they go truffle hunting. Poisonous snakes have always been a problem probably because both truffles and snakes prefer the damper areas in the shade of the plants. A famous Rabbi who lived in Iraq in the 4th century C.E., warned consumers to stay away from truffles that have holes (probably caused by insect larvae). The reason he gave: those holes could be the teeth marks of venomous snakes!

This month's co-author is Elinoar Shavit, a renowned gemologist by trade. She was born in Israel and now lives near Boston. She has an M.A. in Organizational Psychology from Columbia University, and she is a past President of the New York Mycological Society. She has been collecting mushrooms practically from birth, first with her grandmother, then with her mother, and now with her family and friends. In the past few years she has been traveling in the US and abroad, following the growing seasons of morels, truffles and other edible mushrooms, to collect the stories of mushroom hunters, which she intends to publish in a book. You can email her at Elinoar@elinoarshavit.com

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I hope you enjoyed learning about *Terfezia* and *Tirmaina*, the desert truffles. There's always something fun to find if you visit another part of the world. I hope to visit some day to taste these fungi. All we get here are chocolate "dessert truffles" instead of the more delicious "desert truffles."

Learn more about fungi! Go to Tom Volk's Fungi Home Page --TomVolkFungi.net

Return to Tom Volk's Fungus of the month pages listing

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joerg lehmann *Getty*

Images

Truffles

The Hidden Life of

biology

Not just for gourmands, truffles play essential roles in the health of ecosystems

By James M. Trappe and Andrew W. Claridge

These fleshy organs are temporary reproductive structures that produce spores, which eventually germinate and give rise to new offspring. What sets truffles apart from mushrooms is that their spore-laden fruit forms below ground rather than above. Technically, true truffles are those fungi that belong to the Ascomycota phylum of organisms and are marketed as food. But there are trufflelike fungi or "false truffles" in the phy-

Key Concepts

- The truffles that appear on restaurant menus and on the shelves of luxury food purveyors represent only a small fraction of the world's truffle species.
- Truffles figure importantly in ecosystems, sustaining both plants and animals.
- Recognition of the ecological significance of truffles is aiding efforts to conserve threatened species that depend on them.

—The Editors

It's a cool November day near Bologna, Italy.

We are strolling through the woods with truffle hunter Mirko Illice and his little dog, Clinto. Clinto runs back and forth among the oak trees sniffing the ground, pausing, then running again. Suddenly, he stops and begins to dig furiously with both paws. "Ah, he's found an Italian white truffle," Mirko explains. "He uses both paws only when he finds one of those." Mirko gently pulls the excited dog from the spot and pushes through the soil with his fingers. He extracts a yellowish brown lump the size of a golf ball and sniffs it. "*Benissimo*, Clinto," Mirko intones. Though not the finest example of the species, *Tuber magnatum*—which grows only in northern Italy, Serbia and Croatia—Clinto's find will fetch a nice price of about $50 at the Saturday market.

Throughout history, truffles have appeared on the menu and in folklore. The Pharaoh Khufu served them at his royal table. Bedouins, Kalahari Bushmen and Australian Aborigines have hunted them for countless generations in deserts. The Romans savored them and thought they were produced by thunder.

Modern epicures prize truffles for their earthy aroma and flavor and are willing to pay steep prices at the market—recently more than $3,000 per kilogram for the Italian white variety. Yet despite humanity's abiding interest in the fungi, much about their biology has remained veiled in mystery. Over the past two decades, however, genetic analyses and field observations have clarified the origins and functions of these organisms, revealing that they play key roles in many ecosystems. These findings are informing strategies for conserving some endangered species that rely on these denizens of the underworld.

A Fungus among Us

Truffles, like mushrooms, are the fruit of fungi.

James M. Trappe is scientist emeritus at the U.S. Forest Service and a professor of forest science at Oregon State University. He has discovered more than 200 new truffle species on five continents. Jim wonders why anyone would go fishing instead of seeking new truffles. Andrew W. Claridge is a senior research scientist with the New South Wales Department of Environment, Climate Change and Water and a visiting fellow at the University of New South Wales in Australia. He has studied interrelationships among mammals and the fungi they eat for more than 20 years. His favorite hobby is fishing.

All truffles and mushrooms produce networks of filaments, or hyphae, that grow between plant rootlets to form a shared absorptive organ known as a mycorrhiza. Thus joined, the fungus provides the plant with precious nutrients and water, its tiny hyphae able to reach into pockets of soil inaccessible to the plant's much larger roots. The plant, in turn, furnishes its consort with sugars and other nutrients that it generates through photosynthesis—products that the fungus needs but cannot produce on its own because it does not photosynthesize. So beneficial is this partnership that nearly all trees and other woody plants require it for survival, as do the associated fungi.

Most herbaceous plants (those that do not have a permanent woody stem aboveground) form mycorrhizae too, albeit with different fungi. Many fungal species, including all the ones that yield truffles, form a variant of the mycorrhiza called an ectomycorrhiza, in which the fungus envelops the feeder rootlets with a protective outer tissue. The diversity of these ectomycorlum Basidiomycota that function like true truffles. Given these similarities, we refer to all fleshy fungi that fruit underground as truffles.

Scientific efforts to expose the secrets of truffles date to the 1800s, when German would-be truffle growers asked botanist Albert Bernhard Frank to figure out how the delicacies propagated. Frank's studies revealed that the fungi grow on and into the tiny feeder rootlets that trees use to absorb water and nutrients from the earth. On the basis of those observations, he proposed that the organisms have a symbiotic relationship in which each provides nutrients to the other. He further posited that such relationships between subterranean fungi and plants are widespread and that they shape the growth and health of many plant communities. Frank's theories contradicted conventional wisdom about truffles and other fungi—namely, that they all brought about disease and rot in plants—and drew considerable opposition from his peers. But although nearly a century would pass before scholars had definitive evidence, Frank got the story right.

lucy Reading-Ikkanda; Courtesy of Efren Cazares (*Trappe*); Courtesy of Debbie Claridge (*Claridge*)

an alternative dispersal system. And therein lies the complexity of the truffle's scheme. Over millions of years, as truffles retreated underground, mutations eventually led to the formation of aromatic compounds attractive to animals. Each truffle species has its own array of aromatics that are largely absent in immature specimens but intensify and emerge as the spores mature. Of the thousands of kinds of truffles that exist today, only a few dozen appeal to humans. The rest are too small or too tough, or they possess aromas that are unremarkable or downright repugnant. To other animals, however, they are irresistible, their olfactory charms wafting up from the soil. Small mammals such as mice, squirrels and rabbits in the Northern Hemisphere and rat-kangaroos, armadillos and meerkats in the Southern Hemisphere are the main truffle gourmands. But their larger counterparts—deer, bears, baboons and wallabies, among others—also seek out the undercover fungi. Mollusks are attracted to truffles, too. And insects may feed on truffles or lay eggs in them so that their larvae have a ready food source when they hatch.

When an animal eats a truffle, most of the flesh is digested, but the spores pass through unharmed and are defecated on the ground, where they can germinate if the conditions are right. This dispersal system has advantages over the one that mushrooms employ. Feces concentrate spores, in contrast to the more diffuse scattering that occurs with aerial dissemination. In addition, feces are more likely to be deposited in the same kinds of areas where the animals forage for truffles, as opposed to the more random transport of airborne spores. This similarity of environment is beneficial because it increases the likelihood that the spores will land in a spot that has an appropriate plant species with which to establish a mycorrhiza.

Not all truffles rely on scent to attract animals, however. In New Zealand, which lacks native terrestrial mammals, some truffles have evolved rainbow hues that mimic the colors of fruits prized by the local birds. The *Paurocotylis pila* truffle, for one, emerges from the ground as it expands and lies on the forest floor, resembling the plump, red berrylike base of the seeds of *Podocarpus* trees that are a favorite bird food. (Although these colorful fungi do poke above the ground, they are nonetheless considered truffles because their spore-bearing tissues are enclosed in a skin, and they thus depend on animals to disperse their spores.)

Yet another dispersal mechanism has evolved

rhizal fungi is impressive: one of us (Trappe) estimates that some 2,000 species are associated with the Douglas-fir (an evergreen used for timber and Christmas trees), and probably as many or more types partner only with Australia's eucalyptus trees. Numerous other commercially and ecologically important tree species also rely on ectomycorrhizal fungi. Most of these fungi fruit aboveground as mushrooms, but several thousand species produce truffles.

Going Under

Comparisons of the morphology and gene sequences of truffle and mushroom species indicate that most truffles have evolved from mushrooms. But given that truffles require aboveground dispersal of their spores to propagate, why would natural selection favor the evolution of species that hide underground? Consider the reproductive tactic of mushrooms. Although mushrooms exhibit a multitude of structures and colors, they all have fruiting bodies that can discharge spores directly into the air. The airborne spores may then alight nearby or far away to germinate and potentially establish a new colony in association with the roots of a compatible plant host. It is a highly effective approach.

The mushroom strategy is not foolproof, however. Most mushrooms have little defense against environmental hazards such as heat, drying winds, frost and grazing animals. Every day a few spores mature and are discharged. But if inclement weather dries or freezes a mushroom, spore production usually grinds to a halt.

Where such hazards are commonplace, new evolutionary adaptations have arisen. The most successful alternative has been for the fungus to fruit underground. Once the soil is wet enough for the subterranean fruiting body to form, it is insulated from vagaries of weather. The truffle develops with relative impunity, continuing to produce and nurture its spores even when aboveground conditions become intolerable to mushrooms.

At first glance, the truffle's solution might seem facile. The form of a truffle is visibly less complex than that of a mushroom. No longer does the fungus need to expend the energy required to push its spore-bearing tissues aboveground on a stalk or develop a cap or other structure for producing and releasing the spores. The truffle is but a lump of spore-bearing tissue, usually enclosed by a protective skin.

The problem is that the truffles cannot themselves liberate their spores, trapped as they are in their underground realm. That feat demands

truffle eaters

The animals below are among the many that dine on truffles.

Courtesy of Ben Wrigley *National Parks and Wildlife Service* (*long-footed potoroo*); Millard H. SharP *Photo Researchers, Inc.* (*meerkat*); Christophe Courteau *Nature Picture Library* (*kiwi*); Nigel J. Dennis *Photo Researchers, Inc.* (*baboon*)

Lucy Reading-Ikkanda (*map*); Courtesy of james m. Trapp e (*M. glauca*); Courtesy of Matt Trapp e (*R. truncatus*); Courtesy of Michael Castellano and James m. trapp e (*P. pila*)

Southern Hemispheres, despite taking place long after the continents separated. The host plants in these regions are entirely different: whereas pines, beeches and oaks, for instance, partner with truffles in the north, eucalyptus and southern beeches play that role in the south. The truffle and animal species are likewise distinct between hemispheres. Yet the ecosystems and their components—the trees, truffles and animals—function in much the same way.

The greatest known diversity of truffles occurs in temperate areas of Mediterranean Europe, western North America and Australia (although most of Asia, Africa and South America remain unexplored by truffle researchers). These areas have climates with cool, rainy winters and warm, dry summers. Their fungal fruiting seasons are usually spring and autumn, when weather tends to be erratic: some years bring warm, dry spells and others deliver frost; both conditions are inimical to mushrooms. Over time, then, natural selection favored those fungi that sought refuge underground in these regions.

Exactly when the first truffles evolved is uncertain, but scientists have unearthed some clues to their origins. The oldest fossil ectomycorrhizae on record date to around 50 million years ago. And the ancestors of today's pines and other with a few groups of truffles, notably members of the ubiquitous Elaphomycetaceae family and the Mesophelliaceae family, which is endemic to Australasia. Their spores mature into a powder rather than a fleshy, spore-bearing tissue. The powder of *Elaphomyces granulatus*, for instance, is enclosed in a thick rind that is eaten by the animal, freeing the spores. Some of the Mesophelliaceae have a similar structure; others, such as *Mesophellia glauca*, possess a powdery spore mass sandwiched between a thin, hard outer rind and an edible inner core.

Even the spores of uneaten truffles can wander. After maturation, they decay into a slimy, larva-infested suspension in the soil. Invertebrates feed on this rotting tissue or move through it, picking up spores along the way. Truffle spores also travel when predators capture a small truffle-eating species: owls and hawks may carry rodents full of truffles considerable distances to their nests or roosts, where they eat the prey whole or eviscerate and discard the entrails. Either way the spores return to the soil where they may give rise to new truffles.

Together Forever

Evolution's experiments with truffles have been remarkably similar in both the Northern and

invaders and impostors

The black Perigord truffle is under threat from an invader: the Chinese black truffle. Researchers had long been worried that the hearty and adaptable Chinese truffle could spread to the domain of the more finicky Perigord truffle and possibly outcompete it. In 2008 Claude Murat of the University of Torino and his colleagues reported that this fear had been realized: the team detected DNA from both the Perigord truffle and the Chinese truffle in root tips and soil from an Italian truffle plantation. Dishonest purveyors sometimes try to disguise the much more common Chinese truffle as its rarer and tastier counterpart by mixing small amounts of Perigord truffle in with the Chinese variety to give the latter the right scent. DNA analysis has been used to identify Chinese truffles masquerading as the Perigord kind.

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j. n. reichel *Photo Researchers, Inc.*

with imported truffle oil, which he used because it was readily available for purchase, what might the figures be once the odors of native Australian truffles are put to the test? Answering that question is a top priority for his team.

To protect these endangered marsupials and other animals that regularly eat truffles, conservationists will have to ensure the availability of their food. This provision applies not only to those animals that depend directly on truffles but also to their predators. Thus, restoring the habitat of the threatened northern spotted owl in the Pacific Northwest requires meeting the needs of the owl's primary prey, the northern flying squirrel, which eats mostly truffles.

Taming the Truffle

Although researchers have learned much about the ecology of truffles in recent decades, the science of growing them has changed little since the 1960s, when French scientists developed a greenhouse technique for adding spores of the black Perigord truffle into the potting mix of oak and hazel seedlings that are later planted in suitable sites to form truffle orchards, or *truffières.* Under ideal conditions, the *truffières* can produce a crop in four to five years.

After many failed attempts, similar *truffières* were finally established in the U.S. in the 1980s. Today the most productive truffle grower in North America is Tom Michaels of Tennessee Truffles. A former graduate student of Trappe's, Michaels produced an impressive 100 kilograms of Perigord truffles in the 2008–2009 season. To get these results, he pays careful attention to the soil, adding lime every year to keep it friable and well drained. New Zealand and Australia have succeeded in growing Perigord truffles, too.

In stark contrast to the triumphs of Perigord truffle farming, efforts to cultivate the most highly prized truffle species—the Italian white truffle that Mirko and Clinto were hunting, which has an especially intense aroma—have failed. For reasons that remain unknown, this species simply refuses to grow in the greenhouse. To that end, the sequencing of its genome, which is nearing completion, could yield clues to how to coax the king of truffles to grow on command.

Concurrently, truffles may become more prevalent even without cultivation: as the earth warms, the hotter, drier habitats that many truffles favor will spread, setting the stage for increased production and accelerated evolution.

Climate change, then, may yield a benefit for some: more truffles for men and beasts. ■

trees with which truffles form essential relationships arose some 85 million years ago. We can assume, then, that truffles emerged sometime between 85 million and 50 million years ago.

Given this long-standing association between truffles and plants, it is no surprise that the fungi figure importantly in the ecology of many habitats. Not only are they essential to the functioning of numerous plant species, but animals have come to rely on them for food. In the U.S. at least one creature, the Western red-backed vole, depends almost entirely on truffles for sustenance. And the northern flying squirrel, found in North America, eats mostly truffles when available in the wild. On the other side of the globe, in Australia, a marsupial known as the long-footed potoroo subsists on a diet that is about 95 percent truffles. Its fellow marsupials the other rat-kangaroos and bandicoots also bank heavily on truffles. And many other creatures the world over routinely supplement their primary food sources with these fungi.

Scientists' developing knowledge of the intimate relationship between truffles, their plant hosts and their animal carriers is guiding the efforts of cultivators and conservationists alike. In the 1980s in Oregon, Mike Castellano of the U.S. Forest Service, Mike Amaranthus of Mycorrhizal Applications and their colleagues began outfitting nursery seedlings with spores of hearty *Rhizopogon* truffle species to help the seedlings withstand drought and other stressful conditions in plantations. Going forward, cultivators could conceivably augment their returns if they substituted gourmet truffles for *Rhizopogon.* For example, Christmas tree farms in the Pacific Northwest could additionally produce the delicious Oregon white truffle, *Tuber gibbosum.*

Thus far, however, attempts to inoculate trees with this truffle species have produced inconsistent results.

Meanwhile one of us (Claridge) had been using truffles to help determine the population sizes of endangered animals in southeastern Australia—a prerequisite to developing effective protection or recovery programs for these species. He soaks foam pads in olive oil infused with aromatics of the European black Perigord truffle (a favorite of humans) to attract potoroos and other truffle-loving marsupials to stations where they are photographed by motion-sensing digital cameras. This approach has enabled him to detect upward of 50 times as many of these creatures as are counted with the traditional method of cage trapping. If the success rates are this high

➥More To Explore

Field Guide to North American Truffles: Hunting, Identifying, and Enjoying the World's Most Prized Fungi. Matt Trappe, Frank Evans and James M. Trappe. Ten Speed Press, 2007.

Taming the Truffle: The History, Lore, and Science of the Ultimate Mushroom. Ian R. Hall, Gorton T. Brown and Alessandra Zambonelli. Timber Press, 2007.

Trees, Truffles, and Beasts: How Forests Function. Chris Maser, Andrew W. Claridge and James M. Trappe. Rutgers University Press, 2008.

Fast Facts

Black Perigord truffles contain androstenol, a sex hormone found in the saliva of male pigs. The compound is also found in the sweat glands of humans.

Truffle hunters have long used female pigs to locate the fungi underground, but increasingly they are turning to dogs for assistance because the dogs are more willing than the pigs to accept an alternative food reward for their efforts.

Most commercially available truffle oils are flavored synthetically with lab-made compounds such as 2,4-dithiapentane, one of many molecules that give Italian white truffles their distinctive aroma.

Some truffles contain compounds that have potent antituberculosis effects; others exhibit strong anti-inflammatory and anti-oxidant properties.