DETERMINING FACTORS THAT CONTRIBUTE TO THE PROPAGATION, GROWTH AND ESTABLISHMENT OF *BURKEA AFRICANA* TREES

by

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DETERMINING THE FACTORS THAT CONTRIBUTE TO THE PROPAGATION, GROWTH AND ESTABLISHMENT OF BURKEA AFRICANA

I declare that this thesis is my own work and that all the sources I used or quoted have been indicated and acknowledged by means of complete references.

_____ _____ _**15 May 2018**_____

SIGNATURE **DATE**

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DEDICATION

This PhD is dedicated to my mom Vhahangwele Nemadodzi, who refused to attend my MSc graduation and said she will only attend my PhD graduation. Mom, I love you, and yes I wanted to show you that I will make you proud by graduating the highest degree, and I cannot wait for you to witness your daughter graduating the highest degree! I also dedicate this to my son, Tendani Eugene Nemadodzi, never permit anyone to tell you that you can't do this or that, whatever you want to achieve in life is completely dependent on you!! I hope this shows you (Tendani Eugene Nemadodzi) that it all seems impossible until it's done!!

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LIST OF ABBREVIATIONS

A. nomius	Aspergillus nomius
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ARC-ISWC Climate	Agriculture Research Council- Institute of Soil, Water and
ATP	Adenosine triphosphate
B	Boron
B. africana	Burkea africana
BLAST	Basic Local Alignment Search Tool
BMRB	Biological Magnetic Resonance Bank
C	Carbon
Ca	Calcium
CH_3OH-d_4	Deuterated Methanol
Cl	Chlorine
cm	Centimeter
CO_2	Carbon dioxide
Cu	Copper
D_2O	Deuterium Oxide
DNA	Deoxyribonucleic acid
Fe	Iron
G	Gram
GA	Gibberellic acid
GC/MS	Gas Chromatography/ Mass Spectrometry

GPM	Growth-promoting metabolites
H	Hydrogen
H_2O	Water
ha	Hectare
HCI	Hydrochloric acid
HMDB	Human metabolome database
IAA	Indole-3-acetic acid
ITS	Internal transcribed spacer
K^+	Potassium
Kg	Kilogram
KH_2PO_4	Potassium phosphate
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
M	Meter
Mg	Magnesium
MHz	Megahertz
Min	Minute
ml	Milliliter
Mn	Manganese
Mo	Molybdenum
MS/MS	Mass Spectrometry/Mass Spectrometry
N	Nitrogen
N_2	Nitrogen gas
Na	Sodium

NaOD	Sodium hydroxide-d
$NH4^+$	Ammonium
Ni	Nickel
$NO3^-$	Nitrate
Nod	Nodulation
O	Oxygen
OPLS-DA	Orthogonal partial least squares discriminant analysis
P	Phosphorus
PCA	Principal Component analysis
PCR	Polymerase chain reaction
PGPR	Plant Growth-Promoting Rhizobacteria
pH	potential of hydrogen
Pl. richardsiae	Pleurostomophora richardsiae
PPM	Part per million
P-Values	Calculated probability
rRNA	Ribosomal ribonucleic acid
S	Sulfur
SAS	Statistical analysis software
SEM	Standard error of the mean
SOM	Soil organic matter
sp	species
TSP	Trimethylsilylpropanoic acid
Zn	Zinc
^{1}H-NMR	Hydrogen Nuclear Magnetic Resonance

µl	Microliter
µg	Microgram
°C	degrees Celsius

PREFACE

Scientific publications in preparation:

Nemadodzi, L.E., Vervoort, J. and Prinsloo, G. (2017). Differences in the Metabolomic Profile and Chemical Composition of *Burkea* and non-*Burkea africana* Soils

Nemadodzi, L.E., Vervoort, J. and Prinsloo, G. (2017). The microbial diversity that plays a role in discrimination between *Burkea* and non-*Burkea* soils and their effect on growth of *Burkea africana* trees.

Local conferences

Nemadodzi, L.E., Vervoort, J. and Prinsloo, G. (2016). ^{1}H –NMR based metabolomic profile of the soil surrounding *Burkea africana* trees. Diamond route national conference, Johannesburg, South Africa.

Poster presentation

Nemadodzi, L.E., Vervoort, J. and Prinsloo, G. (2017). Investigation and identification of microbial diversity that play a role in discrimination between *Burkea* soils and non-*Burkea* soils and the effects they have on the growth of *Burkea africana* trees.

Nemadodzi, L.E., Vervoort, J. and Prinsloo, G. (2016). ^{1}H –NMR based metabolomic profile of the soil surrounding *Burkea africana* trees. Bloemfontein, South Africa.

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GENERAL ABSTRACT

Burkea africana Hook. (wild syringa) is an average sized leguminous tree, 10-12 m in height occasionally reaching over 20m. This monotypic genus is dominant and co-dominant in Zambia, and is present throughout Africa as far north as Ethopia and west to Nigeria, and south to South Africa especially Limpopo, North West, Gauteng and Mpumalanga. It inhabits dry, non–calcareous sandy soils in savanna and woodlands up to 1500 m altitude or gentle slope of 1080 m elevation. *Burkea africana* produces a relatively large number of seeds, which is unusual for a resprouting species. Several studies conducted on *B*. *africana* trees paid more attention to the medicinal attributes, however little or nothing is known regarding the factors and dynamics that contribute to the growth and existence of these trees, particularly because these trees grow naturally in nutrient-poor savanna soils. Although *B. africana* trees have been in existence for a very long period of time, propagating it through thinning and transplanting of seedlings for regeneration and/ or re-establishment of seedlings to survive until sexual maturity still remains a mystery. It is hypothesized that factors controlling establishment and development of *B*. *africana* trees are related to microbial activities in the soil, very complex and species specific but poorly understood. This study aimed to identify, if there is a symbiotic relationship between the soil and mycorhizal fungi, and rhizobium bacteria or other growth stimulating activities, in the *Burkea* soils, which will accelerate and assist effective growth of *B. africana* trees to reach reproductive stage and produce pods without dying.

The chemical composition of *Burkea* soil and non-*Burkea* soils was analysed using HCl extraction method.). The results indicated the similar values ($p>0.05$) were observed for all micro and macro minerals as well as total nitrogen, pH and organic matter. However, total ions nitrate and ammonium concentration levels of *Burkea* soils were higher ($p<0.05$) than those found in non-*Burkea* soils.

The use of advanced metabolomics tool using ^{1}H-NMR was used to determine and identify soil metabolites which may be responsible for successful growth and establishment of the *Burkea africana* trees. The findings of this study indicated that metabolomic analysis showed different metabolites in the respective soils. Growth-

promoting metabolites (GPM) such as trehalose and betaine were found to be in higher concentrations in the *Burkea* soils. Conversely, acetate, lactate and formate, were found in higher concentrations in the non-*Burkea* soils.

Furthermore, LC-MS was used to determine the soil components present in *Burkea* soil as compared to non-*Burkea* soil using. The results indicated that a total of 22 compounds consisted of essential amino acids such as phenylalamine, threonine, tryptophan, leucine, isoleucine and lysine; conditional essential amino acids such as arginine, cysteine, glycine, glutamine, proline and tyrosine; non-essential amino acids such as citruline, alinine, aspartic acids, asparagine, glutamic acid and serine; nucleobased amino acids such as guanosine, adenine, adenosine, cytindine; dicarboxylic acid such as fumaric acid as well as common non-proteinogenic amino acids such as 4-hydroxyproline compounds were found in both *Burkea* and non-*Burkea* soils.

The study investigated the microbial communities in the soil where *Burkea africana* trees grows successfully (*Burkea* soils) and how it varies from the soils where they do not grow (non-*Burkea* soils). DNA was extracted from the soil and a high throughput sequence bask local assignment search tool (BLAST) was used to analyze the microbial diversity (bacterial and fungal) and composition found in both soils, for a comprehensive understanding of the soil microflora. The results revealed that *Penicillum* sp is prevalent in *Burkea* soils and was the main discriminant between the two soils. On the contrary, non-cultured fungi, which could not be identified, dominated the non-*Burkea* soils. The variances in soil composition suggests that species supremacy play a role in the growth of *B. africana* trees.

Lastly, the current study investigated and also identified what attracts caterpillars known as *Cirina forda* to invade and feed on *B*. *africana* trees. In addition, to determining if there is a symbiotic relationship between the plant-growth metabolites; growth-promoting fungi (*Penicilium* sp) and the caterpillars. The results of the study, revealed that the fungus *Pleurostomophora richardsiae* was predominant in the leaves of *B*. *africana* trees as well as in the caterpillars. It is proposed that *Pl*. *richardsiae* is a volatile compound which attracts caterpillars and makes *B*. *africana* trees susceptible

to caterpillars' outbreaks. The second largest percentage of fungi found in the caterpillars was *Aspergillus nomius*.

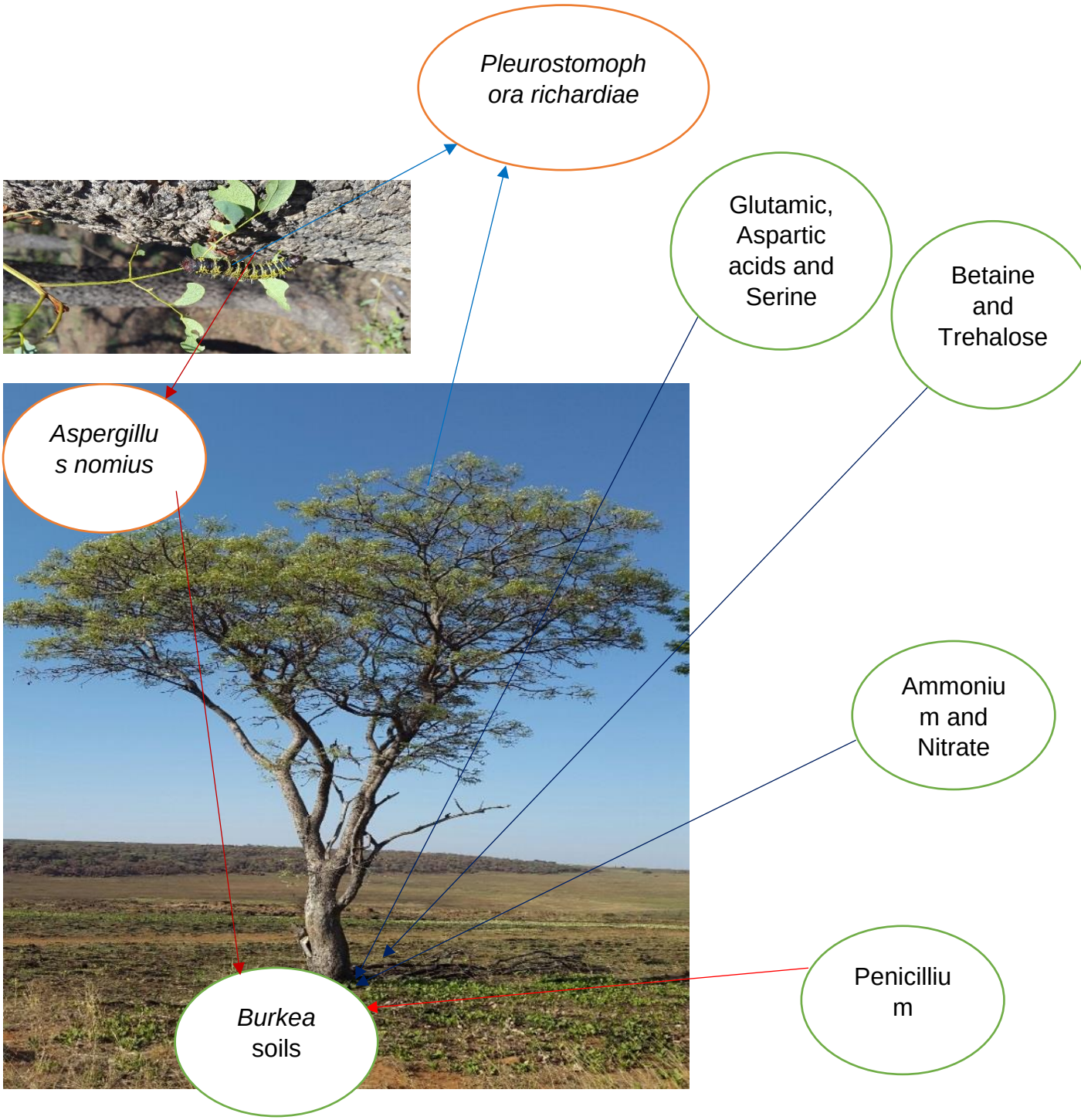

Figure 0: A representative of Burkea soils and their composition.

CHAPTER 1

1.1. GENERAL INTRODUCTION

Trees, which generally live longer than human generation, experience a broad range of environmental conditions during their lifetime, however, a detailed knowledge about their functional attributes or the history of environmental conditions they have experienced is not known (Burke, 2005). One of the main problems facing trees is the allocation of energy to somatic growth, defence mechanisms and reproductive potential where regeneration opportunities/safe sites are available (Clark, 1991a). A study conducted revealed that plant allocations are governed by a combination of broad-scale bioclimatic and historic factors, as well as the local conditions at a particular site (Barbour *et al*., 1987).

Burkea africana Hook. (wild syringa) is an average sized leguminous tree, 10-12 m in height occasionally reaching over 20m. This monotypic genus is dominant and co-dominant in Zambia, and is present throughout Africa as far north as Ethopia and west to Nigeria, and south to South Africa especially Limpopo, North West, Gauteng and Mpumalanga (Wilson & Witkowski, 2003). It inhabits dry, non–calcareous sandy soils in savanna and woodlands up to 1500 m altitude or gentle slope of 1080 m elevation.

Trees bear pods from January to July, and these usually contain large seed, weighing 104 ± 29 mg. The fact that pods do not enter the soil easily is explained by their size and shape (Thompson *et al*., 1993). Witkowski, (unpublished data) conducted a study between 1978 -1999 in Nylsvley Game Reserve, situated in the Northern Province, South Africa. He made an observation that *B. africana* was unusual in that a large proportion of trees above minimum size for fruiting did not produce fruit each season, with 1% of reproductive trees having pods from the previous season but no flower in the following season. In addition, *B. africana* also displays cryptogeal germination also known as plumule burying, which is believed to have arisen in response to frequent burning (Jackson, 1974). The burial of the plumule emanates from the growth of a root crown well below the soil surface, which will consequently protect the buds from fire and other disturbances. This allows *B*. *africana* to resprout when the above ground shoots are burnt off, or damaged from frost (Wilson & Witkowski, 2003). *Burkea*

africana produces a relatively large number of seeds, which is unusual for a resprouting species (Lamont *et al*., 1999). In addition, Lamont *et al*., (1999) discovered that resprouting species tend to produce relatively large seeds with low levels of seed viability. Many studies have been carried out on the factors that lead to *B. africana* survival, but there is still a lot of unknowns concerning growing it effectively until reproduction stage. Yeaton, (1988), conducted a study in Nylsvley, and found that the interaction of porcupine scarring on trees with fire and windstorms played a role in the opening of gaps for regeneration of *B. africana.* Furthermore, he made an observation that large *B. africana* trees does not resprout in response to fire damage of heartwood, even though juvenile non fire-resistant individuals are vigorous resprouters (Yeaton, 1988).

Another study on *B. africana* revealed that there is a strong positive relationship between bark thickness and trunk circumference for plants <400mm trunk circumference but a poor relationship for larger trees (Wilson & Witkowski, 2000). Crawley, (1990) found that plant mortality is generally size dependant, with plants that die as a result of competition and from disturbances such as fire, coming almost exclusively from the smallest size classes.

Scholes, (1991) observed that nutrients such as nitrates, phosphorus, a series of anions and cations and various trace elements are essential to the nutrition of plants, and act as determinants of the composition, structure and productivity. He further indicated that while the base-richness of the parent material is initially important in determining soil fertility, biological activities are important in the creation and maintenance of localised areas of enhanced soil fertility.

Several investigations have found ample evidence in support of soil enrichment under tree canopies, notably with regard to % total N, % organic C and various exchangeable cations like Ca, K, Mg and Na (Hagos, 2001). Furthermore Felker & Clark, (1982; Shearer *et al*., (1983) and Hogberg, (1986) in their studies observed that bird droppings and dung of large animals spending time under trees have also been mentioned as one of the contributing element of soil enrichment. The term “nutrient import” agrees with the findings of Belsky *et al*., (1989) and Hogberg, (1986) who observed that the occurrence of N-fixation due to microbial activities under leguminous trees is a possible source of N enrichment (Scholes, 1991). A study conducted on soil

fertility under trees canopies by Stuart-Hill *et al*., (1987) indicated that leaf litter from leaf fall is a possible source of soil nutrients.

1.1.2 Importance of nutrients on growth and productivity

Scholes, (1993) discovered that nutrients such as nitrates, phosphorus, a series of anions and cations with various trace elements are essential for the nutrition of plants, in addition to performing as determinants for the composition, structure and productivity. The base-richness of the parent material is initially important in determining soil fertility, biological activities are important in the creation and maintenance of localised areas to enhance soil fertility (Werner, 2009). Smit, (1994b) observed that some determinants of vegetative growth of trees includes soil water availability and water stress; soil nutrient availability (Scholes, 1991), carbohydrates reserves and plant hormones (Loescher *et al*., 1990) atmospheric CO_2 concentrations (Polley *et al*., 1992, 1993, 1994), tree age (Agnew & Waterman, 1989; Novellie, 1989), competition (Smit, 1994b) defoliation and shoot pruning (Du toit *et al*., 1990; Ruess & Halter, 1990; Lewis, 1991), and various soil and climatic conditions (Pattern & Ellis, 1995).

1.1.3 Roots absorption of woody plants

The roots systems of some trees and shrubs species are undoubtedly very important factors in nutrient concentrations within the sub-canopy zone (Vetaas, 1992). McNaughton, (1983) discovered that when nutrients are absorbed by the tap roots of woody species, the total nutrient supply to the field-layer is altered, the combination of nutrient reallocation and surface roots turnover and shedding of leaves will together act as nutrients pumps.. The roots of *B. africana* are known to extend for many metres beyond the canopy (Rutherford, 1982) and could therefore constitute a nutrient depletion mechanism. Tree roots may extend well beyond their canopies, the extent depending upon tree species, tree age/size, soil type, and annual rainfall. Soil moisture depletion and trenching studies (Ansley *et al*., 1990; Ansley *et al*., 1991) indicate that shallow, lateral roots can be important in water uptake and maintenance of leaf area and transpiration.

1.1.4 Nitrogen fixation in leguminous trees

Nitrogen is one of the key elements in ecosystem functioning and productivity (Tietema *et al*., 1992). The occurrence of N-fixation due to microbial activities under leguminous trees is a possible source of N enrichment (Felker & Clark, 1982; Hogberg & Kvarnstrom, 1982; Virginia & Delwiche, 1982; Hogberg, 1986). Legumes are unique in their ability to infect roots and form N_2-fixing (nodules) symbioses with members of the Rhizobiaceae (or rhizobia) namely, *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Allorhizobium* (Sprent & Parsons, 2000). Barea *et al*., (2002) and van der Heijden *et al*., (1998, 2006) have showed that the N_2 fixing activity of rhizobia is enhanced in the rhizophere of mycorhizal plants, where synergistic interactions of such organisms with arbuscular mycorhizal fungi have been demonstrated. Requena *et al*., (2001) and Xavier and Germida, (2002) conducted a study in pots and field trials and found that co-inoculation with both symbionts resulted in higher plant biomass and better N and P acquisition. Similarly, Jia *et al*., (2004), Babajide *et al*., (2008) and Wu *et al*., (2009) discovered that tripartite symbiosis of legume-mycorhizal-rhizobium has shown improvements in overall growth of leguminous plants. Zaharan, (1999); Valdenegro *et al*., (2001) and Quatrini *et al*., (2003) also stated that mycorhizal legumes are known for rehabilitation of badly degraded lands and/or desertified habitats emphasizing the ecological significance of this special association.

1.1.5 Trees on nutrient status of the soils

Trees and shrubs have been found to improve the nutrient status of their close surroundings in semi-desert shrub communities (Stachowicz, 2001; Brooker *et al*., 2008) and Southern Savanna (Bate, 1981). Tree litter will unquestionably lead to accumulation of organic matter under and near the trees (Bernhard-Reversat, 1982; Belsky *et al*., 1989). The actual nutrient enrichment, however, will depend on the nutrient content of the leaves and fruits before abscission. Kavvadias *et al*., (2001), observed that the decomposition of tree litter is much slower than the understorey herbaceous litter. Nutrients found in low concentrations throughout the soil profile may be taken up by the roots systems of mature trees and shrubs (Rutherford, 1983). The contribution of bird droppings and dung of large mammals spending time under trees have also been mentioned as a source of soil enrichment (Teague & Smit, 1992). From

this information, it can be concluded that savanna woody plants are essential biological agents that contributes to areas of enhanced soil fertility. However, evidence exists that soil enrichment under tree canopies is a slow process.

Trees may act as nutrient pumps, drawing nutrients from deep horizons and laterally from areas beyond the canopy, depositing them mainly beneath the canopy via litterfall and canopy leaching (Scholes, 1990). This mechanism is supported by the fact that savanna tree roots may extend outward many times the canopy radius and penetrate more deeply into the soil than do grass roots. Tall, aerodynamically rough tree canopies act as an effective trap for atmospheric dust (Bernhard-Reversat, 1982; Escudero *et al*., 1985). The dust contains nutrients, which wash off the leaves during rainstorms and drip into the sub-canopy area. Where trees are sparse it may serve as focal points attracting roosting birds and mammals seeking shade or cover. Herbivores that take refuge in the shadow of trees may enhance the local nutrient cycle (Georgiadis, 1989). Perching birds may enrich soil nutrients (Belsky, 1994) and deposit seeds of other trees and shrubs whose germination and establishment may be favoured in the sub-canopy environment (Ascher, 1995). These findings are supported by McNaughton (1983) and Robinson, (1986) who observed that the concentration of animal dung under big canopies is significant. Blackmore *et al*., (1990) stated that animal droppings may increase soil fertility, and soil nutrients status of the soil, in particular the availability of phosphorus beneath tree canopies for long periods.

A study conducted in Nylsvley in two adjacent savanna with soil differing in nutrient content and water-holding capacity (Knoop & Walker, 1985) showed that the nutrient-rich savannah with fertile, fine-textured soil was dominated by widely spaced *Acacia tortilis* trees. The course-textured, nutrient-poor site was savanna woodland dominated by *B. africana*. Grass removal had no significant effect on the *Burkea* growth but led to increased Acacia trunk radial growth and twig extension. The lack of *Burkea* response suggests that on coarse-textured sites, soil moisture recharge to deeper portions of the soil profile being used by tree roots occurs whether or not grasses are present.

1.1.6 Leguminous tree symbionts micro-organisms

Legumes form symbioses that are not formed by many plants, including *Arabidopsis*. Spaink, (1996); Harrison, (1999) indicated that legume roots are invaded and colonized by rhizobia, and with mycorhizal fungi. A study conducted on mycorhizal fungi by Gosling *et al*., (2006) and van der Heijden *et al*., (2008) discovered that arbuscular Mycorrhizal which form symbioses with majority of plants, influence plant community development, nutrient uptake, water relations, and above ground productivity. Van der Heijden *et al*., (2008) also found that arbuscular mycorrhizas also act as bioprotectants against pathogens and toxic stresses. However, Barea & Azocon-Aguilar, (1983) have demonstrated that arbuscular mycorhizal fungi are known to be one of the most efficient ecological factors in improving growth and N content in legumes. Furthermore, Demir & Akkopru, (2007) stated that arbuscular mycorhizal fungi and rhizobia play a key role in natural ecosystems and influence plant productivity, plant nutrition, and plant resistance.

The occurrence of N-fixation due to microbial activities under leguminous trees is a possible source of N enrichment (Hogberg, 1986). Bacterial geneticists have the ability to specifically nodulate a particular legume, based on the concentration, the types and profile of lipo-chito-oligasacharride nodulation (Nod) factors that these bacteria produce and release into the rhizosphere environment of their host plant. However, Dakora, (1994) found that legume/rhizobia symbiosis involves a two-way molecular conversation; this nodulation specificity is also determined by the quality and quantity of chemical molecules such as flavonoids, betaines and amino acids released by the host plant.

1.1.7 Reproduction of trees and its determinants

According to Smith *et al*., (1999), reproduction encompasses the ability of mature trees to flower and produce viable seeds, and secondly the ability of such seeds to disperse and germinate, and of the newly established seedlings to survive. Ultimately, all living organisms need to survive and reproduce in order to maintain the species. Time to reproductive maturity is a trade-off between the costs (reduced probability of survival, increased generation time) and benefits (prolonged period of reproduction) of delayed maturation (Clark, 1991a). However, Silvertown (1991) suggest that most perennial plants must reach a minimum size before they reproduce. A study conducted in Nylsvley indicated that *B. africana* must reach an appropriate size threshold of 200-400 mm trunk circumference to reach sexual maturity. However, it was also found that even after the size threshold has been reached, trees still do not necessarily reproduce each year and the percentage of reproductive individuals per size class increases with size.

Meyer & Moysen, (1992); Ralowics & Mancino, (1992) observed that seeds of some woody plants have a seasonal dormancy which prevents the seed from germinating under unfavourable conditions. In contrary, a study was conducted Warrag, (1994) who found that dormancy can be due to the seed coat preventing or interfering with water uptake, mechanical restraint or prevention of leaching of inhibitors. Warrag, (1994), discovered that pericarp of seeds of some woody species contain allelochemicals which inhibit seed germination and seedlings of that species. The influence of a diverse range of environmental conditions on the germination of the seeds of many species has been demonstrated by various laboratory experiments, illustrating the influence of varying temperature, light regimes, substrate salinity, pH, soaking in water, seed age, soil type and depth of sowing on the germination of seeds (Cox *et al*., 1993; Sanchez-Bayo & King, 1994; Warrag, 1994).

Of importance to both seedlings and resprouting individuals, is the availability of regeneration opportunities or gap formation in the canopy to allow the plants to establish and enter the tree layer. Assuming a *B. africana* seedling is never suppressed by fire and caused to resprout, sexual maturity (fire-resistant size) would be reached within approximately 29 years.

Several studies conducted on *B*. *africana* trees paid more attention to the medicinal attributes, however little or nothing is known regarding the factors and dynamics that contribute to the growth and existence of these trees, particularly because these trees grow naturally in nutrient-poor savanna soils. It is hypothesized that factors controlling establishment and development of *B*. *africana* trees are related to microbial activities in the soil, very complex and species specific but poorly understood. The focus of this study was to find the differences in the natural soils in which *B*. *africana* trees grows (*Burkea* soils) as compared to soils which have never experienced the growth of *B*. *africana* trees (non- *Burkea* soils).

1.1. Problem Statement

Although *B. africana* trees have been in existence for a very long period of time, propagating it through thinning and transplanting of seedlings for regeneration and/ or re-establishment of seedlings to survive until sexual maturity still remains a mystery. It is due to this reason why it is not listed in the National Forest Act of 1998, not grown commercially or even found in nurseries. Up-to-date, no farmers have successfully propagated *B*. *africana* trees, as they always die 8 months after transplanting.

1.2. Aim of the study

This study aimed to identify, if there is a symbiotic relationship between the soil and mycorhizal fungi, and rhizobium bacteria or other growth stimulating activities, in the *Burkea* soils, which will accelerate and assist effective growth of *B. africana* trees to reach reproductive stage and produce pods without dying.

1.3. Objectives of the study

- To identify physical and chemical properties of the soil where *B. africana* grows effectively (*Burkea* soils) compared to where *B*. *africana* does not grow effectively (non-*Burkea* soils).
- To identify the metabolic profile of the soil surrounding *B*. *africana* trees and determine if the metabolites found in *Burkea* soils that stimulate and influence the growth.

- To generate a comprehensive understanding of below-ground micro-organisms (bacteria or fungi) and whether or not they are able to form symbiotic relationships to aid in the growth and development of *B*. *africana* trees.
- Determine the role of caterpillars (*Cirina forda*)in the growth cycle of *B. africana* trees.
-

1.4. Significance of the study

The results of the study will enable farmers and /or growers to understand, know the growth and transplanting dynamics regarding *B. africana* trees as well as the factors, which should be taken into consideration to enable successful uprooting and transplantation of seedlings outside their natural environment until they transit from vegetative stage to mature trees followed by reproduction stage. The findings of the study will also add greater value in the body of knowledge and open doors for future research in the science environment.

LAYOUT OF THE THESIS

The thesis consists of nine (9) chapters in total, which are distributed as follows:

Chapter 1

This chapter focused on the factors traditionally known to contribute towards the growth of plants, the main aim, problem statement and the objectives of the current study.

Chapter 2

This chapter reviews the literature on the background of B. africana, medicinal benefits provided by different parts of the tree, the additional benefits supplied by the tree. In addition, the study reviews the soil as the main factor affecting plant growth, the soil micro-organisms as the main constituents which made up the soil. Furthermore, the study also discusses the advanced methods which have been used elsewhere to untangle research challenges.

Chapter 3

This chapter investigates, analyses and determines the soil nutrients responsible for the growth and establishment of *B*. *africana*.

Chapter 4

This chapter determines and identifies the metabolites responsible for the growth and establishment of *B*. *africana*.

Chapter 5

This chapter determines and detects the amino acids responsible for the growth and establishment of *B*. *africana*.

Chapter 6

This chapter focuses on the DNA of *Burkea*-soil and their role on the growth and establishment of *B*. *africana*.

Chapter 7

This chapter focuses on the role of caterpillars hosted by *B*. *africana* play in improving the *Burkea*-soil DNA, which in turn influence, promote the growth and establishment of *B*. *africana*.

Chapter 8

The general conclusion and recommendations based on the overall findings of the current study are presented in this chapter.

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CHAPTER 2

2.1. LITERATURE REVIEW

The tree *B. africana* Hook. belongs to the family Leguminosae/ Caesalpiniaceae and is found in large parts of sub-Saharan Africa, with it's medicinal use in a number of cultures well documented (Watt & Breyer-Brandwijk, 1962; Delaveau *et al*., 1979). The name *Burkea* is derived from the surname of the famous collector Joseph Burke, who collected plants in the Magaliesberg area during the 1840s. Africana refers to the continent Africa, where it is widely distributed (Tanko *et al*., 2011). *Burkea* is a monotypic genus, which means that it comprises of only one species (Van Wyk & Van Wyk, 1997). It is widespread in tropical Africa, found in Chad, Sudan, Tanzania, Uganda, Cameroon, Ghana, Guinea, Mali, Niger, Nigeria, Senegal, Togo, Senegal, Mozambique, Zambia, Malawi, Zimbabwe, Botswana, Namibia and South Africa (Tanko *et al*., 2011). It is a common and characteristic tree of sandy soils in dry deciduous bush veld and woodlands (Wilson & Witkowski, 2003) which vary from 1 to 3m in depth. Savanna in Africa is generally regarded as being primarily water-limited (Huntley & Walker, 1982), with nutrients playing a secondary role. The soils are acidic (pH 4.5) and have very little available phosphate and generally low levels of other nutrients (Du Preez *et al*., 1983).

Figure 2.1: *Burkea africana* tree (Photo taken by Nemadodzi L.E) at Telperion Game Reserve

It is commonly known with not less than 30 indigenous names in Southern and Eastern Africa as documented by Watt & Breyer-Brandwijk, (1962). In South Africa, the tree is predominant in Limpopo where it is locally known as Mufhulu in Venda and Mpulu in Tsonga; Mpumalanga and other parts of Gauteng province. *Burkea africana* frequents deep (1-2 m) acidic sandy soils impoverished in most nutrients essential for plant growth (Marschner, 1995), with a particularly low P status. *Burkea africana* is dioecious (separate male and female plants) and produces an annual cohort of seeds. In nutrient-poor ecosystems, seeds generally represent the largest investment a plant makes of scares nutrient reserves (Witkowski & Lamont, 1996).

The bark surface is scaly and fissured, leaves alternate and clustered near the end of twigs, and bipinately compound. The fruit is elliptical with strong pods, flattened at both

sides as shown in Figure 2. The flowers produce nectar collected by honeybees (Dans & Martinez, 2006).

Figure 2.2: *Burkea africana* pods (Photo taken by Nemadodzi L.E)

2.1.1 Medicinal benefits of Burkea africana tree organs

Burkea africana possesses strong medicinal properties as well as biological activities, for instance, in Mali, the bark is used for numerous ailments, comprising headache; migraine; dizziness; pain; inflammation and thrush, in addition to utilization as an antineuralgic, wound-healing (Diallo *et al*., 2002) and tooth-cleaning agent (Mathisen *et al*., 2002). However, pharmacologically, *B*. *africana* is not well known and nothing has been documented yet. A study conducted in Mali revealed that some of the conditions which *B*. *africana* is used to cure might be related to oxidative stress (Mathisen *et al*., 2002). In addition, antioxidants, radical scavengers and inhibitors of enzymeatic lipid peroxidation (Malterud *et al*., 1993; Mathisen *et al*., 1997; Skari *et al*., 1999; Packer *et al*., 1999) is reported to be linked to the bark of *B*. *africana* trees (Correira da Silva & Paiva, 1971). In addition to the above, Mathisen, (2002) discovered that hydroethanol extracts from the bark of *B. africana* are excellent antioxidants, radical scavengers and 15-lipoxygenase inhibitors, and that these effects to a large extent are due to the presence of proanthocyanidins, which may be responsible for its prevalent medicinal use in Africa.

Delaveau *et al*., (1979) reported that an aqueous extract of the stems had weak activity against *Staphylococcus aureus*. The stem bark contains *β*-sitosterol, harman-type alkaloids and tryptamine and it has been proposed that the tryptamine content may be responsible for some of the biological effects ascribed to the bark of *B*. *africana* (Correira da Silva & Paiva, 1971; Ferreira, 1972, 1973a, 1973b). Chemically, Delaveau *et al*., (1979) reported the availability of tannins in bark, fruit husks (Watt and Breyer-Brandwijk, 1962) and twigs. In addition, in Mali, the gum from the bark is edible; and locally considered an aphrodisiac (Tanko *et al*., 2011).

A study conducted showed that the ethanol extract of *B*. *africana* exhibited significant anti-diarrheal activity, due to the inhibition of prostaglandin biosynthesis. Phytochemical screening revealed the presence of flavonoids, tannins, saponins, cardiac glycosides, anthraquinones and triterpenes. Hence, tannins and triterpenes may be responsible for the mechanism of action of *B. africana* anti-diarrheal activity (Knowles *et al*., 2000; Longstreth *et al*., 2006). Furthermore, a survey conducted in Mali revealed that traditional healers cure numerous ailments such as malaria; gastrointestinal diseases; wounds; sexually transmitted diseases; insects and snakebites using *B*. *africana* (Malterud, 2017).

2.1.2 *Burkea africana* as wood

Its wood is hard and heavy, particularly useful for constructional work such as bridges, sleepers, fences or for tool handles (Neya *et al*., 2004). The heartwood is dark brown or reddish brown and described to be very resistant to fungi (Aubreville, 1950; Irvine, 1961; Dalziel, 1937). The presence of biocidal compounds in extracts, which are shown to inhibit mycelium growth of several brown rot and white rot fungi on malt agar medium, and strong hydrophobic character of *B. africana* heartwood plays an important part in the dimensional stability of *B. africana* wood (Neya *et al*., 2004).

2.1.3 *Burkea africana* as a host

Burkea africana is the host for caterpillars known as *Cirina* forda as shown in Figure 2, which feeds on the leaves. These caterpillars are referred commonly to as Mashonzha by VhaVenda, VaTsonga and Bapedi tribes in the Northern Province of South Africa. These caterpillars are collected fresh, killed, preserved by adding salt and dried, thereafter sold in the streets markets by street vendors who are mostly women. The caterpillars are considered a delicacy, eaten as a side dish (after boiled

and fried) with pap normally known as vhuswa, which is a hard porridge, made of ground maize. *Cirina forda* caterpillars are known to be a high source of protein.

Figure 2.3: *Cirina forda* feeding on *B. africana* leaves (Photo taken by Nemadodzi L.E).

2.2 Soil, the main factor affecting plant growth

Soil provide an essential ecosystem function and services such as food production, regulation of atmospheric concentrations of greenhouse gases, prevention of soil erosion, regulation of the quality and quantity of water availability, and the maintenance of animal, plant and microbial biodiversity (Ciais *et al*., 2013; Diaz, *et al*., 2006; Pimentel, 2000; Tilman *et al*., 1997). There are several characteristics which soils tend to develop after transitioning from native vegetation to another environment such as loss of aggregate stability and increased erosion; acidification; over-supply or insufficient replacement of N and P relative to crop removal; changes in the molecular composition of plant biomass input; reductions in both composition and abundance of functional biodiversity of local plant, animal and micro-organisms and soil organic matter loss (Don *et al*., 2011; FAO, 2015; Flynn *et al*., 2009; Matson *et al*., 1997; Pimentel *et al*., 1992; Sala *et al*., 2000; Smith *et al*., 2016; Vitousek *et al*., 2009). The above-mentioned alterations on the soil could be the reasons why *B*. *africana* seedlings never grow if excavated and transplanted outside its native habitat. The findings of this study will enable the build-up of knowledge on soil microbial communities, viability and activities, and what should be taken into consideration to

ensure the same molecular composition of the soil and abundance of functional biodiversity.

2.3 Soil microorganisms as a nutrient acquisition for plants

Soil covers almost all of the terrestrial area on earth and have an indispensable ecological function in the global cycle of carbon, nitrogen and sulphur (Urich *et al*., 2008). A single gram of soil has been estimated to contain thousands to millions of different bacterial, archaeal and eukaryotic species (Torsvik *et al*., 2002; Gans *et al*., 2005). Although Eukaryotes are major players in soils and strongly influence the prokaryotic community structure, their diversity and abundance has reviewed comparably little attention in molecular studies (Fierer, 2007). Soil microorganism play an important role in nutrient acquisition for plants (Susan, *et al*., 1997). The abundance and activities of soil microorganisms are influenced by various environmental (e.g. soil type, nutrient status, pH, moisture) as well as plant factors (e.g. species, age). Furthermore, soil is a habitat for a vast, complex and interactive community of naturally-occuring soil organisms, whose activities largely determine the physico-chemical properties of the soil. Moreover, soil microbes perform important functions in agroecosystems including their role in plant growth promotion through mineral nutrition and control of phytopathogenic microbes. From seed germination until a plant reaches maturity, it lives in close association with soil organisms (Lynch, 1983). There are reports that have demonstrated a substantial increase in plant growth following single, dual or three member association of rhizospheric microorganism (Zaidi *et al*., 2003; Zaidi & Khan, 2007).

Owing to their immense physical, chemical, and biological heterogeneity, soils are considered the most microbial diverse environments on earth (Daniel, 2005). For instance, 1 gram of soil can contain up to 10^9 microbial cells, representing more than 10, 000 genomes (Torsvik & Ovreas, 2002). Soils are structured media where the mineral and organic matter components are organized into aggregates that vary in size, porosity, pore size and continuity, and composition (Hansel *et al*., 2008). Environmental factors that influence microbial community composition and diversity include (but are not limited to) pH (Eichorst *et al*., 2007), particle size (Sessitsch *et al*., 2001), organic carbon content (Zhou *et al*., 2002), nutrient availability (Fierer *et al*.,

2003) water content (Treves *et al*., 2003) and oxygen concentration (Ludemann *et al*., 2000).

Microbial communities are responsible for a broad spectrum of biological activities in virtually all natural environments including oceans (Delong, 2005), soil (Daniel, 2005) and human-associated habitats (The Human Microbiome, 2012). Metagenomics shotgun sequencing provides a uniquely rich profile of microbial communities, with each data set yielding billions of short reads sampled from the DNA in the community (Segata *et al*., 2012).

Microbial communities affect directly or indirectly the physico-chemical properties of soils through their metabolic activities. Rhizosphere microorganisms including fungi can enhance plant growth by different mechanisms (Khan *et al*., 2010). Microorganisms play a fundamental role in the biogeochemical; cycling of elements including P in natural ecosystems (Gerretson, 1948). Furthermore, microbial activity in the rhizosphere could dissolve sparingly soluble inorganic P and increase plant growth. The microbial biomass in soil also contains a significant quantity of immobilized P that is potentially available to plants (Brookes *et al*., 1984; Oberson *et al*., 2001). Fungi are associated with improved growth of many plant species due to increased nutrient uptake, production of growth promoting substances, tolerance to drought, salinity and transplant shock and synergistic interaction with other beneficial soil microorganism such as N-fixers and P-solubilizers (Rai *et al*., 2013).

2.4 Plant Growth Promoting Metabolites in the soil

Metabolites are the end products of a variety of cellular processes and metabolomics provides high-throughput characterization and quantification of living organisms, and as a result, is increasingly applied to the areas of system biology, drug discovery, pharmaceutical research, early disease detection, toxicology and food science (Gowda *et al*., 2008). The ultimate goal of metabolomics is to measure all the metabolites in an organism both qualitatively and quantitatively, which can provide a clear metabolic picture of a living organism under certain conditions (Kim *et al*., 2010). Metabolomics analyses have shed light on unique chemical defense mechanisms and production of secondary metabolites (Goulitquer *et al*., 2012). Untargeted metabolomics is a rapidly-growing and robust method that has become an important approach in biomedical science by providing comprehensive data-driven metabolism

analyses of complex extracts (Fiehn, 2002; Garcia *et al*., 2008; Baran *et al*., 2009, 2013). As mass spectrometry (MS) instruments such as Fourier Transform Ion Cyclotron Resonance (FTICR/MS) (Hirai *et al*., 2004), capillary electrophoresis (CE/MS) (Soga *et al*., 2003; Edwards *et al*., 2006), and liquid chromatography (LC/MS) (Baran *et al*., 2011, 2013) are commonly used, they are not widely available.

In contrast, GC/MS is a widely-used and generally more accessible instrument to microbiologists and soil scientists due to its low operational costs, availability of curated metabolite spectral databases, broad analytical scope with good coverage of metabolite classes (carbohydrates, alcohols, sterols, amino acids, fatty acids) as well as widespread application o phospholipid fatty acid analysis (Buyer & Sasser, 2012).

Understanding the soil biochemistry requires identifying the soil metabolites such that they can be linked to enzymes and microorganisms (Swenson, *et al*., 2014). Unfortunately, it is important to note that, the field of soil metabolomics still lags behind, but is emerging as an equally important area of study. This is due to the fact that in the past decade, metabolomics has been developed as an important field of plant sciences and natural products chemistry (Ward *et al*., 2007).

Most secondary metabolites produced by soil microbes appear to be secreted and play a role in controlling biotic interactions. Other roles of secondary metabolites, such as facilitating symbiosis with insects, plants and higher animals are documented by Demain & Fang (2000). However, little research has focused on soil metabolites and their role to promote or inhibit growth of trees.

For instance, the sugar trehalose is common in micro-organisms where it acts as a protective agent against various stresses (Crowe *et al*., 1998). In tobacco, trehalose have improved growth under drought stress (Romero *et al*., 1997), implying that the protective properties of trehalose also apply to plant cells. Betaine has also been reported to improve growth and yield of water-stressed *Nicotiana tabacum* (Aghoma *et al*., 1997b), *Glycine max*; (Aghoma *et al*., 1997c), *Triticum aestivum*; (Borojevic *et al*., 1980), *Zea mays*; (Agboma *et al*., 1997a), and salt and high temperature stressed plants such as *Lycopersicon esculentum* (Makela *et al*., 1998a,b) salt stressed plant such as *Oryza sativa*; (Harinasut *et al*., 1996, Lutts, 2000) and *Gossypium hirsutum* (Naidu *et al*., 1998 & Gorham *et al*., 2000). In contrast, it was found in other studies

that betaine did not improve growth and yield of stressed *Gossypium hirsutum*; (Meek *et al*., 2003),*Triticum aestivum*; (Agboma *et al*., 1997a) and *Brassica napus* (Makela *et al*., 1996) and might indicate that other factors also influence the effect of these compounds.

2.4.1 The advantages of using NMR

NMR analysis in most cases, ^{1}H-NMR is sufficient to generate metabolomics data of a sample within a relatively short time (5-10 min for 64-128 scans). It reflects the amount of all metabolites in the extract because the NMR signals are directly in proportional to the molar concentration independent of the characteristics of a compound (Kim *et al*., 2010). NMR is a very suitable method to carry out an analysis because it allows the simultaneous detection of diverse groups of secondary metabolites (flavonoids, alkaloids, terpenoids) beside abundant primary metabolites (sugars, organic acids, amino acids). Moreover, in an NMR spectrum, signals are proportional to their molar concentration, making the direct comparison of concentrations of all compounds possible, without the need for calibration curves of each individual compound. In addition, NMR is a very useful technique for structure elucidation (Ward *et al*., 2007). It is also robust and reproducible, this means that the same samples replicates will give the same metabolites regardless of where the analysis is conducted. Another advantage of ^{1}H-NMR has been reported as an advanced analytical method which is non-destructive and highly reproducible (Nicholson *et al*., 1999). The number of peaks generated by a metabolite, as well as their location and ratio of heights are reproducible and uniquely determined by the chemical structure of the molecule (Zheng *et al.,* 2011). In addition, it was found than an ^{1}H-NMR spectrum displays chemical shifts in parts per million versus intensities of the signals (Ernst *et al*., 1990).

2.4.2 The advantage of using LC-MS for analysis

For LC-MS based targeted metabolomics, it is desirable to detect as many metabolites as possible using their retention times and accurate masses. High-resolution mass spectrometers have the potential to quantitate broad range known analytes (targeted analysis) while simultaneously detecting untargeted data on all ions present, including ones arising from unanticipated compounds (untargeted analysis) (Lu *et al*., 2010). The key characteristics of the resulting LC-MS method include fast analysis due to the

use of a small particle column. MS-based techniques are more sensitive, in particular when using liquid chromatography (LC) connected to a tandem MS/MS for quantitative analysis in the multiple reaction mode (Figueira *et al*., 2017)

2.4.3 Advanced methods used to analyse soil metabolites

Nuclear Magnetic Resonance (NMR) was used in this study to identify untargeted soil metabolites due to its low operational costs and availability. In addition, triple-Quadrupole Liquid Chromatography-Mass Spectrometry (LC-MS) was used in identification of untargeted amino acids, although it is high in operational costs, requires an expert and not widely available in South Africa. Findings from this study will provide key insights into the primary soil metabolites, which influence the growth and establishment of *B. africana.* This work will lay a technical foundation and enable wide range of other researchers to grasp the value of soil metabolomics in answering difficult research questions.

2.5 Macro-elements required for growth

2.5.1 Nitrogen

Limited nitrogen (N) supply causes reduced plant growth and morphological changes such as increased root length relative to shoot growth to explore a larger soil volume (Niu *et al*., 2006). Cycling of mineral nutrients, i.e. retranslocation in the phloem from shoot to roots, and translocation of cycled nutrients back to the shoot in the xylem, is important for plant growth, especially under stressed conditions (Marschner *et al*., 1997). Deciduous trees remobilize nitrogen from senescing leaves into woody tissues, from where it is available for the growth of new tissues in the following season (Taylor, 1967; Tromp, 1983; Titus & Kang, 1982). The storage of N can be particularly important for the early seasonal growth of young trees, which make substantial demands upon soil nutrient reserves for canopy growth, compared to mature trees which are far less dependent upon uptake of soil reserves (Attiwell, 1979; Miller, 1981). Internal cycling of nitrogen has been shown to be a major source of nitrogen used for the seasonal growth of both evergreen and deciduous trees providing up to 90% of N needed for leaf growth of some species (Millard, 1995). It is also known as the nutrient having limited forest productivity (Cole, 1981). The amount of N stored by a tree, depends upon both tree growth and development, and environmental factors such as

soil fertility which determines the availability of N for uptake. In most soils, the proportion of inorganic N present as nitrate increases as total N increases (Attiwill *et al*., 1996).

2.5.2 Phosphorus

Phosphorus (P) is one of the most essential macro-elements required for growth and development of plants (including photosynthesis, energy and sugar production) and also promotes N_2 fixation in legumes (Saber *et al*., 2005). Phosphorus is the second most important macronutrient after nitrogen that is critical for plant growth, stability and continued existence of life, in addition, it contributes up to about 0.2% dry mass (Rai *et al*., 2013). There are many elementary and principal roles of P in many plant physiological processes, such as photosynthesis, utilization of sugar and starch, and energy transfer (Rai *et al*., 2013). Furthermore, P is an indispensable structural component of numerous molecules, including nucleic acids, which are the building blocks of genes and chromosomes in the cell nucleus and are obligatory for cell division and formation of meristematic tissues (Tisdale *et al*., 1985). For maximum yield, plants are in need for an ample amount of P from the very early stages of growth (Grant *et al*., 2005). P is an integral component of ATP and ADP molecules, phospholipids and nucleic acid, which are important in cellular membranes, and provides compounds for photosynthesis in plants and respiration in animal.

In soils, of the total P (0.5%), only 0.1% is plant available (Scheffer & Schachtschabel, 1988). The deficiency of P in turn severely restricts growth and yield in plants. The repeated and injudicious applications of chemical P fertilizers, have been reported to lead to the loss of soil fertility (Gyaneshwar *et al*., 2002) by disturbing microbial diversity, and consequently reduces plant growth. Nitrogen and phosphorus are the two major plant nutrients and combined inoculation of N_2 fixers and P solubilizers may benefit plants better than either group of organisms alone (Khan *et al*., 2010).

Total soil P typically ranges from 100 to 2000 mg kg^{-1} soil, which corresponds to about 350 to 7000 kg ha^{-1} P on an area basis, although only a small fraction of this P is available for crop uptake (Morel, 2002).

2.5.3 Potassium

Potassium (K^+) is one of major nutrients considered essential for crop growth and yield development, although it is not an integral component of any cellular organelle or structural part of the plant. It is the most abundant cation in plants and is associated or involved with many of the physiological processes supporting plant growth and development (Pettigrew, 2008). The total amount of potassium absorbed by the crop during the growing season depends upon the crop species being grown, the amount of native soil K^+, the amount of fertilizer K^+ applied, K^+ availability in the soil, environmental conditions during the growing season and the management practices employed (Eakin, 1972; Mengel & Kirkby, 1987; Mullins & Burmester, 1998).

Potassium is also involved directly or indirectly in plant protein metabolism (Blevins, 1985). This involvement can begin with the stimulation of NO^{3-} uptake and transport within the plant, as K^+ serves as the accompanying counter cation (Blevins et al., 1978a, 1978b). Mengel, (1980), also demonstrated that the transport of amino acids is enhanced by higher K^+ levels, especially the transport of amino acids to developing seeds. The number of pods per plant (Bharati *et al*., 1986; Jones *et al*., 1977; Nelson *et al*., 1945) and the weight of individual seeds (Bharati *et al*., 1986) increased in response to K^+ fertilization.

Out of all the mineral nutrients, potassium plays a critical role in plant growth and metabolism, and it contributes greatly to the survival of plants that are under various biotic and abiotic stresses (Wang *et al*., 2013). In addition, K^+ plays essential roles in enzyme activation, protein synthesis, photosynthesis, osmoregulation, stomatal movement, energy transfer, phloem transport, cation-anion balance and stress resistance (Marschner, 2012).

In the current study, metagenomics was used to determine the soil DNA, diversity of microbial communities (e.g. Fungi and Bacteria) from where *B. africana* grows versus where they do not grow effectively. This was done on the hypothesis that micro-organisms present in the soil may have major influence on the growth and establishment of *B*. *africana* trees and a change of environment could be detrimental to the survival and effectiveness of microorganisms which are known to influence growth. The results of this study will be of greater practical value towards the

successful growth and development of *B*. *africana* trees if grown outside their natural habitat, and if inoculation of plant growth promoting microbe(s) should be done alone or in combination with other plant growth promoting metabolites, to ensure overall performance of *B*. *africana* to reach their maturity.

However, in spite of its exceptional medicinal benefits of *B*. *africana*, it has a reputation for being difficult to grow outside its natural environment, and no studies had been reported on the reasons of its difficulty to propagate or grow and what can be done to grow them successfully until they reach maturity. Farmers who have tried growing it, reported that it dies after a period of 8 months of being propagated. This study aimed to discover and identify factors, which contribute to the growth, and establishment of *B*. *africana* trees. The findings of this study will be used as a point of reference and incorporated when growing or transplanting *B*. *africana* seedlings out of their natural habitat. Furthermore, this research will not only add to the body of knowledge with regard to *B. africana* trees but will also formulate areas for further research and review areas of controversy.

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CHAPTER 3

ANALYSIS OF MACRO AND MICRO ELEMENTS IN *BURKEA* SOIL VERSUS NON-*BURKEA* SOILS

Abstract

The purpose of this study was to investigate the soil factors responsible for the successful establishment of *Burkea africana* trees. Fifteen samples were collected from three (3) different sites where *B. africana* grows (*Burkea* soil) and where it does not grow (non-*Burkea* soil) to determine the chemical composition of the soil using HCl extraction method. Data collected was analysed using the statistical analysis software (SAS, 2010). The results indicated the similar values ($p>0.05$) were observed for all micro and macro minerals as well as total nitrogen, pH and organic matter. However, total ions nitrate and ammonium concentration levels of *Burkea* soils were higher ($p<0.05$) than those found in non-*Burkea* soils.

Keywords: *Burkea africana*, soil, nitrate, ammonium

3.1. INTRODUCTION

Soil is a complex mixture of numerous inorganic and organic constituents that vary in size, shape, chemical constitution and reactivity, and hosts numerous organisms (Medeiros *et al*., 2006). The biological, chemical and physical processes involved in soil organic matter (SOM) turnover can partly be deduced from the dynamics of soil carbohydrates (Amelung *et al*., 1999). Total sugars have been estimated to constitute 10% on average of SOM, occurring in living and decaying organisms, as well as in extracellular materials (Mehta *et al*., 1961). Soil carbohydrates have been known to influence soil structure, chemical processes, plant nutrition and microbial activity (Medeiros *et al*., 2006). Trees and shrubs have been found to improve the nutrient status of their nutrient status of their close surroundings in semi-desert shrub communities (Garcia-Moya & Mckell, 1970).

Currently, there are 17 elements in the Periodic Table known to be required by all higher plants (Ascher, 1991). Of these essential elements, 9 are macronutrients (C, H, O, N, K, Ca, Mg, P and S) that are normally present in plant tissues at concentrations of greater than 0.1% dry weight. Eight micronutrients (defined as

essential elements that are required in plant tissues at concentrations of less than 100 µg/g dry weight) are now recognized as essential for all higher plants namely B, CI, Cu, Fe, Mn, Mo, Ni, and Zn. Other elements are considered to be essential for certain plant species or for some species that are grown under specific environmental conditions (Welch, 1995).

Burkea africana Hook. grows in savannas and woodlands up to 1500 m altitude. It inhabits dry, acidic sandy soils impoverished in most nutrients essential for plant growth (Marschner *et al*., 1995), with a low P status. Nevertheless, *B*. *africana* has proven challenging to propagate outside its natural habitat until it reaches reproductive stage, and its growth has remained a mystery due to absence of information on re-establishment. Furthermore, no information on the chemical properties, macro and micro element constituents of the soils where *B*. *africana* trees grows effectively have been reported yet. This chapter looked at the differences in mineral elements found in *Burkea* soils versus non-*Burkea* soils and their influence on the growth and establishment of *B*. *africana*.

3.2 MATERIAL AND METHODS

3.2.1 Study site

The study was conducted at Telperion Game Reserve which is situated in Mpumalanga, South Africa at three different sites within the reserve namely site 1 (S 25°42’40.00’’; E 029°00’21.6’’); site 2 (S 25°41’26.6”; E 029°01’46.7”) and site 3 (S 25°39’49.4”; E 029°01’59.7”). The reserve is approximately 1 000 ha in size and the vegetation cover is described as Highveld grassland and savannah with large rocky outcrops present throughout the area (Swanepoel, 2006). The reserve stocks a variety of large mammal species, including plains zebra, greater kudu, eland, baboons and waterbucks as well as smaller antelope species such as impala. During the study, it was observed that the area is dominated by sandy loam soils, rocks and characterised by grass, shrubs, different trees and diversity of wild animals.

3.2.2 Soil sample collection

Soil was collected from 3 different sites, with two sampling regions for each site representing 3 areas where *B. africana* grows (*Burkea* soils) as well as 3 areas where *B. africana* does not grow (non-*Burkea* soils). At each site, 15 samples were collected at a depth of 0-60 cm deep. The soils were collected and placed in brown bags, transported and stored at room temperature until use. Chemical composition of the soils was analysed at Agriculture Research Council-Institute for Soil, Climate and Water (ARC-ISWC) laboratory for carbon, total nitrogen, phosphorus, organic matter, pH, potassium, iron, magnesium, manganese, calcium, nitrate and ammonium.

3.2.3 Laboratory soil analysis

Exchangeable cations and anions were extracted with 1 M ammonium acetate (1: 10, soil: extractant ratio), shaken for 2 hours and analysed for C, Ca, Mg, K, Fe, total N and Na using automatic absorption spectrophotometry (Pharmacia LKB-Ultrospec III). Exchangeable anions were extracted with distilled water (1: 5, soil: H_2O), shaken for 2 hours and NO_3^- and NH^{4+} ions in extracts were subsequently analysed by ion chromatography (Dionex DX 120). The pH was determined with a pH meter (Micro pH 2001, Crison) in a 1: 10 w/v suspension of 5 g of each sample. Organic matter content was estimated from the determination of carbon using the combustion method with the elemental analyser (Euro EA).

3.2.4 Statistical analysis

Data was analysed using a one-way analysis of variance with SAS statistical analysis software (SAS, 2010). When ANOVA indicated a significant *P*-value, means were separated using the Duncan multiple range test.

3.3. RESULTS

3.3.1. Similarities between *Burkea* soils versus non-*Burkea* soils

3.3.1.1. Total Nitrogen

The two soils showed similar total N concentrations, no significant differences were observed between *Burkea* soils versus non-*Burkea* soils as shown in Table 3.1.

3.3.1.2. Phosphorus

The two soils showed similar P concentrations, no significant differences were observed between *Burkea* soils versus non-*Burkea* soils as shown in Table 3.1.

3.3.1.3. Potassium

There was no significant difference found in K concentrations in *Burkea* soils versus non-*Burkea* soils as illustrated in Table 3.1.

3.3.1.4. pH

There was no significant differences found in the pH between *Burkea* soils versus non-*Burkea* soils.

3.3.1.5. Magnesium

The findings of the study indicated that Mg concentrations revealed no significant differences between the two soils as shown in Table 3.1.

3.3.1.6. Sodium

The results of the current study revealed that Na concentrations showed no significant differences between *Burkea* soils versus non-*Burkea* soils.

3.3.1.7. Carbon availability

Both *Burkea* soils and non-*Burkea* soils showed the same amount of C availability as indicated in Table3.1.

3.3.1.8. Manganese

There was no significant differences found in Mn in *Burkea* soils versus non-*Burkea* soils as indicated in Table 3.1.

3.3.1.9. Iron

The results of the study revealed that there was no significant differences in the Fe concentration between *Burkea* soils versus non-*Burkea* soils as shown in Table 3.1.

3.3.1.10. Organic matter

There was no significant differences found in the OM between *Burkea* soils versus non-*Burkea* soils as illustrated in Table 3.1.

3.3.2. Differences found in *Burkea* soils versus non-*Burkea* soils

3.3.2.1. Nitrate

The results of the study revealed that nitrate levels in *Burkea* soil was significantly higher ($p<0.05$) than those found in non-*Burkea* soils as illustrated in Table 3.1.

3.3.2.1. Ammonium

In this study, ammonium level in *Burkea* soil was significantly higher ($p<0.05$) than those found in non-*Burkea* soils as shown in Table 3.1.

Table 3.1: Chemical composition of Burkea versus non-Burkea soils

	Soils		
Chemical composition (mg/kg)	***Burkea***	**Non-*Burkea***	**SEM**
Fe	30.26	20.27	6.070
Mn	27.38	24.76	5.964
pH	4.72	4.81	0.047
P	5.82	7.04	2.844
C	52.86	55.05	8.536
Mg	35.89	23.4	6.081
K	64.65	74.74	7.830
Na	1.78	2.56	0.917
Total nitrogen	0.13	0.16	0.001
N- NO_3^-	7.06[a]	5.35[b]	2.423
N-NH_4^+	9.40[a]	7.11[b]	0.912
Organic matter	3.40	2.57	0.964

[a,b] Means with different superscripts are significantly different ($p<0.05$)

SEM: standard error of mean

3.4 DISCUSSION

Interest in plant micronutrients and macronutrients has escalated within the last few decades because of research demonstrating that they play important roles in plant disease resistance and in root-stress resistance because plant foods are significant sources of these essential elements for animals and humans (Nielsen, 1992; Van Campen, 1991; Miller *et al*., 1991; Graham & Webb, 1991). The concentrations of micronutrients in plants can vary widely depending on many factors, including plant

species, genotype, growth conditions, and between different organs and tissues of the same plant species.

The results of the current study showed that micro elemental analysis showed no significant differences between *Burkea* and non-*Burkea* soils, with the exception of macro elements such as ammonium and nitrate which indicated higher levels in the *Burkea* soils. Several studies revealed that the relative amounts of ammonium and nitrate is critical for growth and morphogenesis of plant cells (Halparin & Wetherell, 1965; Ramage & Williams, 2002). When ammonium and nitrate are both available in the soil, at the same place, immobilization depletes first or exclusively the ammonium pool with nitrate being immobilized after ammonium has been exhausted. Consequently, the nitrate is potentially more available in soil, particularly for plant uptake (Recous & Mary, 1990; Lopez-Bucio *et al*., 2003).

Furthermore, nitrate appears to be a good substrate as ammonium for assimilation by soil microorganisms. Nitrate immobilization i.e. microbial nitrate uptake is closely dependent on ammonium availability. In addition, ammonium has been found to be the preferred form of N for assimilation by microbes in many cultivated soils (Azam *et al*., 1993). Consequently, the rate of nitrification, which controls the residence time of ammonium and nitrate are the two major factors, which determine the proportion of nitrate and ammonium immobilized in the soils (Recous & Mary, 1990). Jansson, (1958; Robertson & Groffman, 2007) pointed out that the microbial immobilization of N is a major process in the soil which is largely influenced by nitrate and ammonium.

Availability of nitrogen as a plant mineral nutrient is the strongest limiting factor to plant growth and yield (Stewart *et al*., 2005). Most plants species are able to absorb and assimilate nitrate, ammonium, urea and amino acids as nitrogen sources, but the response to a particular form of nitrogen varies from species to species (Haynes & Goh, 1978). In general, most crop plants prefer a mixture of ammonium and nitrate and will take up a higher proportion of ammonium to nitrate than is present in the soil solution. The results of the study revealed that *B*. *africana* is dependent on ammonium and nitrate as a source of nitrogen for effective growth and establishment. The assumption from the current study is confirmed by (Hayne & Goh, 1978) who discovered that the main nitrogen sources under natural conditions are the ions, ammonium and nitrate.

Furthermore, nitrate is an important macronutrient which serves as a signal for growth as plants respond to nitrate by altering their metabolism and inducing genes in the nitrate assimilation pathway (Crawforda & Glass, 1998; Wang *et al*., 2012). In the soil solution, nitrate is carried towards the root by bulk flow and is absorbed into the epidermal and cortical symplasm (Glass & Siddiqi, 1995). The absence of nitrate ammonium in the non-*Burkea* soils may be the reason *B*. *africana* is unable to grow when transplanted to non-*Burkea* soils outside their natural habitat.

On the other hand, stunted root growth and leaf chlorosis are the main phenotypic markers of ammonium toxicity in plants (Britto & Kronzucker, 2002; Li *et al*., 2014). Depending on conditions and the plants specific and varietal characteristics ammonium behaved as a stressor (Britto & Kronzucker, 2002) and an antagonist to the stress (Fernandez-Crespo *et al*., 2012). It has been observed that after transplanting *B*. *africana*, the growth continued for a period of at least eight months, the leaves turned brown, and ultimately died (Unpublished data). It is therefore presumed that inoculating non-*Burkea* soils with ammonium and nitrate will enrich the soils with macronutrients which will contribute towards the nitrogen needed for the effective growth of *B*. *africana*.

3.5. CONCLUSION

Nitrogen is the mineral nutrient that plants need in the greatest quantities and one that most frequently limits growth and crop yields. There are different forms in which the plant absorbs nitrogen such as nitrate and ammonium needed for plant growth. In the absence of these two ions, growth of the plant could be compromised, which can lead to stunted roots, leaf chlorosis and ultimately the death of the plants. The ability of nitrate and ammonium to influence the growth and as antagonists to stress should be taken into account when growing *B. africana* trees outside their natural environments. Growing and establishing *B*. *africana* trees outside their native soils may lead to environmental stress caused by alterations in the nitrate and ammonium concentrations levels. Based on the results presented herein, inoculation of the soil with both nitrate and ammonium to enable *B*. *africana* grow and survive environmental

3.6. . REFERENCES

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CHAPTER 4

^{1}H-NMR based metabolomics to identify soil metabolites which influences the growth and establishment of *Burkea africana* trees.

Abstract

The aim of the study was to determine the soil metabolites responsible for successful growth and establishment of the *Burkea africana* trees. The findings of this study indicated that ^{1}H-NMR metabolomic analysis showed different metabolites in the respective soils. Growth-promoting metabolites (GPM) such as trehalose and betaine were found to be in higher concentrations in the *Burkea* soils. Conversely, acetate, lactate and formate, were found in higher concentrations in the non-*Burkea* soils.

Keywords: *Burkea soils*, trehalose, betaine, growth

4.1. INTRODUCTION

Metabolites are the end products of a variety of cellular processes and metabolomics provides high-throughput characterization and quantification of living organisms, and as a result, is increasingly applied to the areas of system biology, drug discovery, pharmaceutical research, early disease detection, toxicology and food science (Gowda *et al*., 2008). Most secondary metabolites produced by soil microbes appear to be secreted and play a role in controlling biotic interactions. Demain & Fang, (2000), documented other roles of secondary metabolites, such as facilitating symbiosis with insects, plants and higher animals. For instance, the sugar trehalose is common in micro-organisms where it acts as a protective agent against various stresses (Crowe *et al*., 1998). Betaine has been reported to be an osmoprotectant in salt and high temperature stressed plants such as *Lycopersicon esculentum* (Makela *et al*., 1998a, b) and salt stressed plants such as *Oryza sativa*; (Harinasut *et al*., 1996; Lutts, 2000) and *Gossypium hirsutum* (Naidu *et al*., 1998; Gorham *et al*., 2000). Nuclear Magnetic Resonance (NMR) spectroscopy is widely used for high-throughput characterization of metabolites in complex biological mixtures (Zheng *et al*., 2011). The field of metabolomics studies small molecules, such as amino acids, nucleic acids, lipids or carbohydrates as well as other more complex secondary metabolites which are

present in cells and/or extracellular fluids of biological organisms (Wishart *et al*., 2007). However, little research has focused on soil metabolites and their role to promote or inhibit growth of plants.

Databases such as the human metabolome database (HMDB) (Wishart *et al*., 2007); BioMagResBank (BMRB) (Ulrich *et al*., 2008) and commercial databases such as the Chenomx NMR suite (Weljie *et al*., 2006) are used to annotate NMR specific characteristics which are also known as values for a variety of metabolites.

Burkea africana trees grow in a nutrient-poor soil environment, however, the dynamics of transplanting the seedlings and growing them in different soils outside their natural environment has been proven challenging and not successful to date. This chapter investigated the variance in the chemical composition of the *Burkea* soils in comparison with non-*Burkea* soils using advanced ^{1}H-NMR technique for a comprehensive understanding of soil metabolites with reference to the factors which contribute to the growth and establishment of the *B. africana* trees.

4.2. MATERIAL AND METHODS

4.2.1. Sampling site

The study site is described in section 3.2.1 in Chapter 3.

4.2.2. Soil collection

Soil collection was done and stored as described under section 3.2.2 of soil collection in Chapter 3.

4.2.3. Chemicals and buffer preparation

Deuterated methanol-d4 (CH_3OH-d_4), non-deuterated KH_2PO_4, sodium deuterium oxide (NaOD), trimethylsilyl propionic acid-d4 sodium salt (TSP) and deuterium oxide (D_2O) was supplied by Sigma-Aldrich. The buffer was prepared by adding 1.232 g KH_2PO_4 to 100 ml of D_2O with 100 mg TSP (0.01%) added as a reference standard.

4.2.4. Extraction method and NMR Measurement

The protocol designed by Kim *et al*., (2010) was implemented for the extraction procedures, with a few adjustments. A 500 mg soil sample were transferred to 2 ml Eppendorf tubes. The samples were extracted with 750 µl deuterated methanol and 750 µl KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.01% TSP. The Eppendorf tubes were vortexed for 1 minute at room temperature and then, ultra-sonicated for 20 minutes without heating. The solutions were centrifuged for another 15 minutes at 10 000 rpm to separate the supernatant from the precipitate. The supernatant was transferred to standard 5 mm NMR tubes and subjected to ^{1}H- NMR analysis. The ^{1}H-NMR measurements were performed on a Varian 600 MHz spectrometer with a frequency of 599.74 MHz. The acquisition time of each ^{1}H- NMR spectrum was 7 min, which consisted of 32 scans with a width of 20 ppm.

Gradient shimming was used to improve magnetic field homogeneity. All spectra were phase corrected and binned at 0.04 ppm using MestReNova as prescribed by Maree & Viljoen, (2012) before statistically analysed with SIMCA 13.0.3 (Umetrics, Umea, Sweden). Metabolites detection and quantification were carried out using Chenomx NMR suite (evaluation edition) using TSP as reference and the built-in spectral library of metabolites. The Human Metabolome Database (HMDB) (Wishart *et al*., 2013) was also used to annotate our findings.

4.2.5. Statistical analysis

The pre-processed data binned at 0.04 ppm was exported to SIMCA 13.0.3 for statistical analysis. The data was scaled using Pareto and a PCA and an OPLS-DA model were used to illustrate the distinctive separation between the 3 sampling sites where soils were collected.

4.3. RESULTS

4.3.1. Annotation of compounds

4.3.1.1. Metabolites in *Burkea* soils

The statistical model OPLS-DA showed a clear separation between *Burkea* soils versus non-*Burkea* soils, which indicates the differences in the metabolites found in each respective soils as shown in Figure 4.1.

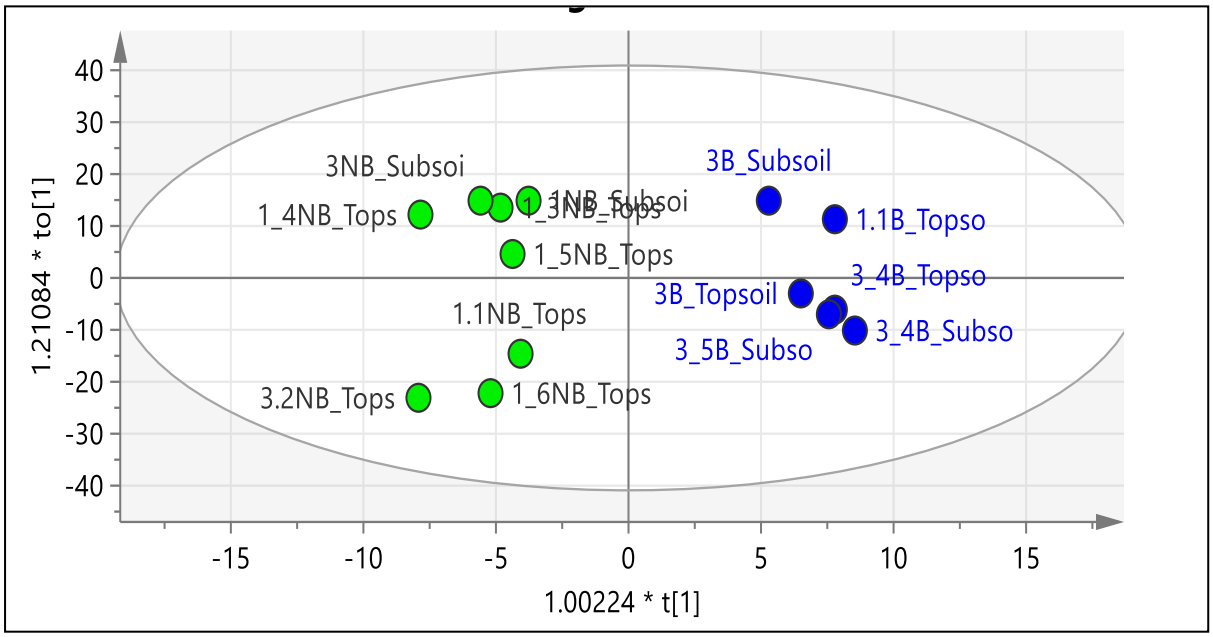

Figure 4.1: OPLS-DA used as a statistical model to show separation between *Burkea* soils (blue) versus non-*Burkea* soils (green). Top=top soil and Sub = sub soils (R2X at 0.938 and R2Y at 0.593).

Furthermore, it was possible to detect the difference in the metabolic profile in *Burkea* versus non-*Burkea* soils using a contribution plot (Figure 4.2).

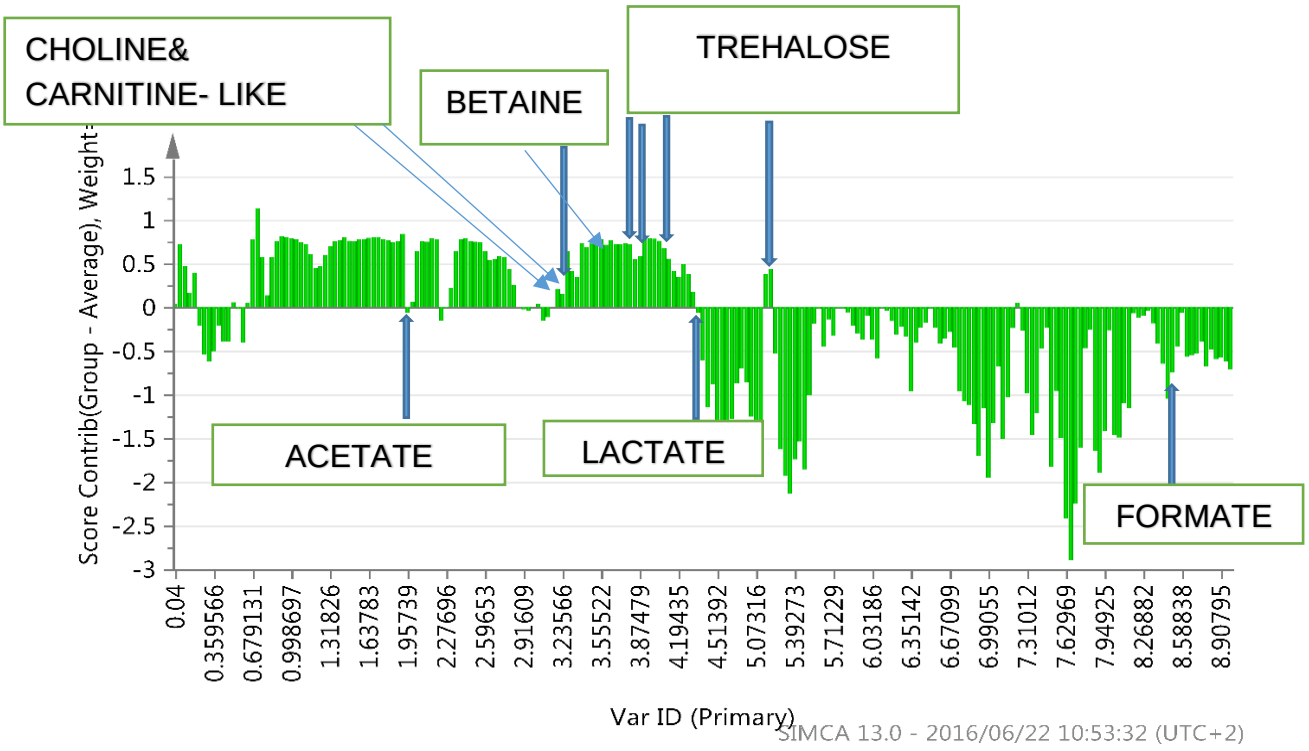

Fig 4.2: Contribution plot representing the difference between *Burkea* versus non-*Burkea* soils showing regions with positive correlation with *Burkea* soils for trehalose (specific values 3.6, 3.8, 3.9 and 5.2 ppm), betaine (3.3 and 3.9 ppm), and negative association on non- *Burkea* soils for acetate (1.9 ppm), lactate (4.1 ppm) and formate (8.4 ppm).

NMR regions from the contribution plot were used to annotate compounds and the NMR values for the compounds were compared with the NMR values found in the HMDB and Chenomx databases. Growth promoting metabolites trehalose (3.6, 3.8, 3.9, 5.2 ppm) and betaine (3.3, 3.9 ppm) as well as two choline-related and carnitine-related compounds showed a positive correlation and were prevalent in the *Burkea* soils with a negative and/or zero association in non-*Burkea* soils (Table 4.1).

Table 4.1: Annotated compounds showing different NMR regions found in *Burkea* soils versus *non-Burkea* soils compared to Chenomx and Human Metabolome database values

		Database	
Metabolite	**NMR region (ppm)**	**Chenomx**	**HMDB**
***Burkea* soil**			
Trehalose	3.46, 3.65, 3.83, 3.86, 3.88, 5.2	3.6, 3.8, 3.9, 5.2	3.42, 3.44, 3.65, 3.83, 3.87, 5.18 (Wilshart *et al.,* 2009)
Betaine	3.27, 3.9	3.3, 3.9	3.25, 3.89 (Wilshart *et al*., 2009)
Choline-like	3.20	N/A	N/A
Carnitine-like	3.22	N/A	N/A
***non-Burkea* soil**			
Acetate	1.92	1.9	1.91(Wilshart *et al*., 2013)

Formate	8.46	8.4	8.44 (Wilshart *et al*., 2009)
Lactate	4.1	4.1	4.1 (Wilshart *et al*., 2009)

Identification was done by spiking samples with trehalose, betaine, choline or carnitine. Trehalose and betaine were positively identified, choline and carnitine were almost identical to the observed chemical shifts but the small difference was sufficient to label the two observed resonances in *Burkea* soils as choline-like and carnitine like.

4.3.1.2. Metabolites in non-*Burkea* soils

Acetate (1.9 ppm), lactate (4.1 ppm) and formate (8.4 ppm) were positively associated with the non-*Burkea* soils. The annotation of these metabolites was done using Chenomx Profiler as prescribed by Weljie *et al*., (2006) and supported by previously published data on Human metabolomics data base (HMBD) (Wilshart *et al*., 2009) as presented in Table 4.1 . Moreover, in view of the splitting patterns and chemical shifts the acetate, lactate and formic acid molecules can be positively identified.

4.3.2. Identification of annotated metabolites compounds

Identification was of the annotated metabolic compounds based on spiking NMR samples with candidate metabolites.

4.4. DISCUSSION

Soil is a complex mixture of numerous inorganic and organic constituents that vary in, size, shape, chemical constitution and reactivity, and hosts numerous organisms (Medeiros *et al*., 2006). The results of this study demonstrated that trehalose and betaine as well as are metabolites found to be prevalent in *Burkea* soils. It is proposed that trehalose and betaine are growth-promoting metabolites required to sustain the growth of *B*. *africana* if grown outside their native soils. Trehalose is responsible for signalling growth whilst betaine is responsible for improving growth and therefore

probably play a role in growth promotion. The absence of these growth-promoting metabolites in non-*Burkea* soils is a clear indication that different soils possess different primary and secondary metabolites, which are likely to contribute to the growth of plants dominant in these respective soils. Unfortunately, until recently, no work has been recorded on soil metabolites, which makes this study the first to report such findings.

Growing *B. africana* has always been unsuccessful as it has proven difficult to transplant and grow until it reaches pod-bearing stage. The results of this study suggests that it could be due to the metabolites composition in the soils that ensures growth and survival.

The findings of this study is in agreement with other researchers, who found that trehalose have improved growth in tobacco (Romero *et al*., 1997). In addition, trehalose in rhizobia was found to be a key compound for signalling plant growth and yield in leguminous plants (Reina-Bueno *et al*., 2012). A study conducted on *Escherichia coli* revealed that increasing the production of trehalose resulted in increased growth under osmotic stress (Purvis *et al*., 2005). Betaine has also been reported to improve growth and yield of water-stressed *Nicotiana tabacum* (Aghoma *et al*., 1997b), *Glycine max*; (Aghoma *et al*., 1997c), *Triticum aestivum*; (Borojevic *et al*., 1980), *Zea mays*; (Agboma *et al*., 1997a) and *Bacillus subtilis* (Holtmann & Bremer, 2004).

Trehalose is known to play variety of roles, from being an energy source to a stress protectant (Gancedo & Flores, 2003), during osmostress and carbon starvation (Niederer *et al*., 1992; Sillje *et al*., 1999). Whereas, many naturally occurring betaines serves as organic osmolytes, substances synthesized or taken up from the environment by cells are used for protection against osmotic stress, drought, salinity or high temperature. Intracellular accumulation of betaines permits water retention in cells, thus protecting from the effects of dehydration (Wilshart *et al*., 2003). Furthermore, trehalose is also known and recognized as a fungal carbohydrate as discovered by Martin *et al*., (1988); Niederer *et al*., (1989). Both betaine and trehalose are known as reserve carbohydrates in microorganisms, primarily fungi (Martin *et al*., 1988; Sillje *et al*., 1999).

The results of the study also revealed that several compounds such as acetate, formate and lactate which are present in non-*Burkea* soils, are known to be produced by bacteria as reported by Babel *et al*., (1983). The compounds lactate, formate and acetate are therefore strongly linked to bacterial metabolism and probably evident of the bacterial presence in non-*Burkea* soils. Growth inhibition by formate has been reported for several methylotrophs, including yeasts (Swartz & Cooney, 1981; Babel *et al*., 1983) autotrophic bacteria (Dijkhuizen *et al*., 1977), and RuMP-type bacteria (Puhar *et al*., 1982). Furthermore, it has been reported that high concentrations of formate could reduce the pH gradient, and as a result, inhibit the growth of cells (Baronofsky *et al*., 1994; Herrero *et al*., 1985).

It is suggested that the presence and composition of soil microbes is important for producing the metabolites which may play an important role in protecting plants from different stress conditions or create unfavourable conditions for establishment and growth of seedlings. The results of this study suggests that *Burkea* soils probably contains microorganisms (fungi/bacteria) which release or metabolise growth promoting metabolites which influence growth, survival and protects the plants from adverse stress conditions. In agreement with the above, Mehta *et al*., (1961) discovered that the sources of sugars such as betaine and trehalose in soils are plants (the primary source), animals (the minor source) and microorganisms (fungi, bacteria algae). However, future research is recommended to determine the relationship between the growth promoting metabolites and soil microbial composition with regards to their role in growth as it has not been addressed before. In addition, there is a need to identify the microorganisms and understand their dynamics in soils as growth promoting metabolites releasers. On the contrary, non-*Burkea* soils lack microorganisms that produces metabolites, which are known to promote growth, osmoregulate stress, hence *B. africana* trees are unable to grow in these soils.

In contrast, other studies are in disagreement with the findings of this study which indicated that betaine did not improve growth and yield of stressed *Gossypium hirsutum*; (Meek *et al*., 2003), *Triticum aestivum*; (Agboma *et al*., 1997a) and *Brassica napus* (Makela *et al*., 1996). It is consequently suggested that it shoud be investigated if soils should be enriched with betaine and trehalose to ensure that *B. africana* seedlings are not subjected to any form of stress, triggered by the absence of these

metabolites, which may eventually cause their ultimate death before they reach the pod bearing stage.

4.5. CONCLUSION

Although metabolomics are applied in various studies to address numerous challenges, soil metabolomics studies are not that common. This study focused on soil metabolites and their effects on chemical composition of soils collected from different locations on the same reserve. Furthermore, the study also showed the difference in chemical composition of these soils and their possible significant contribution on the growth, propagation as well as re-establishment of *B*. *africana* trees. NMR was employed to investigate and detect untargeted metabolites in *Burkea* soils and non-*Burkea* soils. The results of this study demonstrated the availability and prevalence of metabolites such as trehalose and betaine known as growth-promoting metabolites in *Burkea* soils, probably contributing to the growth and survival in nutrients poor soils. The absence of these compounds as well as the presence of acetate, lactate and formate that are presumed to inhibit growth were only found in higher concentrations in non-*Burkea* soils.

Inoculation of soils when growing *B*. *africana* for commercial purposes, with growth-promoting metabolites (trehalose, betaine) to amend the soil for successful growing and establishment of *B*. *africana* is proposed and recommended.

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CHAPTER 5

LC-MS analysis of soil where *Burkea africana* grows

Abstract

The focus of the study was to determine the soil components present in *Burkea* soil as compared to non-*Burkea* soil using LC-MS analysis. LC-MS revealed presence higher concentration of numerous compounds known as essential amino acids and non-essential amino acids were found in higher dominance in *Burkea* soils versus non-*Burkea* soils.

Keywords: *Burkea soils*, essential amino acids, non-essential amino acids, growth

5.1. INTRODUCTION

Amino acids have traditionally been considered as precursors and constituents of proteins. Several studies have been conducted on amino acids and their role in plants and have been documented to be acting as osmolytes, regulating ion transport, modulating stomatal opening and detoxification of heavy metals. In addition, their roles also affect synthesis and activity of some enzymes, gene expression and redox-homeostasis (Rai, 2002).

A key driver of the use of liquid chromatography-mass spectrometer (LC-MS) for metabolomics is the technological advances in instrumentation (Lu *et al*., 2010). Metabolomic data provide a signature of the physiological state and reflect specific biochemical processes that were altered between mutant and wild-type lines or strains (Tolstikov *et al*., 2003). Metabolomics, in its most ambitious global form, tries to comprehensively analyse all known and unknown metabolites in a given biological sample (Griffiths *et al*., 2003).

This requires the ability to differentiate the targeted analytes from other interfering compounds, which may be achieved based on mass-to-charge ration on a mass spectrometer (MS) and retention time in chromatography. Mass spectrometry when combined with effective sample preparation and chromatographic separation, has high sensitivity and specificity, as well as a good dynamic range (Kimball *et al*., 2006; Want

et al., 2007; Rabinowitz, 2007; Rabinowitz & Kimball, 2007). In addition, mass spectrometry is a uniquely powerful technique for identifying and quantifying low-abundance components in complex mixtures (Bhattacharya *et al*., 1995; Castrillo *et al*., 2003; Villas-Boas *et al.*, 2005).

The key characteristics of the resulting LC-MS method include fast analysis due to the use of a small particle column, effective quantification of a broad range of known cellular metabolites, and simultaneous detection of unanticipated metabolites via untargeted analysis (Lu *et al*., 2010). MS-based techniques are more sensitive, in particular when using liquid chromatography (LC) connected to a tandem MS/MS for quantitative analysis in the multiple reaction mode (Gouveira-Figueira *et al*., 2014).

Tremendous progress in mass spectrometry based metabolomics, is the main reason it was used in this study as an advanced analytical tool to differentiate types of amino acids and other compounds found in *Burkea* soils versus non-*Burkea* soils, and to determine their effects towards the growth and re-establishment of *B*. *africana* trees.

5.2. MATERIAL AND METHODS

5.2.1. Sampling site

The study site is described in section 3.2.1 in Chapter 3.

5.2.2. Soil collection

Soil collection was done and stored as described in section 3.2.2 in Chapter 3.

5.2.3. Extraction method

Soil samples (10 µL injection) were analyzed on an Agilent Infinity 1290 LC equipped with a Waters BEH C18column (2.1 mm × 150 mm, 130˚A, 1.7 m particle size) coupled to an Agilent 6490 Triple Quadrupole system with Funnel Technology (Agilent Technologies, Santa Clara, CA, USA). Related lipid metabolites (NAEs and fatty acid glycerol esters) were analyzed in positive electrospray ionization mode, were analyzed in negative mode. The stable isotope dilution method was used to quantify the analytes, in which two types of internal standards were used mainly (i) deuterated internal standards (ii) 12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid (Yang *et al*., 2009).

5.2.4. Statistical analysis

Data was analysed using a one-way analysis of variance of SAS, statistical analysis software (SAS, 2010). When ANOVA indicated a significant P-values, means were separated using Duncan multiple range test.

5.3. RESULTS

5.3.1. Identification of compounds

5.3.2. Differences in metabolites found in *Burkea* soils versus non-*Burkea* soils

Higher ($p<0.05$) concentrations of aspartic acid, serine, 4-Hydroxyproline, glutamine, threonine, glutamic acid proline, lysine, guanosine, tyrosine, adenosine, isoleucine, phenylalanine , tryptophan and citrulline were shown in *Burkea* than in non-*Burkea* soils as shown in Table 5.1.

5.3.3. Similarities between *Burkea* soils and non-*Burkea* soils

The concentration levels of glycine, cytidine, adenine, leucine, acetyl carnitine and fumaric acid were similar ($p>0.05$) in *Burkea* and non-*Burkea* soils as indicated in Table 5.1.

Table 5.1: Metabolites concentrations detected in *Burkea* and non-*Burkea* soils using LC-MS quantification

	Soils		
Metabolites	***Burkea***	**Non-*Burkea***	**SEM**
Aspartic acid	54572[a]	16087[b]	1739.845
Serine	366826[a]	170977[b]	14905.66
4-Hydroxyproline	10540[a]	7643[b]	447.987
Glycine	54636	50491	6662.825
Glutamine	130176[a]	60259[b]	6662.825
Threonine	66867[a]	16025[b]	1224.934
Glutamic acid	82005[a]	13149[b]	4022.601
Citrulline	22565[a]	3089[b]	947.719
Proline	128680[a]	26096[b]	4530.320

Lysine	49075[a]	14903[b]	2984.130
Guanosine	81611[a]	36711[b]	2731.467
Cytidine	35296	34131	1692.545
Adenine	134064	125639	19526.543
Tyrosine	33072[a]	16720[b]	1379.953
Adenosine	768010[a]	547474[b]	18855.132
Isoleucine	322050[a]	72972[b]	6094.5899
Leucine	282818	257661	7148.160
Phenylalanine	137183[a]	35000[b]	7738.684
Acetylcarnitine	75057	63587	366.627
Tryptophan	41518[a]	15248[b]	1480.327
Fumaric acid	336355	3359 03	4209.594

a,b Means with different superscripts are significantly different (P<0.05)

SEM: standard error of mean

5.4. DISCUSSION

LC-MS results indicated that a total of 22 compounds consisted of essential amino acids such as phenylalamine, threonine, tryptophan, leucine, isoleucine and lysine; conditional essential amino acids such as arginine, cysteine, glycine, glutamine, proline and tyrosine; non-essential amino acids such as citruline, alinine, aspartic acids, asparagine, glutamic acid and serine; nucleobased amino acids such as guanosine, adenine, adenosine, cytindine; dicarboxylic acid such as fumaric acid as well as common non-proteinogenic amino acids such as 4-hydroxyproline compounds were found in both *Burkea* and non-*Burkea* soils, although in different concentrations as shown in Table 5.1.

Amino acids are basic structural units of proteins and peptides. Their presence in soils and organic matter (humus) is due to the breakdown of native proteins derived from plants, animals and microorganisms. They serve as substrates for soil enzymes involved in N turnover in soils. Amino acids may also serve as energy sources for soil microorganisms (Ivarson & Sowden, 1966) and as important sources of N for plants (Broadbent, 1984; Kumari *et al*., 1987). Zhang *et al*., (1999) discovered that glutamine can serve as an alternative nitrogen source, however, high concentrations of glutamine can inhibit growth. It is therefore, assumed that glutamine serve the same purpose as ammonia and nitrate which were found to be significantly predominant in *Burkea* soils and used as nitrogen sources as discussed in section 3.4 in Chapter 3. Addition of glutamine, which is relatively nontoxic (Grambor *et al*., 1968), would enable the cells to maintain a high growth rate for a longer period. The current findings are in agreement with Lipson & Monson, (1997), who discovered that amino acids may be an important N-source for plants in the form of nitrate and ammonium. Amino acids contain both C and N and are good substrates for microbial growth (Alef & Kleiner, 1988). The microbes in the soils are presumed to be important for the release of metabolites such as trehalose and betaine, which are known as growth promoting metabolites as discussed previously in Chapter 4.

No documented information is available on the effect of amino acids towards the growth of *B*. *africana* trees. Such information is needed to enable farmers to grow these trees successfully outside their natural habitat.

It is therefore determined that one of the many options to ensure survival of *B*. *africana* trees when grown outside its native soils, the new soils must be inoculated with compounds such as aspartic acids, serine, 4-hydroxyproline, glutamine, glutamic acid, threonine, citrulline, lysine, guanosine, isoleucine, phenylalanine and tryphtophan as nitrogen source as well as energy source required for growth. This will enable plants to adapt their metabolic process to the new conditions and enhance the growing mechanisms, which are not completely understood. In addition, amino acids will also protect *B*. *africana* trees against environmental stress such as osmotic pressure and salinity. Inoculating the new soils with glutamine in particular, which is equal to ammonium and nitrate in supporting growth as observed by Gamborg, (1970) would enable *B*. *africana* to maintain high growth rate for a longer period or until it reaches sexual maturity stage of pod bearing age. In addition, there is also a growing evidence that activity of amino acids such as glutamine plays an important role in regulation of microbial metabolism of N in soil ecosystems (McCarty, 1995).

Burkea africana is a leguminous tree, and symbiotic nitrogen fixation is an important source of nitrogen for growth. However, it has been proven that *B*. *africana* is known for its inability to fix nitrogen (unpublished data). The presence of amino acids, together with nitrate and ammonium has been known to inhibit nitrogen fixation, although the mechanism of this inhibition is still unclear (Vessey & Waterer, 1992). Mansour, (2000) also concluded that accumulation of nitrogen containing compounds such as amino acids under saline stress correlated to salt tolerance of plants.

All effects released by amino acids on different processes related to nitrogen utilization and plant growth can be integrated in a model, in which N uptake and plant growth would be regulated in a homeostatic way. Under normal growth condition, an adequate availability of free amino acids is necessary in order to meet any changes in protein synthesis and growth rates (Barneix & Humberto, 1996).

Some reports are available where accumulation of other free amino acids under drought has been shown such as aspartic acid, glutamic acid and glutamine in cotton (Hanower & Brzozowska, 1975), serine and aspartic acid in maize (Slukhai & Shvedova, 1972) and proline and glutamic acid in detached rice leaves (Yang *et al*., 2000). In addition, increase of proline content increased K^+ content and alleviated salt stress effects in growth of *Vigna radiate* cultures (Kumar & Sharma, 1989). The

presence of amino acids such as aspartic acid, glutamic acid and serine have been reported to promote flowering due to iron uptake by the plants (Tanaka *et al*., 1987). Based on the above findings, it is proposed that aspartic acid, glutamic acid, and serine which are highly dominant in *Burkea* soils are responsible for regeneration of growth in the event where *B*. *africana* trees is susceptible to drought.

5.5. CONCLUSION

The overview of findings of this study showed that plant growth is largely dependent upon environmental factors that change very fast, including nutrient and water supply, temperature and light intensity. LC-MS was able to detect 22 compounds in both *Burkea* and non-*Burkea* soils. Out of the ones identified, glutamine, threonine, glutamic acid, citruline, proline, guanosine, tyrosine, phenylalanine, threonine etc as shown in Table 5.1 were found to be significantly prevalent in *Burkea* soils. The role of these compounds in the soil is equivalent to that of nitrate and ammonium as N source to improve growth, serve as energy source, mitigating drought and salt stress and enhance soil microbe development. Maintaining the new soils and ensuring sustenance of these compounds will probably ensure the continuity of *B*. *africana* growth and establishment until they reach pod-bearing stage. It is hypothesized that the presence of these compounds found higher concentrations in *Burkea* soils could be attributed to different soil microbes from non-*Burkea* soils.

5.6. RECOMMENDATIONS AND FUTURE STUDIES

It is most likely that *Burkea* soils have all the necessary nutrients and microorganisms necessary for growth, which regress after 8 months and/or get lost through excavations and uprooting of seedlings. Excavating *B*. *africana* as well as disturbing the *Burkea* soils may lead to disturbance of microorganisms which are assumed to be the source of different types of amino acids. Changes in the soil environment may alter the concentrations and availability of essential, non-essential as well as conditional essential amino acids which are required in the regulation of many processes related to nitrogen metabolism of the plant. Inability to maintain the composition of *Burkea* soils maybe the reason which lead to ineffective growth of *B*. *africana*.

Inoculating the new soils with these amino acids will ensure that *B*. *africana* seedlings not only have continuous growth but reaches flowering stage. Suggestions for future studies to confirm the assumption arose from the current study include identifying the source of different types of amino acids prevalent in *Burkea* soils, determining the concentrations levels of the compounds found to be significantly higher in *Burkea* soils, the stage or time of inoculating as well as subsistence methods which should be used to ensure longer sustainability in the soils.

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CHAPTER 6

THE ROLE OF FUNGAL MICROBES SPECIES IN *BURKEA* SOILS

Abstract

The study investigated the microbial communities in the soil where *Burkea africana* trees grows successfully (*Burkea* soils) and how it varies from the soils where they do not grow (non-*Burkea* soils). DNA was extracted from the soil and a high throughput sequence bask local assignment search tool (BLAST) was used to analyze the microbial diversity (bacterial and fungal) and composition found in both soils, for a comprehensive understanding of the soil microflora. The results revealed that *Penicillum* species is prevalent in *Burkea* soils and was the main discriminant between the two soils. On the contrary, non-cultured fungi, which could not be identified, dominated the non-*Burkea* soils. The variances in soil composition suggests that species supremacy play a role in the growth of *B. africana* trees.

Keywords: *Burkea* africana, fungi, *Penicillium* sp, growth

6.1. INTRODUCTION

Soil cover almost all the terrestrial area on earth and have an indispensable ecological function in the global cycles of carbon, nitrogen and sulphur (Ulrich, 2008). Soils have arguably the highest diversity of microbial life on earth and represent a rich but still poorly explored reservoir of genetic resources with considerable potential for downstream industrial applications (Mocali & Benedetti, 2010).

A single gram of soil has been estimated to contain thousands to millions of different bacterial, archaeal and eukaryotic species (Torsvik *et al*., 2002, Gans *et al*., 2005 interwoven in extremely complex food webs. Communities of soil microbes carry out a multitude of small-scale processes that underlie many environmentally important functions. Microbial communities affect directly or indirectly the physico-chemical properties of soils through their metabolic activities. For instance, rhizosphere microorganisms including fungi can enhance plant growth by different mechanisms (Khan et *al*., 2009). Plants are in constant contact with a community of soil biota that contains fungi ranging from pathogenic to symbiotic (Broeckling *et al*., 2008).

Interactions between plants and soil microbes are highly dynamic in nature and based on co-evolutionary pressures (Dobbelaere *et al*., 2003; Duffy *et al*., 2004; Hamilton & Frank, 2001; Klironomos, 2002; Klironomos, 2003, Morgan *et al*., 2005; Morrissey *et al*., 2004; Reinhart & Callaway, 2006). With metagenomics technologies, new dimensions in the characterization of complex microbial communities have been reached. Metagenomics approaches are used to explore these resources through deep sequencing and cloning techniques (Vogel *et al*., 2009; Lefevre *et al*., 2008).

It was observed that *B. africana* trees are found and grow in clusters, normally in nutrient impoverished sandy soils, indicative of a soil interaction favouring the establishment and growth of trees in specific areas. It has always been a challenge to distinguish *Burkea* soils versus non-*Burkea* soils in addition to what propel *B*. *africana* trees to grow effectively in certain soils but fails to grow on other soils.

To date, no studies have investigated the presence and/or absence of microbes in *Burkea* soils, and their role in plant growth promotion thereof. Therefore, the aim of the chapter was to investigate and determine the microbial community diversity in *Burkea* soils (soils where *B. africana* trees grow) compared to non-*Burkea* soils (soils where *B. africana* trees does not grow) for a comprehensive understanding of their microbial composition and the contribution in the soils towards growth promotion of plants.

6.2. MATERIALS AND METHODS

6.2.1. Study site

The study site is described in section 3.2.1 in Chapter 3.

6.2.2. Soil collection

Soil collection was done and stored as described under section 3.2.2 of soil collection in Chapter 3.

6.2.3. Soil DNA extraction and fungal community analysis

Soil samples (500 mg) were subjected to DNA extraction using a NucleoSpin Soil DNA kit (Mo Bio Laboratories) according to the manufacturer's instructions. The soil DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and results confirmed with agarose gel before sending for PCR

(amplification and cloning of DNA) and sequencing at Inqaba Biotechnology industry, situated in Pretoria, South Africa.

6.2.4. PCR and sequencing

16S rRNA: regions were amplified in a 25 µl reaction tube using Q5 ® Hot start High-Fidelity 2x Master Mix (New England Biolabs, USA). An Amplicon library PCR was separately performed in replicate extractions. The DNA primers used was Truseq Tailed 341F and 785R. The Thermocycler settings for PCR amplification were as follows: (1) initial denaturation at 95°C for 2 minutes (2) 30 cycles of 95°C for 20 s (3) 55°C for 30 s (4) 72°C for 30 s and final elongation at 72°C for 5 minutes. The PCR products were purified using a Zymoclean gel DNA recovery kit (Zymo Research, USA). Purified amplicons were barcoded using the NEBnext Multiplex oligos for illumina indices. The indexed amplicon libraries were purified using the Agencourt® Ampure® XP bead protocol (Beckman Coulter, USA). Library concentration was measured using Nebnext Library quant kit (New England Biolabs, USA) and quality validated using Agilent 2100 Bioanalyser (Agilent Technologies, USA). The samples were pooled in equimolar concentrations and diluted to 4nM based on library concentrations and calculated amplicon sizes. The library pool was sequenced on a MiSeqTM (Illumina, USA) using a MiSeqTM Reagent kit V3 600 cycles PE (Illumina, USA). The final pooled library was at 10pM with 20% PhiX as control. Data of 2x300bp long reads per sample were produced. Different primers were used for bacteria and fungal sequence, the list of primers used for bacterial sequencing were: Truseq 341 FTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCTACGGGNGGCWGCAG and Truseq 785R ACACTCTTTCCCCACACGACGCTCTTCCGATCT GACTACHVGGGTATCTAATCC.

The list of primers used for fungal sequence were Truseq ITS1 FTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTTGGTCATTTAGAGGAAGT AA. In addition, ITS4 Truseq used was ACACTCTTTCCCCACACGACGCTCTTCCGATCTTCCTCCGCTTATTGATATGC.

On the High throughput sequence, BLAST search program (http: // blast.ncbi.nlm.nih.gov) was used to compare the nucleotide sequence similarity of the ITS region in related fungi. On the basis of the sequence similarity, the fungal isolates

were identified. This was also done to indicate the relatedness of microbial diversity found in *Burkea* and non-*Burkea* soils, using 16S rRNA gene sequencing for the all-inclusive understanding of the soil DNAs.

6.3. RESULTS

6.3.1. Bacterial community composition

Burkea and non-*Burkea* soils possess the same taxonomical kingdoms comprising of bacteria (99%), as the most abundant, followed by Archaea (0.05%), protozoa (0.01%) and unknown (which could not be identified or cultured) taxonomical kingdom (0.00%). Furthermore, the results also revealed that the main dominant phylum that ranked first consisted of unknown, unidentified and uncultured bacteria (99.03%) which could not be grouped with the sequence of known bacteria phylum classification. The second leading phylum in *Burkea* soils was *Proteobacteria (3.52%)*, which were distributed as *Alphaproteobacteria* (0.10%)*, Betaproteobacteria* (0.02%), *Deltaproteobacteria* (0.01%) and (0.01%*) Gammaproteobacteria.* The *Actinobacteria (1.33%)*, was ranked third, followed by *Acidobacteria* (0.76%), *Planctomycetes* (0.20%), *Firmicutes* (0.14%) and *Verrucomicrobia* (0.01%). The main and only bacterial difference between *Burkea soils* and non-*Burkea* soils was the presence of *Chloroflexi* phylum, which formed part of the phylum classification in non-*Burkea* soils.

6.3.2. Fungal diversity

6.3.2.1. Taxonomical kingdom and Phylum Classification

The results of the fungal sequence found in *Burkea* soils showed that kingdom classification was assigned as plantae (63.83%); fungi (29.75%); protozoa (4.06%); bacteria (0.99%) with (1.35%) reading counts remained unknown as shown in Figure 6.1.

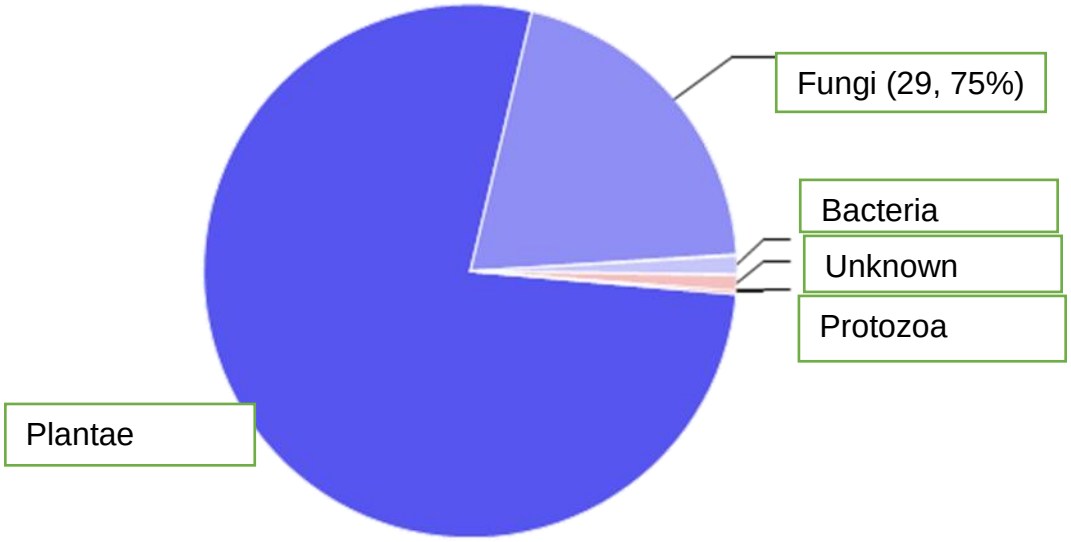

Figure 6.1: Representation of taxonomical kingdom in *Burkea* soils

In addition, the current study revealed that phylum classification had diverse soil communities, with Tracheophyta identified as the dominant phylum comprising of 63.82% in *Burkea* soils. The second prevalent was Ascomycota which is a fungal phylum with 16.63%, followed by Ciliophora which is a bacterial phylum with 4.06%. Furthermore, 15.04% of unknown phyla could not be categorised under any phylum.

On the contrary, kingdom classification in non-*Burkea* soils was dominated by Fungi (73.15%), followed by Bacteria (10.09%), Protozoa (9.78%) Unknown (5.52%) and lastly Plantae (1.45%). In addition to the above findings, the phyla classification showed a variety of soil microorganisms representing the different kingdoms mentioned above. Fungi Ascomycota (44.15%) proved to dominate, followed by unknown fungal phyla (36.36%), Ciliophora (9.78%), Basidiomycota (7.63%), Tracheophyta (1.36%), Glomeromycota (0.43%) and *Actinobacteria* (0.38%). Other phyla found in these soils made up of Bryophyta, Zygomycota, Chytridiomycota and Bacteriodetes (0.48%).

6.3.3.2. . Order and Family Classification

The current study states that Asparagales (63.81%) dominated *Burkea* soils followed by unknown fungal species at 16.82%. Fungi Coniochaetales and Hypocreales

showed percentages of 7.74% and 7.89% respectively. Furthermore, Spathiididae also formed part of the top order classification with 4.06%.

The occurrence of family Orchidaceae (51.90%) in *Burkea* soils revealed to be prevailing, with 33.29% unknown family as the second dominance. Bionectriaceae was ranked the third (16.01%), followed by Coniochaetaceae and Spathidiidae (1.39%). Several other families were found at the lower percentages namely Trichocomaceae, Hyaloscyphaceae, Netriaceae and Amphisphaeriaceae (0.49%) respectively.

On the contrary, non-*Burkea* soils showed that Eurotiales took dominance with 84.52%, and the family classification had unknown with 48.55% followed by Trichocomaceae (18.98%) and Sphathidiidae (14.65%).

6.3.2.3. Identification of species

The results of the present study, revealed that *Penicillium* sp highly dominated *Burkea* soils with 72.17%, followed by 22.53% *Clonostachys candelabrum* and Uncultured fungal species as demonstrated in Figure 6.2.

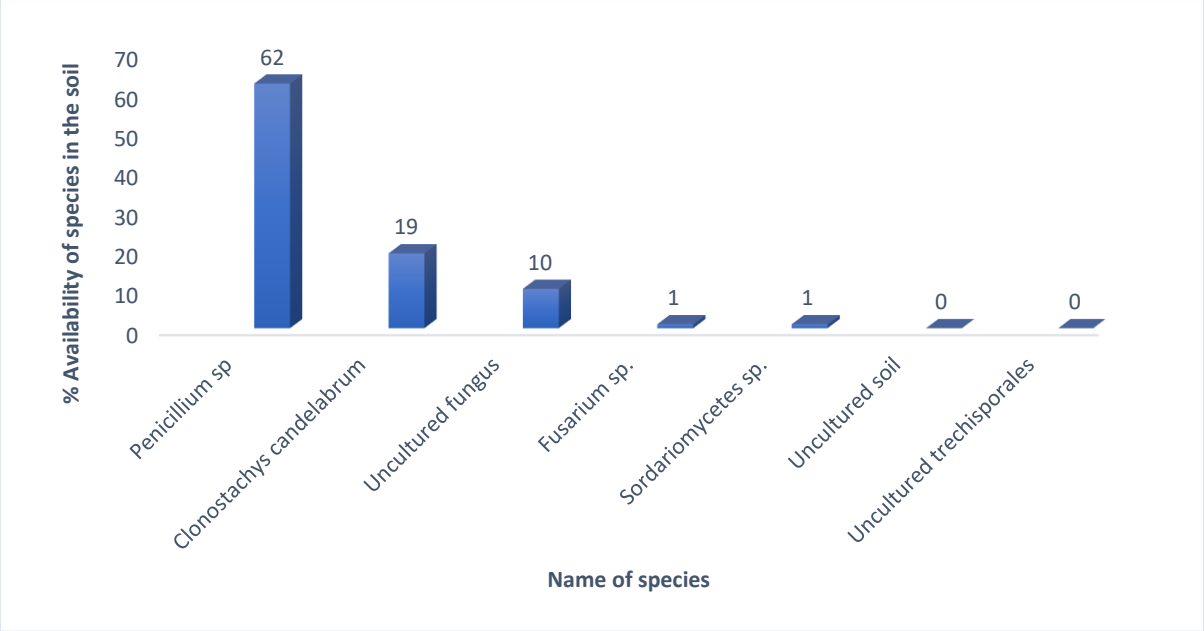

Figure 6.2: Representation of species in *Burkea* soils

On the contrary, non-*Burkea* soils was dominated by Uncultured fungi and Uncultured soils, which could not be categorized under any fungal species as shown in Figure 6. 3.

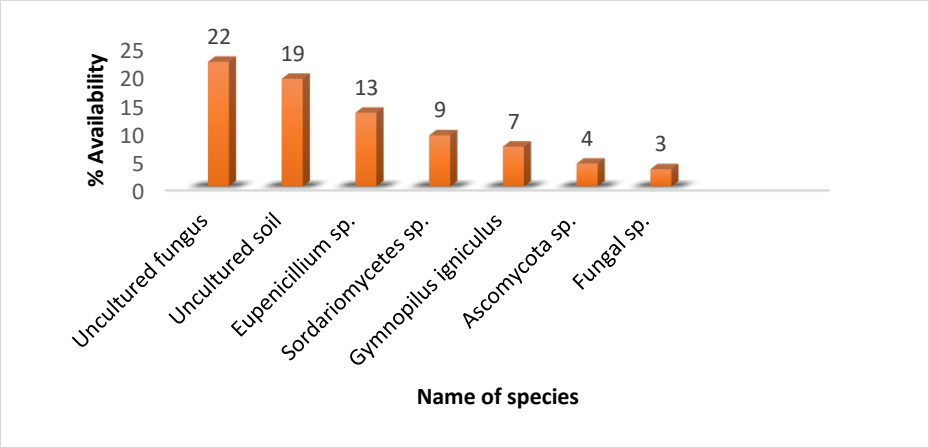

Figure 6.3: Representation of species in non-*Burkea* soils

6.4. DISCUSSION

Soil is a habitat for a vast, complex and interactive community of naturally occurring soil organisms, whose activities largely determine the physico-chemical properties of

the soil. Moreover, soil microbes perform important functions in agroecosystems including their role in plant growth promotion through mineral nutrition and control of phytopathogenic microbes. From seed germination until a plant reaches maturity, it lives in close association with soil organisms (Khan *et al*., 2009). Over the years, growing, transplanting and establishing *B*. *africana* seedlings outside their natural environment has proven to be difficult as they never reach maturity and eventually dies. The metagenomics approach has enabled the isolation of all untargeted groups of eukaryotes from soil samples collected where *B. africana* trees grow successfully and categorize them at different taxonomic, phylum, order, family, and species classification levels.

The microbial biodiversity between *Burkea* and non-*Burkea* soils investigated was compared to comprehend and identify soil microflora and their importance in the growth of *B*. *africana* trees. The results of the study showed that the bacterial community profile found in *Burkea* soils were remarkably similar to non-*Burkea* soils. The similarity appears at the phylum level, where 94.03% of bacterial community found were unknown and seem to be in abundance. The results of this study are in agreement with Amann *et al*., (1995); Torsvik *et al*., (1990) who revealed that soil microbial diversity is vast, and it is estimated that 99% of species remain unidentified.

The second dominant bacterial phylum was *Proteobacteria* (3.52%), followed by *Actinobacteria (1.33%), Acidobacteria* (0.76%), *Planctomycetes* (0.20%), *Firmicutes* (0.14%) and *Verrucomicrobia* (0.01%) in both soils. A study conducted by Jansen, (2006) stated that the most abundant bacterial phyla were *Proteobacteria* (39%) and *Acidobacteria* (19%), followed by *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomyceytes*, *Gemmatimonadetes*, and *Firmicutes*. Furthermore, numerous researchers discovered *Acidobacteria* as the most common in forest soils, possibly because the *Acidobacteria* species are slow-growing bacteria fit to nutrient-limited soils (Ward *et al*., 2009). Another study revealed that a dominance of *Proteobacteria* occurs in disturbed soils (Nusslein & Tiedgie, 1999). Furthermore, another study by Borneman & Triplet, (1997) revealed a different bacterial community which was dominated by phylum *Firmicutes*/*Clostridium* (22%), followed by *Acidobacteria* (18%), *Planctomycetes* (16%), and *Proteobacteria* (12%). From these results, it is proposed that different soils contain different bacterial composition, which may benefit or detriment the growth of plants. Therefore, the findings of the current study suggests

that soil bacteria present in *Burkea* and non-*Burkea* soils are not plant growth promoting rhizobacteria (PGPR) skilled enough to promote plant growth by colonizing the plant root (Cleyet-Marcel *et al*., 2001), and there is no symbiosis relationship with the roots of *B. africana* trees.

An important goal in ecology is to identify the strength of plant associations with their physical environments in order to understand the factors underlying the distribution and abundance of species (Schreeg *et al*., 2010). In this current study, 16S rRNA was used in metagenomics approach to investigate the microorganisms in understanding the below-ground patterns of biodiversity that exist across the soils, which may be worthwhile in comprehending the reasons for the existence and survival of *B. africana* trees, their growth promoters and why they are difficult to propagate. It was observed that, the degree to which soil nutrient and element associations are similar amongst closely related species is also of practical importance for phylogenetic investigations into the community structure.

A vast majority of biodiversity studies have recorded short-term effects, yet, only focused on grassland ecosystems (Reich *et al*., 2012). However, ecosystem is naturally dominated by woody plants, and it is estimated to cover nearly half of the land surface on earth (Tedersoo *et al*., 2016), and yet little information is known about the biodiversity of microorganisms in the soils. Compared with grassland plants, trees individuals live for several centuries, and are more widely spaced, creating a heterogeneous patch stem flow via, accumulation of roots and leaf litter (Nadrowski *et al*., 2010).

Species classification and identification clearly quantified the differences in microbial community and their composition in *Burkea* and non-*Burkea* soils respectively. A dominant fungal strain *Penicillium* sp was identified by means of BLAST analysis from 16S rRNA extracted from *Burkea* soils. *Penicillium* sp is a well-known and most common fungi occurring in a diverse range of habitats, from soil to vegetation to air, indoor environments and various food products (Visagie *et al*., 2014). Its main function in nature is the decomposition of organic materials, where species cause devastating decompositions as pre-and postharvest pathogens on food crops (Frisvad & Samson, 2004, Pitt & Hocking, 2009 , Samson *et al*., 2010) as well as producing a diverse range of mycotoxins (Frisvad *et al*., 2004). Its biggest impact is the production of penicillin

(antibacterial), which revolutionised medical approaches to treating bacterial diseases (Fleming, 1929; Chain *et al*., 1940; Abraham *et al*., 1984; Thom, 1945). Several reports have suggested that *Penicillium* sp interact with roots of crop plants to enhance the plant growth (Hyakumachi, 1994; Shivanna *et al*., 1994; Khan *et al*., 2008). This may imply that when excavating *B*. *africana* seedlings from their *Burkea* soils, *Penicillium* may remain in the soils, which will cause the death of roots as the symbiosis between soil and roots would have ceased or disturbed.

Soil is a primary source of fungal growth, and is associated with the roots of all plant species (Radhakrishnan *et al*., 2014). Fungal species provide different benefits to their hosts (van der Heijden & Kuyper, 2003) and more diverse communities are further efficient in the uptake of organic phosphorus (Baxter & Dighton, 2005). A study conducted showed that fungal species have demonstrated to respond to different primary and secondary plant metabolites that may function as carbon substrates and/or growth modifying signals (Broeckling *et al*., 2008). In addition, fungi supply inorganic nutrients to plants, such as ammonium, nitrate, as stipulated in section 3.3.2 under Chapter 3 of the current study, and phosphate (Seastedt *et al*., 2008), which are used as biofertilizers. These findings implies that the role of fungi in the *Burkea* soils is not only limited to promoting growth through an interaction with the roots of *B*. *africana*, but also to avail the anions needed as N source, in addition to carbon required for growth-promoting metabolites such as trehalose and betaine as stipulated in section 4.3.1.1 under Chapter 4.

Waqas *et al*., 2014, agrees with the findings of the current study, when they discovered that fungi produces a wide range of bioactive metabolites, which can improve plant growth (Waqas *et al*., 2014). In addition, they are also known as potent plant growth-promoting fungi, and secrete the plant hormones, indole-3-acetic acid (IAA) and GA, and are also involved in phosphate solubilization, which may be a reason they increase the plant growth (Khan *et al*., 2008; Radhakrishnan *et al*., 2014). Furthermore, some species of *Penicillium* are well known for their antagonistic activity against pathogens by producing antibiotics and induce resistance in plants by activating multiple defence signals (Hossain *et al*., 2007). In addition to the above functions, *Penicillium* can survive under environmental stress condition such as saline soil and promote plant growth against salt stress (Ahmad *et al*., 2011). The results of the present study coupled with other research findings suggests that *Penicillium* sp

performs the same role in the *Burkea* soils as trehalose and betaine as discussed previously in section 4.4 under Chapter 4, by producing antibiotics and induce resistance in the soils which enable plants to survive under environmental stress conditions such as saline soils and promote plant growth against salt stress as indicated by Ahmad *et al*., (2011).

Recently, Sartaj *et al*., (2011) reported that *Penicillium* EU0013 inoculation is capable of enhancing growth and protecting tomato plants against *Fusarium* wilt. The results of this study suggest that there is a positive synergetic relationship between fungi in the soil and the roots of *B*. *africana* trees, which promote and influence their growth from seedling stage to maturity. Excavating *B*. *africana* seedlings from their natural habitat may interfere and disturb their interaction with plant growth-promoting *Penicillium* sp, which may cause death of these trees if transplanted elsewhere due to the absence of *Penicillium* sp in the soil.

Several reports are in agreement with the findings of the current study, where the reports suggested that plant growth is enhanced by the application of soil fungi (Ruanpanum *et al*., 2010; Tchameni *et al*., 2011; Dutt *et al*., 2013).

In addition to the above mentioned benefits, Whiteside *et al*., 2012, has ascertained the role of fungal essential amino acids such as glutamine, non-essential amino acids such as aspartic acid as well as nucleobased molecule such as adenosine which are abundant in *Penicillium* sp culture medium, on seedlings growth. The above amino acids were found to be higher in *Burkea* soils as discussed in section 5.3.2 of Chapter 5.

6.5. CONCLUSION

Burkea africana tree productivity is mainly dependent on the symbiotic interaction with microorganisms. The lack of differences in bacterial diversity between *Burkea* soils and non-*Burkea* indicate that the bacterial composition is not affecting the growth and survival of *B. africana* trees. Overall, the main dissimilarity was found in fungal species known as *Penicillium* sp which was highly prevalent in *Burkea* soils. *Penicilllium* sp play numerous roles, such as a plant-growth promoter and enhancer by supplying inorganic nutrients (ammonium and nitrates); as sources of nitrogen; a releaser of plant-growth metabolites (trehalose and betaine); amino acids producing-fungi which

enhance seedlings root growth and a tolerant which makes plants to survive salinity and environmental stress. The results of the study suggests that treating the seeds and/or inoculating the non-*Burkea* soils with *Penicillium* sp might improve the growth and establishment of *B*. *africana* seedlings when grown outside their natural environment. It is therefore suggested that the absence of *Penicillium* fungal species could be the main reason for farmers not being able to grow *B*. *africana* seedlings until maturity stage.

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CHAPTER 7

The role of caterpillars *(Cirina forda)* in the growth and establishment of *B. africana* trees

Abstract

Burkea africana is known to be the only host for the caterpillars known as *Cirina forda*. The current study was therefore undertaken to determine what attracts *Cirina forda* to feed on *B*. *africana* trees and to determine if there is a symbiotic relationship between the plant-growth metabolites; growth-promoting fungi (*Penicilium* sp) and the caterpillars. The results of the study, revealed that the fungus *Pleurostomophora richardsiae* was predominant in the leaves of *B*. *africana* trees as well as in the caterpillars. It is proposed that *Pl*. *richardsiae* is a volatile compound which attracts caterpillars and makes *B*. *africana* trees susceptible to caterpillars' outbreaks. The second largest percentage of fungi found in the caterpillars was *Aspergillus nomius*.

Keywords: growth, *Pleustoromophora richardsiae*, *Aspergillus nomius*, leaves, symbiotic relationship

7.1. INTRODUCTION

Burkea africana trees occurs in various types of woodlands over a wide range of altitudes and habitants, but is most characteristic for hot, low-lying areas (Coates Palgrave, 1997). The leaves fall from May to September and new leaves flush from August to December (Fichtler, 2004). Flowers appear from August to November whereas fruit ripen from February to October but can remain on the tree for a very long time (Coates Palgrave, 1997; Storrs, 1995). In addition, *B*. *africana* is famous for being the host of caterpillars known as *Cirina forda* from November to January (Figure 7.1).

Figure 7.1: Caterpillars (*Cirina forda*) feeding on the leaves of *B*. *africana* trees (Photo taken by Nemadodzi L.E, 2016).

No information is available on why the caterpillars are attracted and feed on the leaves of the trees, and their role towards the growth and establishment of *B*. *africana* trees. The aim of the current study was to determine if there is a role of *Cirina forda* towards the growth and establishment of *B*. *africana* trees.

7.2. MATERIALS AND METHODS

7.2.1. Study area

The study area is described under section 3.2.1 of chapter 3.

7.2.2. Leaves and Caterpillars collection

The leaves and caterpillars were randomly collected from *B*. *africana* trees in November 2017. Samples were placed in bottles, and kept at -80°C before subjected to DNA extraction, purifying and sequencing.

7.2.3. Genomic DNA PCR and sequencing

Isolation of DNA and genomic DNA was sent to Inqaba Biotechnical Industries, a commercial NGS service provider, for sequencing. Briefly, genomic DNA samples were PCR amplified using a universal primer pair (341F and 785R - targeting V3 and V4 of the 16S rRNA gene). Resulting amplicons were gel purified, end repaired and illumina specific adapter sequence were ligated to each amplicon.

Following quantification, the samples were individually indexed, and another purification step was performed. Amplicons were then sequenced on illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit.

20Mb of data (2x300bp long paired end reads) were produced for each sample. The BLAST-based data analysis was performed using an Inqaba in-house developed data analysis pipeline.

7.3. RESULTS

7.3.1. Taxonomical classification of Caterpillars

The results of the study showed that the Fungal kingdom was most prevalent (96. 78%), followed by an unknown kingdom (3.17%) which could not be classified under any kingdom. Bacteria, Plantae and Protozoa had the least percentage of 0.03 and 0.01 respectively.

7.3.2. Phylum classification

The fungi Ascomycota was the most prevalent (94.02%), followed by an unknown phylum (5.90%). Other phyla such as Tracheophyta, *Proteobacteria*, Ciliophora had the lowest prevalence with almost undetectable levels.

7.3.3. Family classification

Pleurosmataceae dominated (60.08%), followed by *Trichocomaceae* (32.91%). The third and fourth family were unknown and not assigned to any classification, and were recorded at 6.08 and 0.45% respectively.

7.3.4. Species classification

The species which took predominance was the fungi, *Pleurostomophora richardsiae* (60%); the second dominant was *Aspergillus nomius* (32%) as demonstrated in Figure 7.2.

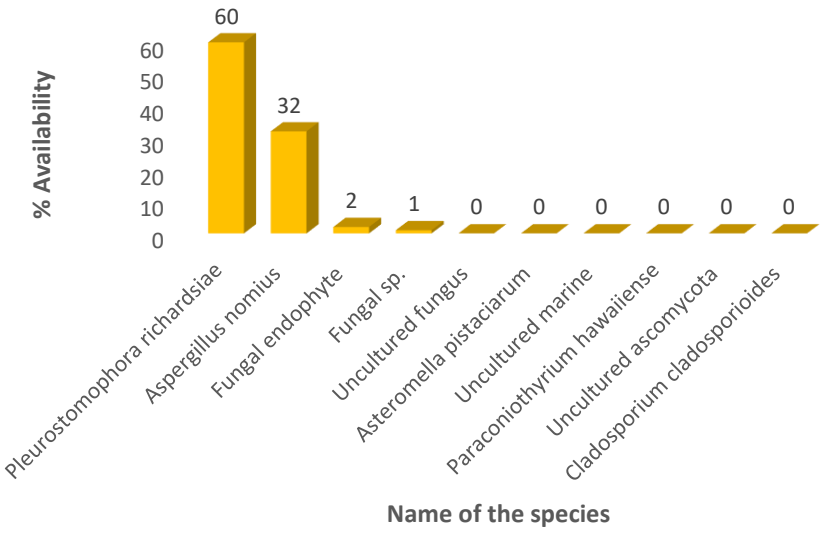

Figure 7.2: Representative of fungal species found in caterpillars (*Cirina forda*)

7.3.5. Taxonomical classification of the leaves

The results of the study showed that the Fungal kingdom was most prevalent (99.47%), followed by an Unknown kingdom (0.46%) which could not be classified under any kingdom, and Protozoa had the lowest percentage of 0.7%.

7.3.6. Phylum classification

The leaves of *B. africana* was dominated by fungi Ascomycota (94%), followed by an unknown phylum (5%), with other phyla such as Tracheophyta, *Proteobacteria*, Ciliophora at almost undetectable levels.

7.3.7. Family classification

Pleurosmataceae (72%) was found to be the most prevalent family, followed by *Togniniaceae* (14%), and *Polyporaceae* (6.05%).

7.3.8. Species classification

The species which took predominance was the fungi, *Pleurostomophora richardsiae* (72%); the second dominant was *Phaeoacremonium scolyti* (14%) as demonstrated in Figure 7.3.

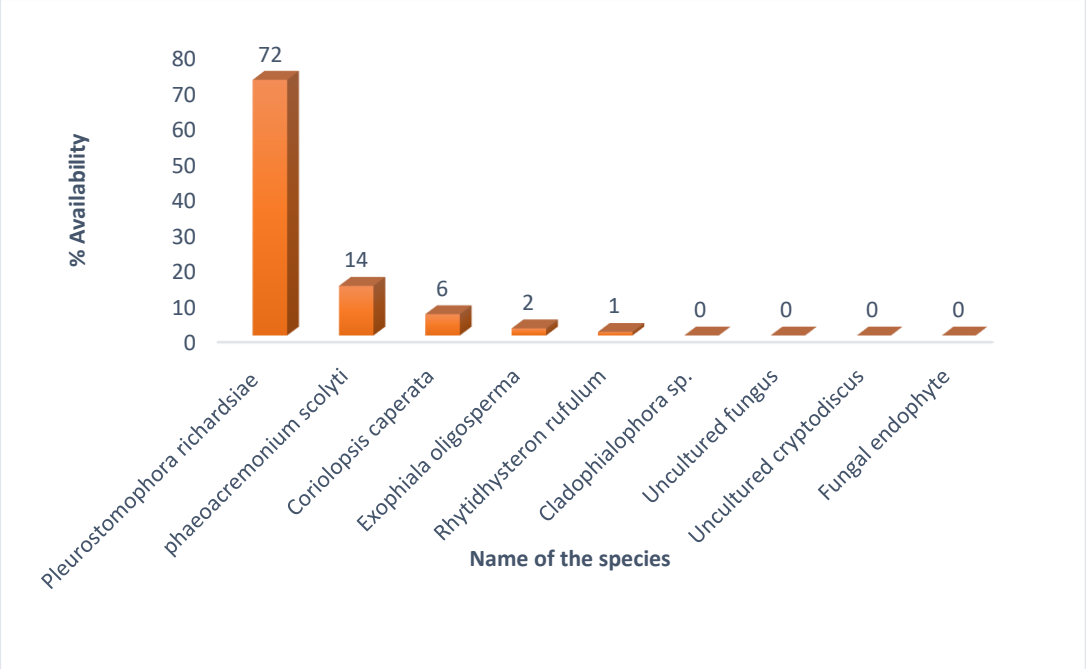

Figure 7.3: Representative species found in the leaves of *B. africana* trees

7.4. DISCUSSSION

Although a range of fungi were isolated from the caterpillers (*Cirina Forda*), two species *Pl. richardsiae* and *A. nomius* were consistently isolated with high percentages. *Pleurostomophora richardsiae* was abundantly isolated from the *Cirina forda* caterpillars. Interestingly, the same species was also found in high frequencies

in the leaves of *B*. *africana* trees. Therefore, it is suggested that this fungal species should be considered a leaf pathogen of *B*. *africana* trees.

Pleurostomophora richardsiae is an emergent fungal pathogen that has been associated with esca and petri disease in California (Rolshausen *et al*., 2010), and caused vascular discoloration after field and glasshouse inoculations similar to that seen In Petri-diseased grapevines in South Africa (Halleen *et al.*, 2007). *Pleurostomophora richardsiae* is a rare dematiaceous (dark-walled) fungus that was previously known as *Phialophora richardsiae* but has been recently renamed (St-Germain, 2011). It was first isolated from a patient with a *phaeomycotic* cyst in 1968 (Schwartz, 1968). It is found in the soil, decaying wood and vegetation (Levenstadt *et al*., 2012). Levenstadt *et al*., (2012), conducted a study which revealed that *Pl*. *richardsiae* was found to be dominant in the leaves. It is suggested that *Pl*. *richardsiae* found in the leaves of *B*. *africana* is the main reason of caterpillars pervading this tree. This is the first study to report that *Pl*. *richardsiae* is also found to be prevalent in the caterpillars (*Cirina forda*) which feeds on the leaves of *B*. *africana* trees.

This species is also considered the most aggressive pathogen among several other fungi (Olmo *et al*., 2015). Its aggressiveness maybe be related to the concentration level found in the leaves, which in turn causes severe mechanical damage during and after the caterpillars' invasion. The current study represents the first report of *Pl*. *richardsiae* to be the main factor of caterpillars infesting *B*. *africana* trees. However, there is no report of any damage to trees due to the presence of this fungus in the leaves of *B. africana*.

Aspergillus is an ubiquitous group of filamentous fungi spanning over 200 million years of evolution (Galagan *et al*., 2005). Among the over 185 aspergilli, there are several that have an impact on human health and society, including 20 human pathogens as well as beneficial species used to produce foodstuffs and industrial enzymes (Timberlake & Marshall, 1989).

Furthermore, *Aspergillus* is exceptional among microorganism in being both a primary and opportunistic pathogen as well as a major allergen (Casewdevall & Priofski, 1999; Denning, 1998; Greenberger, 2002). Its conidia production is prolific and so human respiratory tract exposure is almost constant (Latge, 1999). *Aspergillus nomius* produce carcinogenic secondary metabolites known as aflatoxins (Cotty *et al*., 1994;

Dorner *et al*., 1984; Kurtzman *et al*., 1987) responsible for hepatotoxic and immunosuppressive properties in humans and other animals (Williams *et al*., 2004) which may render agricultural products unusable as feeds and can lead to significant economic loss (Stoloff *et al*., 1991). *Aspergillus nomius* originally was considered rare, but recent studies indicate that *A*. *nomius* is widely distributed and might be of economic importance (Ehrlich *et al*., 2007; Olsen *et al*., 2008; Doster *et al*., 2009). The species is often associated with insects such as alkali bees (Hesseltine *et al*., 1970; Kurtzman *et al*., 1987) and Formosan subterranean termites (Rojas *et al*., 2001) and is frequently isolated from insects' frass in silkworm-rearing houses in Eastern Asia (Ito *et al*., 1998; Perteson *et al*., 2001). It is important to note that this is the first study to isolate and relate *A*. *nomius* to the caterpillars which feeds on *B*. *africana* leaves, although its origin of genetic diversity is yet unknown and therefore calls for further research. The assumption arose from the current study is also confirmed by Peterson *et al*., (2001) who reported that *A*. *nominius* is found in dead or diseased insects.

In addition soil populations in agricultural fields (Horn & Dorner, 1998; Ehrlich *et al*., 2007) suggest that *A*. *nomius* might contribute to aflatoxin contamination of crops. Aflatoxins are polyketide-derived furanocoumarins with known toxic, mutagenic, carcinogenic and immunosuppressive properties (Coulombe, 1991; Turner *et al*., 2003). It is due to these properties mentioned above that the presence of *A*. *nomius* in the caterpillars, produces aflatoxins which suppress the defense mechanism towards herbivores attack, which ultimately leads to caterpillar invading the trees and severely feed on the leaves. *Aspergillus nomius* has been reported from tree nuts (Olsen *et al*., 2008; Doster *et al*., 2009), sugarcane (Kumeda *et a*l., 2003) and an assortment of seeds and grain (Kurtzman *et al*., 1987; Pitt *et al*., 1993; Kumeda *et al*., 2003). A human case of ocular infection by *A*. *nomius* also has been documented (Manikandan *et al*., 2009). For instance, several aflatoxin outbreaks in humans, following consumption of contaminated grain, have been documented (Krishnamachari *et al*., 1975; Azziz-Baumgartner *et al*., 2005).

Crops infected with aflatoxigenic fungi produced by *A*. *nomius* are the main source for replenishment of soil populations, especially when colonized plant material is deposited onto the soil (Horn, 2007). This is most likely to occur in the *Burkea* soils where caterpillars drops onto the soils after feeding on the leaves and ultimately, their colonized dead bodies forms part of the microorganisms which play a huge role in

differentiating *Burkea* soils from non-*Burkea* soils. This could also be the reason why *B*. *africana* trees grows in clusters and yet refuses to grow from soils outside their natural environment. What could be seen as an attack through the infestation of *B*. *africana* trees by caterpillars is assumed to play a huge role in the microorganisms found in *Burkea* soils which in turn are known to influence the growth of *B*. *africana* trees.

The biggest question during the current study has been the source of infection of *Cirina forda* caterpillars which invade *B*. *africana* trees. Dicke & Guthrie, (1988); Ingram, (1994); King, (1994); Dowd, (1998) reported that adults of major insect pests of corn transport primary inoculum from the soil to plant foliage and developing fruits where eggs are laid. The above mentioned may explain the origin of *Pl*. *richardsiae*, which was found on the leaves, it is therefore proposed that it may be the fungal attractant to caterpillars. During feeding on the leaves of *B*. *africana*, the caterpillars may eventually become colonized by *A*. *nomius* and later fall to the ground, decay and in the process becomes a primary inoculum in the *Burkea* soils. This source of infection remains hypothetical and is yet to be confirmed with future research.

Aspergillus and *Penicillium* are two of the most economically important genera of fungi, which belongs to the family *Aspergillaceae*, a family belonging to the phylum *Ascomycota* (Houbrake & Samson, 2011), as mentioned in section 7.3.2 of the current chapter under results on 7.3. On April 14, 2012, the international commission on *Penicillium* and *Aspergillus* (ICPA) met in Utrecht, the Netherlands, and discussed the implications of the single-name nomenclature on *Aspergillus* and *Penicillum* taxonomy. Furthermore, *Penicillium* is most closely related to *Asperigillus* and these two genera share certain physiological features with each other and therefore are heat-resistant, and are able to grow on low water activity products (Houbraken *et al*., 2014). Dierckx, (1901) proposed the first infrageneric classification of *Penicillium* and introduced the subgenera *Aspergilloides*. Pitt, (1980) also agreed with the above proposal based on a combination of phenotypic characters and physiology. Houbraken & Samson, (2011) showed that *Penicillium* could be divided into two subgenera (*Penicillium* and *Aspergilloides*). *Penicillium* and *Aspergillus* are sister genera, due to sharing a common ancestor (divergence) (Crous et al., 2007).

It is due to the above mentioned, that it is proposed that *Aspergillus* found to be predominant in the caterpillars, is the same fungi as *Penicillium* which was prevalent in the soil as discussed in section 6.3.2.3 under Chapter 6. In addition, this implies that *Aspergillus* and *Penicillium* plays a massive part as a primary inoculums in the soil, and influence the growth and establishment of *B*. *africana*. The findings of the current study also presume that the presence of these two fungal species contribute tremendously to the difference between *Burkea* and non-*Burkea* soils. In the absence of a continuous introduction of inoculum into the soil by caterpillars, the fungal species are probably not maintained in the soil. The absence of *Aspergillus* and *Penicillium* would mean different soil composition which will not be conducive and favourable for continuous growth of *B*. *africana* thus ultimately causes the slow death of *B*. *africana* seedlings.

7.5. CONCLUSION

Pleurostomophora richardsiae predominantly found on the leaves of *B*. *africana* is assumed to the fungal attractant to caterpillars. *Aspergillus nomius* was correspondingly dominant in the caterpillars. Furthermore, *P*. *richardsiae* was also found in the caterpillars, which is attributed to the feeding on *B*. *africana* leaves. Based on the findings of the current study, it is concluded that *A. nomius* found in the caterpillars which invade *B*. *africana* is considered to play a tremendous substantial role in the growth and establishment of *B*. *africana* trees. In addition, *A*. *nomius* which is also known as *Penicillium* is assumed to be the main variance in soil composition between *Burkea* and non-*Burkea* soils. It is therefore, proposed that the presence of caterpillars on *B*. *africana* trees serves as an inoculation of *Burkea* soils through colonization of their dead bodies which in turn contribute to the microorganisms required to influence the growth and establishment of *B*. *africana*.

7.6. REFERENCES

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CHAPTER 8

GENERAL CONCLUSION AND RECOMMENDATIONS

Burkea africana grow in poor nutrients soils, particularly sandy-loam soils. Interestingly, *B*. *africana* grow effectively in its natural soils (*Burkea* soils) and is unable to grow in soils outside its natural habitat (non-*Burkea* soils). Over the years, numerous farmers and tree growers have struggled to grow these trees in vain (unpublished data). It is due to this reason that *B*. *africana* is not grown commercially and not found in nurseries. The current study was conducted to determine the factors that contribute to the successful growth and establishment of *B*. *africana*.

From the results of the current study, it is concluded that *B*. *africana* depends on ammonium and nitrate as forms of nitrogen essential for growth. Ammonium is a reduced form of nitrogen, whereas nitrate is an excess form of nitrogen. The combination of the two are the essential nutrients which were found to be predominant where *B*. *africana* trees grows (*Burkea* soils) and absent where they do not grow (non-*Burkea* soils). The absence of these two ions could lead to the growth of *B*. *africana* being distracted, underdeveloped roots, leaf turning yellow and ultimately death. In addition to inducing growth, nitrate and ammonium serve as protector against stress.

These ions are produced by growth-promoting metabolites known as trehalose and betaine. Trehalose serves as an indication for growth whilst betaine is responsible for improving growth. Furthermore, the two ions are necessary for mitigation of stressful conditions caused by salinity, drought related conditions, and excess freezing condition. Both betaine and trehalose are known as reserve carbohydrates in microorganisms, primarily fungi. It is presumed that the absence of these growth-promoting metabolites in non-*Burkea* soils is an indication that diverse soils possess different metabolites which may contribute or prevent growth of *B*. *africana*. Compounds such as acetate, formate and lactate were found to be leading in non-*Burkea* soils and are intensely associated with bacterial metabolism and possibly apparent of the bacterial existence in non-*Burkea* soils.

The results of this study also revealed a total of 22 compounds consisted of essential amino acids such as phenylalamine, threonine, tryptophan, leucine, isoleucine and lysine; conditional essential amino acids such as arginine, cysteine, glycine,

glutamine, proline and tyrosine; non-essential amino acids such as citruline, alinine, aspartic acids, asparagine, glutamic acid and serine; nucleobased amino acids such as guanosine, adenine, adenosine, cytindine; dicarboxylic acid such as fumaric acid as well as common non-proteinogenic amino acids such as 4-hydroxyproline compounds were found in both *Burkea* and non-*Burkea* soils, although in different concentrations. It is presumed that these different types of amino acids serve as energy sources for soil microorganisms, probably fungi and also as important of nitrogen for effective growth of *B*. *africana* trees. Glutamine is specifically known to serve the same purpose as nitrate and ammonium in addition to maintaining high growth rate *B*. africana for a longer period. It is therefore determined that one of the many options to ensure survival of *B*. *africana* trees when grown outside its native soils, the new soils must be inoculated with compounds such as aspartic acids, serine, 4-hydroxyproline, glutamine, glutamic acid, threonine, citrulline, lysine, guanosine, isoleucine, phenylalanine and tryphtophan as nitrogen source as well as energy source required for growth. It is anticipated that aspartic acid, glutamic acid, and serine which are highly dominant in *Burkea* soils are responsible for regeneration of growth in the event where *B*. *africana* trees is susceptible to drought. This will enable plants to adapt their metabolic process to the new conditions and enhance the growing mechanisms, which are not entirely understood.

Furthermore, it was identified and determined that soil microorganisms played a tremendous contribution towards the growth of *B*. *africana*. The fungal species *Penicillium* known to influence growth; produces stress reducing metabolites needed to regulate stress during the plant growth and secrets amino acids was found to be prevalent in *Burkea* soils. The results of this study suggest that there is a positive synergetic relationship between fungi in the soil and the roots of *B*. *africana* trees, which promote and influence their growth from seedling stage to maturity. Excavating *B*. *africana* seedlings from their natural habitat may hinder and disrupt their interaction with plant growth-promoting *Penicillium* sp, which may cause death of these trees if moved elsewhere due to the absence of *Penicillium* sp in the soil.

The current study also revealed that *Penicillium* is also known as *Aspergillus nomius* which is a fungal species detected in the caterpillars (*Cirina forda*) which infests *B*.

africana. It is therefore supposed that when caterpillars drops onto the soils, their dead bodies are deposited into Burkea soils where they add to the inoculation of the soils with *A. nomius* which in turn aids as a contribution factor in the growth and establishment of *B*. *africana*.

Aspergillus nomius, and *Penicillium* are the most important genera of fungi detected in the current study. Not only do they belong to the same family (*Aspergillaceae*), and phylum *Ascomycota* (Houbraken & Samson, 2011), but they also share diverse physiological properties and produce similar structures (Houbraken *et al*., 2014). It is therefore put forward that *Aspergillus nomius* and *Penicillium* is one fungus with different names.

In addition, the results of the current study revealed that the presence of caterpillars which invade *B*. africana trees is attributed to fungi *Pleurostomophora richardsiae* found in high prevalence in the leaves of *B*. *africana.* It is proposed that *Pl*. *richardsiae* serves as a volatile attractant fungi to caterpillars, which infest and feed on the leaves of *B*. *africana* trees.

In conclusion, the positive interactions mentioned above are all interconnected to the presence of fungi *Penicillium sp in Burkea* soil*. Penicilllium* is known to serve various roles, such as a plant-growth promoter and enhancer by supplying inorganic nutrients (ammonium and nitrates); as sources of nitrogen; a releaser of plant-growth metabolites (trehalose and betaine); amino acids producing-fungi which augment seedlings root growth as well as a tolerant which makes plants to survive salinity and environmental stress. It is therefore supposed that the presence of ammonia, nitrate, betaine, trehalose, amino acids, *Penicillium* is what characterize and distinguish *Burkea* soils from non-*Burkea* soils. The results of the current study consequently accept the initial hypothesis which assumed that there is a dissimilarity in the microbes composition between *Burkea* soils versus non-*Burkea* soils, which ultimately impact and positively contribute to the growth and establishment of *B*. *africana* trees. In addition, all objectives of the study as were achieved.

RECOMMENDATIONS AND FUTURE WORK

For effective growth and establishment of *B*. *africana* trees outside their natural environment, it is recommended that inoculating non-*Burkea* soils with *Penicillium* as a primary inoculum is recommended. Future work will involve advance research on the source of *Penicillium,* the concentration levels required in the soils as well as ways and means of ensuring the presence of *Pl*. *richardsiae* in the leaves of *B*. *africana* for attraction of caterpillars.