Cancer bush-microbe symbiosis and their nutrient cycling efficiency in Limpopo Province

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DECLARATION BY CANDIDATE

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TITLE OF PROJECT: Cancer bush-microbe symbiosis and their nutrient cycling efficiency in Limpopo Province

I, Thobile Perseverance Mkhwanazi, hereby declare that the dissertation titled 'Cancer bush-microbe symbiosis and their nutrient cycling efficiency in Limpopo Province' for Master of Science degree in Agricultural Science to be awarded is entirely my own work and has not previously been submitted in any institution of learning or another university for assessment of any postgraduate qualification. Sources that are used in fulfilment of the dissertation have been acknowledged.

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DEDICATION

To my loving parents, Mr BP and Mrs BI Mkhwanazi, grandmother, N Mngomezulu and all my siblings.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine Triphosphate
AMF	*Arbuscular mycorrhizal* Fungi
BNF	Biological Nitrogen Fixation
DAFF	Department of Agriculture, Forestry, and Fisheries
ECM	*Ectomycorrhizal* Fungi
FAO	Food and Agriculture Organization
GABA	Gamma Amino Butyric Acid
HIV	Human immunodeficiency virus
LCOs	Lipochitooligosaccharides
*Nod*D	Nodulation Factors (Nodulation protein D)
PGPR	Plant Growth-Promoting Rhizobacteria
PSM	Phosphorus Solubilizing Microbes
PSF	Phosphorus Solubilizing Fungi
SOM	Soil Organic Matter
SOC	Soil Organic Carbon

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ABSTRACT

Cancer bush (*Sutherlandia frutescens* (L.) R. Br) is one of most important medicinal plant native to Southern Africa. It is currently facing extinction threats due to overharvesting from the wild. In the wild, cancer bush grows mostly in arid terrains characterized by nutrient poor soils. As a results, the plant establishes symbiotic relationship with beneficial microorganisms such as the root-nodulating bacteria which through N-fixation and other nutrient cycling abilities is able to enhance growth and development of the plant. However, the cancer bush symbionts remain unknown, together with their specific roles. The current study intended to investigate the diversity of microbes in the root nodules of cancer bush across two sites (Tubatse and Makgupheng) in Limpopo Province over two seasons, winter and summer (Objective 1) and examine the rhizosphere microbial enzyme activities on N-fixation, C-cycling, and P-solubilisation, together with potential of soil physicochemical properties in influencing the above (Objective 2). To achieve Objective 1, the bacteria were extracted from cancer bush root nodules and their morphological and molecular characteristics were determined. Morphological characteristics were described based on colony color, shape, elevation, surface and margins. Molecular analysis was based on the isolation and sequencing of the bacterial 16S RNA ribosomal gene. The bacteria were further grown on Simmons citrate and Pikovskaya's media to test their ability to fix nitrogen and solubilize phosphorus, respectively. To achieve Objective 2, the calorimetric analysis method was conducted to determine rhizosphere soil bacteria extracellular enzyme activities on carbon (using the glucosidase and β-glucosaminidase tests), nitrogen (using the nitrate reductase test) and phosphorus (using phosphatase alkaline and phosphatase acid test). Soil chemical tests were further conducted to determine the total extractable micro-and macro nutrients in cancer bush rhizosphere soil, organic carbon, organic matter as well as soil pH. Based on morphological analysis, the study isolated a total of 30 bacteria species in winter and 70 species in summer

which were further classified into 16 and 13 different morphological taxa, respectively. Of the 100 isolates combined, a total of 19 symbiotic bacteria were successfully characterized based on molecular characteristics and were affiliated with 12 different genera: *Cellulosimicrobium*, *Sphingobacterium*, *Rhizobium*, *Bacillus*, *Micrococcus*, *Lysinibacillus*, *Alcaligenes*, *Stenotrophomonas*, *Enterobacter*, *Leucobacter*, *Serratia* and *Kosakonia*. In Makgupheng a total of 3 genera: *Serratia*, *Leucobacter* and *Sphingobacterium* were detected, while in Tubatse 4 genera: *Micrococcus*, *Alcaligenes*, *Rhizobium* and *Bacillus* were detected. A total of 5 genera: *Lysinibacillus*, *Stenotrophomonas*, *Cellulosimicrobium*, *Kosakonia* and *Enterobacter* occurred at both study sites. The *Stenotrophomonas* and *Leucobacter* spp. emerged as the dominant genera for Makgupheng, while *Stenotrophomonas* spp. was dominant in Tubatse for the winter season. During the summer season, *Bacillus* emerged as the dominant group in Tubatse, while *Serratia* was dominant in Makgupheng. The comparisons of the neighbour-joining (NJ) tree indicated a strong homology between the generated DNA sequences with those from NCBI database with 80 to 100% homology of the species identified. A maximum homology 99.66% was found with *Serratia* sp. (CP055161.1) and minimum homology 78.33% with *Lysinibacillus* sp. (FJ528593.1). The Shannon-Wiener diversity index (H′) showed that both localities had diversity indices greater than 1. The H′ value observed in Makgupheng was 1.61 in summer and 2.40 in Winter. The H′ value of Tubatse was 2.00 in summer and 2.04 in winter. The Sampson indices (Ds) in Tubatse were 0.94 and 0.93 for winter and summer, respectively. The Ds values observed in Makgupheng were 0.91 and 0.85 for winter and summer, respectively. Both populations were evenly distributed with a Pielou's evenness (*J*) value closer to 1. In addition, Tubatse, with 94% average had highest number of bacteria that tested positive (+) for nitrogen cycling test compared with Makgupheng, 90% average. The enzyme activities of bacteria in the soil were not significantly different. Moreover, no correlation was observed between soil edaphic factors, particularly primary nutrients and enzyme activities, except for P

alkaline with soil pH and K. No correlation was observed between season and edaphic factors, except pH. A strong correlation was observed between location and edaphic factors (soil nutrient availability). The soil analysis report showed that Makgupheng had low pH (5.43) (overly acidic) in summer and pH neutral (7.23) in winter. In Tubatse there were no significant differences in pH between seasons, the pH range was between 6.82-7.14. Soils from Tubatse had a higher Ca (> 10 $Cmol_c$/kg) and ECEC (15 - 25 $Cmol_c$/kg), slightly higher Mg (> 4 $Cmol_c$/kg), higher K (0.2 - 0.6 $Cmol_c$/kg) and clay (> 40%) content compared with Makgupheng which had low Ca (< 4 $Cmol_c$/kg), ECEC (< 15 $Cmol_c$/kg), Mg (0.5 - 4 $Cmol_c$/kg), K (< 2 $Cmol_c$/kg) and clay (< 15%). The P levels (0 - 5 mg. Kg^{-1}), organic carbon and organic matter percentage were relatively low (< 4%) for both sites in both seasons. Both site had low C: N, Makgupheng (12.57 :1) and Tubatse (11.09: 1), lower than the general C: N of legume plant soils (20: 1 or < 25: 1). Moreover, plants growing in Makgupheng derived most of their N from the atmosphere than plants in Tubatse as indicated by a higher percentage nitrogen derived from the atmosphere (%NDFA) in the leaves.

In conclusion, diverse group of PGPRs were isolated from the root nodules of cancer bush plants in both sites. Bacteria occurrence, abundance and diversity were more site (nutrient status and pH) specific rather than due to seasonal shift. Season indirectly influenced the abundance and diversity by altering soil edaphic factors (particularly pH). Soil pH was the major driving factor of bacterial diversity followed by total soil nutrient (carbon, nitrogen and with phosphorus to less extent). Neutral pH seems to support optimal growth and functioning of most organisms and also influenced enzyme activity. High ECEC increase nutrient availability in the soil, increase soil fertility thus supporting/providing good conditions for plant growth and microbial functioning. Lastly, the study reports on the potential role of these microbes in improving plant growth through nutrient cycling and acquisition mechanisms confirmed by the high % NDFA levels, specifically in plants from Makgupheng.

Key words: Extracellular enzymes, edaphic factors, medicinal plants, microbial community, nutrient acquisition, plant adaptation, PGPRs, *Sutherlandia frutescens*, symbiosis.

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CHAPTER 1

INTRODUCTION

1.1 Background to the study

'A healthy functioning soil is one that ensures nutrient cycling for optimum plant growth, however, agricultural productivity is often limited by available soil nutrients, especially nitrogen' (Mahmud, Makaju, Ibrahim & Missaoui, 2020). Soil nutrient availability is one of the major and crucial factors affecting ecosystem structure and function by altering biodiversity and richness (Fernandez-Martinezl *et al*., 2014). For instance, nitrogen (N) and phosphorus (P) availability in savannah grasslands influences the abundance of woody plants (Makhaye, Valentine, Tsvuura, Aremu & Magadlela, 2018).

Barber (1962) defines nutrient availability in soil as the concentration of available nutrients and the rate at which they move from the soil to the root surface of plants. Nutrient deficiency has a major effect on plant growth with low P availability having a greater impact on legume production,casing poor plant growth and development (López -Arredondo, Leyva-González, González-Morales, López-Bucio & Herrera-Estrella, 2014). Zhang, Liao and Lucas (2014) reported that legume plants have more demand for P as it is needed for optimal N-fixation and plays a major role in N transformation and regulation of enzymatic activities to enhance nodule formation. Researchers observed a significant correlation between the concentration of P in nodules and N-fixation (Mitran *et al*., 2018). Hence, the distribution rate of P may play an important role in determining the symbiotic efficiency as well as the degree of legume adaptability under deficient nutritional conditions (Sulieman & Tran, 2015).

Cancer bush (*Sutherlandia frutescens* (L.) R. Br.) is one of the leguminous medicinal plant of the Fabaceae family, well-known for its immense human medicinal importance (Prinsloo & Street, 2012). Cancer bush has been reported to treat several human health conditions such as fever, wounds, stomach-ache, internal cancer and type II diabetes (Prinsloo & Street, 2012).

Cancer bush is a widespread drought-tolerant plant, common in drier parts and mining areas of Southern Africa (Fu, 2012). These arid areas are characterised by nutrient deficiency soils and long periods of limited water supply (Emran, Rashad, Gispert & Pardini, 2017). As a results, majority of plants growing in these areas tend to develop strategies that enables them to grow under these stressful conditions and one of these ways is establishing beneficial symbiotic associations with soil microbes that help with nutrient acquisition (Sánchez-Castro, Ferrol & Barea, 2012). The association with specific rhizobia helps these plants to convert atmospheric nitrogen (N_2) into soluble nitrogen form (NH_3^+) that can subsequently be available for plant uptake and use (Zullo & Ciafardini, 2020; Datta, Singh & Tabassum, 2015). This symbiotic relation between soil microorganisms and legumes is reported to give a maximum contribution of the global N-fixation (Shengepallu, Gaikwad, Chavan & Anand, 2018).

As a legume, cancer bush has a symbiotic relationship with root nodulating bacteria in the soil that biologically fix nitrogen for its growth and development. The root nodulating bacteria together with other symbionts are often referred to as the plant growth promoting rhizobacteria (PGPR). The PGPRs present in the soil can directly facilitate plant growth through N-fixation, P-solubilisation and increased nutrient uptake through phytohormone production (Karthik, Oves, Thagabalu, Sharma, Santhosh & Arulselvi, 2016). The indirect mechanisms involve their bio-control properties such as antibiotic and lytic enzymes production. The PGPR in cancer bush remain unknown, hence the current study intended to identify and characterize the symbionts of cancer bush, their growth promotion abilities under two natural populations in Limpopo Province and determine the nutrient cycling efficiency by quantifying the extracellular enzyme activities in the soil.

1.2 Research problem

There is an increasing interest for farming cancer bush in smallholder farming systems and yet, limited investigations have been done on this plant and its cultivation (Makgato *et al*., 2020). Several studies of the interaction between plants and their microbiome have been done in search for growth-promoting organisms to use as biological inputs for agriculture (Zuluaga, Milani, Goncalves & Oliveira, 2020). The legumes-rhizobia symbiosis is the most fascinating mutualisms that makes a huge contribution towards terrestrial ecosystems and restoration of African soils (Teixeira & Rodríguez-Echeverría, 2015). It represents an alternative source of important nutrients such as N for plant uptake, use and maintaining their availability in the soil for sustained use (Teixeira & Rodríguez-Echeverría, 2015). However, only a small number of legumes have been analysed for nodulation, especially in natural areas without agriculture. Studies show that only about 57% of 650 genera of legume species have been studied for nodulation (Shengepallu *et al*., 2018). This lack of information is even more critical when the species is threatened. Cancer bush is among the many South African indigenous medicinal plants in which their diversity is threatened due to overharvesting from the wild that could possibly cause extinction in the near future, and therefore means to improve cultivation to preserve the plant are crucial (Masenya, Mashela & Pofu, 2022; Raimondo *et al*., 2009 cited in SANBI, 2010-2012. This highlight need to bring more of these species into cultivation to conserve them for future generations. To effectively preserve the plant through commercial production, an understanding of its symbiotic association with rhizosphere microorganisms, role played by microbes in enhancing the plant growth is required.

1.3 Motivation for the study

Exploring more legume plants can lead to the identification of many more beneficial microbes that can be used as agricultural inputs, to achieve sustainable agriculture (Shengepallu *et al*.,

2018). Most farmers have limited knowledge of the value of PGPRs and therefore, rely more on chemical fertilizers as a result. Zahran (2017) reported that the N-fixing rhizobia-legume symbioses have a great potential to improve yields and reduce the need for inorganic fertilizer use. Characterization and mapping of the soil microbial diversities of cancer bush will provide an understanding of the interaction between the organisms, accountable for nitrogen availability and other essential nutrients such as P needed for plant growth (Makgato *et al*., 2020). Some of the rhizobia bacteria not only fix nitrogen but also have solubilizing capabilities (Bouizgarne *et al*., 2015).

1.4 Purpose of the study

1.4.1 Aim

Identification and characterisation of cancer bush root nodulating bacteria in Limpopo Province, determination of their nutrient cycling efficiency and quantifying the soil extracellular enzyme activities.

1.4.2 Research objectives

i. To investigate the diversity of microbes in the root nodules of cancer bush across two localities (Tubatse and Makgupheng) in Limpopo Province over two seasons.
ii. To examine the effect of soil physicochemical properties on rhizosphere microbial enzyme activities of C-cycling, P-solubilisation and N-fixation.

1.4.3 Research hypotheses

i. It is hypothesized that there will be variation in the microbial diversity within the root nodules of Cancer bush plants between the two different localities (Tubatse and Makgupheng) in the two seasons study.

ii. It is hypothesized that there will be variation in the activities of rhizosphere microbial enzyme of N-fixation, P- solubilization and C- cycling and soil properties across the two different localities (Tubatse and Makgupheng) in Limpopo Province.

1.5 Reliability, validity and objectivity

Reliability is defined as the ability of a measuring instrument to give similar or consistent results when used at different times or used repeatedly and when the variables being measured have not changed (Sürücü & Maslakci, 2020).The differences that may exist at the time the measuring instrument is used and changes that may exist in the population or sample make it very difficult to get similar results every time the instrument is being used. However, a strong positive correlation between the measuring instrument is an indication of reliability (Sürücü & Maslakci, 2020). The present study used appropriate levels of statistical significance (LSD) at 5% probability during mean separation for measuring variability in the various experiments and E- values that were zero or less confirmed a strong confidence that the database match is a result of homologous relationships.

Validity shows whether the measuring instrument do measure what it is intended to measure, and how well (accuracy of results) it performs its function (Sürücü & Maslakci, 2020). To ensure validify, the present study used randomization and also increased replication of treatments (i.e. 1 000% bootstrap support) and appropriate models (maximum composite likelihood) to confirms the species or genus names to increase validity.

Objectivity thrives to avoid bias by basing the facts, research findings or judgements on verifiable data (Eisner, 1992). The results obtained from the study were discussed based on empirical evidence as shown by statistical analysis and compared with findings from other studies, to eliminate subjectivity.

1.6 Bias

Bias is as a form of systematic error that can affect scientific investigations and distort the measurement process (Sica, 2006). Simundic (2013) defines bias as any form of deviation from the truth in data collection, analysis and interpretation that could results to false conclusion. As suggested by Simundic (2013) and Gomez and Gomez (1985), to ensure that selection bias was minimized or reduced the study used randomization and increased replication of treatments. Randomization allows treatments/subjects an equal chance of being assigned to similar conditions, under which the experiment is conducted (Simundic, 2013).

1.7 Scientific contribution

The identification of PGPR strains of cancer bush, testing of their plant growth promoting abilities and efficiency in nutrients cycling will be a silver bullet when developing an effective nodulation bacterium which can be used as a potential bio-fertilizer or bio-control agent, thus reducing the need to use inorganic fertilisers that are expensive and environmentally unfriendly (Zuluaga *et al*., 2020). Shomi, Uddin and Zerin (2021) reported that these isolates can be used as bio-fertilizer candidates for soil fertility restoration and better crop response. Studies recommend the use of these defensive and growth promoting species on agricultural crops to achieve yield enhancement, environmental restoration and reduce the need for agrochemical inputs in regulating various pests and diseases (Duhan *et al*., 2020).

1.8 Structure of dissertation

Each chapter in this dissertation is a stand-alone chapter with its own sample of references formatted in Harvard referencing style as recommended by the University of Mpumalanga. Chapter 1 provides a general introduction to the research problem. Chapter 2 provides the work that has been done on the problem and work not yet done. An investigation on the diversity of

microbes in the root nodules and rhizosphere of the cancer bush, and their plant growth promoting traits are presented in Chapter 3. Chapter 4 presents the physicochemical characteristics of the collected soil and their influence on extracellular enzyme activities. Chapter 5 summarises the findings of all chapters and concludes the dissertation. This chapter also provides some recommendations for future research.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Sutherlandia frutescens (L.) R. Br commonly known as cancer bush is a legume plant that is indigenous to South Africa and other parts of Southern Africa (Nguyen, 2018; Ojowole, 2004). It is a member of the Fabaceae family which has 600 genera of plants with over 1200 species distributed all over the world (Ngcobo, Gqaleni, Chelule, Serumula & Assounga, 2011; Egbichi, 2009; Fernandes *et al*., 2004). Cancer bush obtained its name from reports by Khoi San and Cape Dutch folks, dating back to 1895 because of its use against internal cancer (Fu, 2012). The aerial parts of the plant such as the flowers, leaves, pods and barks as well as the underground part (roots) are boiled in water to create water infusion (tea) and decoction which is used as treatment for fever, cancer, diabetes, kidney/liver problems, rheumatism, stomach ailments, HIV/AIDS, many infectious or inflammatory diseases (Aboyade, Styger, Gibson & Hughes, 2014). This perennial shrub is mostly found in the Cape Floristic Region, specifically in the Fynbos Biome which is the home of many flowering plant species (Aboyade *et al*., 2014). Fabaceae (cancer bush included) is restricted to the arid terrains and rocky sandy parts along coastal areas of Southern Africa which are Lesotho, South Africa, Botswana, Namibia and Zimbabwe (Fu, 2012). Major distributions in South Africa are in the Western Cape region, however, the plant can also be found in certain parts of KwaZulu-Natal, Limpopo, Mpumalanga (Chen *et al*., 2016), Northern Cape and Eastern Cape Provinces (Aboyade *et al*., 2014).

Recently, there has been a higher demand for cancer bush-based products and plant parts, which increased harvesting of this plant from natural populations (Raselabe, 2017). This growing demand has triggered the increased interest in the cultivation of the medicinal plants by small-scale farmers to improve their livelihoods and sustain these resources for future

generation (Nwafor & van der Westhuizen, 2020). However, several challenges are still constraining farmers' involvement in the cultivation of cancer bush. Low germination rates and plant establishment have been identified as a major constrains when cultivating medicinal/wild plants (Canter *et al*., 2005) with dormancy as a major barrier to seed germination (Tsiantis, 2006). Previous studies investigated some of the agronomical tactics that can be implemented to improve cultivation of the plant, and one of these were to find ways to improve seed germination and crop establishment include the use of pre-sowing treatments (i.e. seed scarification) that break seed dormancy and stimulate germination (Mkhwanazi *et al*., 2023; Korth, 2021; Shaik, Dewir, Singh & Nicholas, 2008). Shaik, Dewir, Singh and Nicholas (2010) explored the biotechnological tactics which involved micropropagation of cancer bush from vegetative plant parts with the aim to reduce wild harvesting while improving *ex-situ* cultivation and resources of acclimatized plants. Raselabe (2017) investigated the effect of cultural practices (pruning and fertilizer application) on growth, biological activities, and chemical properties of the cancer and these were found to improve plant growth. Masenya et al. (2022) investigated the effect of rhizobia inoculation (both native and commercial strains) on growth and chemical composition of cancer bush. Their findings showed that native strain supports the plant growth than the commercial strain and have a potential of being used in cancer bush husbandry. A better understanding of the strategies to optimally cultivate and successfully commercialize cancer bush are very important. Currently, there is limited information about the symbiotic rhizosphere microbe interactions of cancer bush, growth promotion of strains and nutrient cycling efficiency of strains. As previously mentioned, cancer bush is a wild medicinal plant common in savanna region. These arid terrains are characterized by nutrient poor soils and prolonged water deficit (Colling *et al*., 2010). As a legume, cancer bush has formed a symbiotic relationship with the PGPRs in the soil that enables it to grow in such stressful conditions (Raselabe, 2017). This study intends to identify and characterize

cancer bush symbionts, their role in nutrient acquisition and plant growth improvement. Furthermore, the study aimed at investigating the impact of soil enzyme regulation and nutrient availability, especially nitrogen and phosphorus on legume (the cancer bush) production. The study also looked at how edaphic factors regulate or influence enzyme activities.

2.2 Characterization of nodulating bacteria in leguminous plants

Several studies on rhizosphere bacteria identification have been conducted using morphological, biochemical, and molecular approaches (Table 2.1). Rhizobia bacteria have been found to be the most common bacteria and isolated mostly from cultivated legume crops which include but not limited to soybean (*Glycine max* L.), cowpea (*Vigna unguiculata* L.), chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L) and red clover (*Trifolium pretense* L) (Ndusha, 2011). These bacteria include species belonging to the genera: *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Methylobacterium, Burkholdera, Cupriavidus, Devosia, Herbaspirillum, Ochrobactrum* and *Phyllobacterium* (Ndusha, 2011). All the listed genera belong to the Rhizobiaceae family which consist of 61 species distributed among 13 genera. According to Ndusha (2011), the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium, Allorhizobium* and *Agrobacterium* have the rising number of species, 53 species described, within the rhizobia genera. The group of micro-organisms within this genus are categorized together by virtue of their ability to colonize and nodulate roots of plants in the Leguminosae (Fabaceae) family (Ndusha, 2011).

Besides rhizobia, other non-rhizobial species have also been isolated from tissues of legumes, mostly in medicinal leguminous plants and these have been found belonging to *Aerobacter, Pseudomonas, Agrobacterium, Bacillus, Chryseomonas, Curtobacterium, Enterobacter, Erwinia, Flavimonas,* and *Sphingomonas* (Rajendran, Patel & Joshi, 2012), *Actinobacteria,*

Proteobacteria, *Acidobacteria*, *Azotobacter*, *Alcaligenes*, *Flexibacter*, *Cronobacter*, *Brevibacillus* and *Klebsiella* (Sumbul, Ansari & Mahmood, 2020; Dubnath *et al*., 2016; Singh, 2015; Shi, Yaun, Lin, Yang & Li, 2011). Most studies report that more than one rhizobia /non rhizobial strains occupy a single nodule, which is defined as dual (or multiple) nodule occupancy (Ndusha, 2011). Characterization of rhizosphere bacteria with such capabilities offer vital information in the development of effective local bacteria strains for maximizing productivity of plants (Gyogluu *et al*., 2018).

Makgato *et al*. (2020) investigated the potential of commercial *Rhizobium* inoculation on N-fixation, phytochemical profile and the effect on rhizosphere soil microbes of cancer bush. They observed that inoculation with commercial rhizobium did not have any significant effect on the plant biomass and N-fixation, however, the phenolics and flavonoids were significantly improved (Makgato *et al*., 2020). The antioxidant activities of the shoot extracts increased with increased levels of *Rhizobium* inoculation. The effect of the *Rhizobium* on the rhizosphere carbon source utilization profiles did not vary, depicting weaker ability in converting or degrading C, P and N profiles. Soil microbial enzyme activity describe the potential of soil microbe to degrade or convert substrates from an organic form into plant-available nutrients. Therefore, the lower microbial/ enzyme activities in the soil reported suggest a slower release of nutrient from organic substrates to substance that can be assimilated by plants (Makgato *et al*., 2020).

Table 2.1: Characterization of plant growth promoting microorganisms and their relationship with their host (medicinal plants)

Plant growth promoting microorganism	Host specificity	Relationship	References
Bacillus sp.	*Matricaria chamomilla* L., *Calendula officinalis L*., and *Solanum distichum Schumach.*	Biological control	Koeberl, Schmidt, Ramadan, Bauer & Berg, 2013
Actinobacteria, Proteobacteria Acidobacteria	*S. Saponaria*, *Fritillaria thunbergii*, *Rhododendron arborem*.	Nitrogen fixation	Shi *et al*., 2011; Garcia *et al*., 2016; Dubnath *et al*., 2016
Azotobacter chroococcum	*Chlorophytum borivillianum.*	Nitrogen fixation, Phosphate solubilization and Growth-hormone production	Solanki, Kumar & Sharma, 2011; Sumbul *et al*., 2020
Bacillus subtilis, *Pseudomonas putida*, *Alcaligenes* sp., *Bacillus megaterium*, *Enterobacter* sp., *Bacillus thuringiensis*, *Bacillus firmus*, *Pseudomonas rhizosphaerae*, *Flexibacter* sp., *Cronobacter sakazakii*, *Bacillus cereus*, *Brevibacillus agri* and *Klebsiella pneumonia*	*Ocimum sanctum*	Nitrogen fixation	Singh *et al*., 2015

2.3 Symbiotic plant-microbe relationships in indigenous plants that help with nutrient acquisition

Plants live in association with microorganisms such as bacteria, protozoa, fungi and nematodes which occur in both below and aboveground surface. Some of these microbes are beneficial to the plant while others have detrimental effects on plant health, growth and development (Kushwaha *et al*., 2020). The most dominant and known groups of microbes that have beneficial impact on plant growth belong to four families namely the Rhizobaceae (rhizobia species), Glomeromycota (*Arbuscular mycorrhizal* fungi, AMF), Hypocreaceae (*Trichodesmium*) and Basidiomycota (*Serendipita indica*) (Prasad, Chhabra, Gill, Singh & Tuteja, 2020). They can colonize roots of plants and establish a relationship that is beneficial to either the plant or both the organisms and the plant (i.e., can either solubilize P only, fix N or do both). Organisms from these groups possess either one or more than one factor of growth promotion. However, there are other groups (such as *Bacillus*, *Frankia*, *Burkholderia* etc.) that also possess the same functions, but they are not as dominant as organisms belonging to the above-mentioned groups (Thomas & Singh, 2019).

According to King (2021), plants form symbiotic relationships with these beneficial microbes for them to adapt well in their environment and for accumulation of nutrients as well as sustaining growth. The rhizosphere bacteria enhance plant productivity through various plant growth promoting activities such as nitrogen fixation, suppressing the growth of harmful microbes, solubilizing phosphorus, 1-Aminocyclopropane 1-carboxylic acid (ACC) deaminase activity, production of phytohormones as well as siderophore production (Singh *et al*., 2015; Ahmed *et al*., 2014), summarized in Figure 2.1. The diversity of the rhizobacteria is always plant-specific, implying that the rhizosphere bacteria community of legumes is influenced by the difference in plant species and soil characteristics. The most studied symbiotic microbial interaction is the endo-symbiotic interaction where plants develop root nodules in legumes

which is triggered by rhizobia (gram negative bacteria), and alpha proteobacteria are the most common microbial species that associate with legumes of the Fabaceae family (Hunter, 2016). Several symbiotic interactions that plants have developed with different microorganisms in the soil are further explained below:

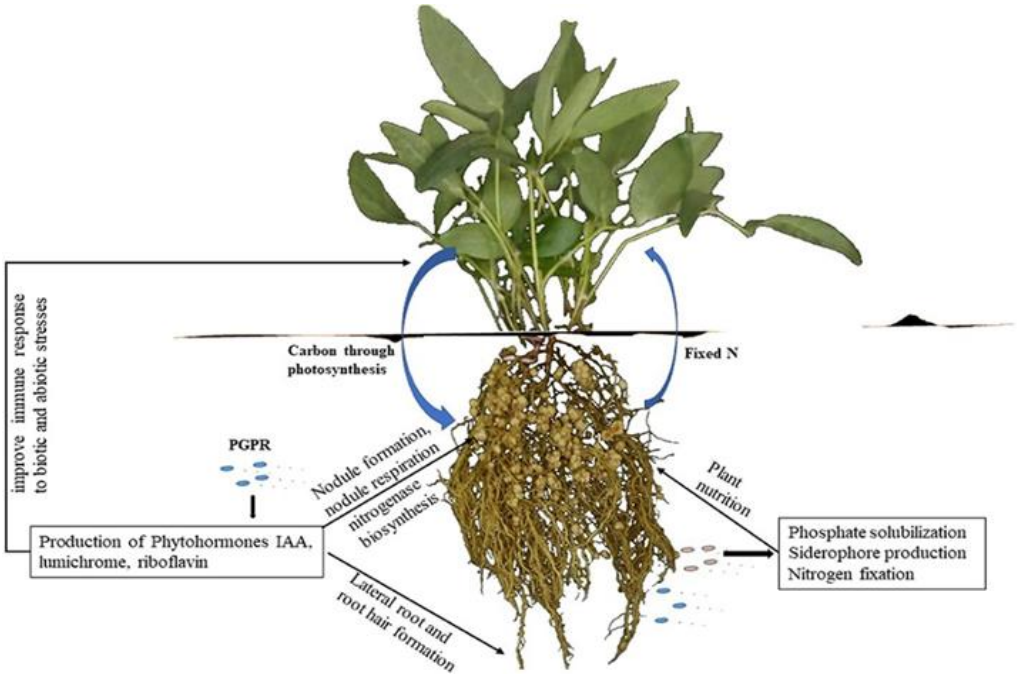

Figure 2.1: Summary of the symbiotic interactions between rhizobia bacteria and legume plants and the mechanisms of growth promotion (Jaiswal, Mohammed, Iby & Dakora, 2021).

2.3.1 Symbiotic biological nitrogen fixation

Biological nitrogen fixation (BNF) is a process that involves the reduction of the inert N_2 into reactive compounds (specifically, ammonia-$NH3^+$) that can be assimilated by plants (Figure 2.2 & 2.3) (Mabrouk *et al*., 2018). In this process, N_2 is combined with H^+ from water molecules to form ammonia (Soumare *et al*., 2020). Biological nitrogen fixation is a high energy demanding process whereby 16 ATP molecules are needed to break one molecule of N_2 and an additional 12 ATP molecules for assimilation of NH^{3+} (Soumare *et al*., 2020). Until this gaseous substance is reduced, plant cannot use it. The process is mediated by enzyme

activities that are bound with soil bacteria called the nitrogenase enzyme as illustrated in Figure 2.2 (Bellenger, Darnajoux, Zhang & Kraepiel, 2020). Nitrogenase is a protein complex composed of enzymes with metal co-factors. There are three of these enzymes that serve as catalytic components, according to their active site co-factor binding metal. Molybdenum MoFe-cofactor for Mo-nitrogenase or the conventional enzyme is the most significant in terms of nitrogen fixation and is found in all diazotrophs while some other photosynthetic bacteria such as the Azotobacter and cyanobacteria their cofactors are Iron (FeFe-cofactor) and Vanadium (FeV-cofactor) for Fe-nitrogenase and V-nitrogenase, respectively (Bellenger *et al*., 2020; Bhat, Ahmad, Ganai, Haq & Khan, 2015). These metal factors are encoded by *nif* genes, D and K for Mo and H for Fe (Soumare *et al*., 2020). The dinitrogenase reductase (Fe-protein) produce high power reducing electrons that the dinitrogenase (metal-cofactor) then uses to reduce N_2 into NH_3 (Soumare *et al*., 2020).

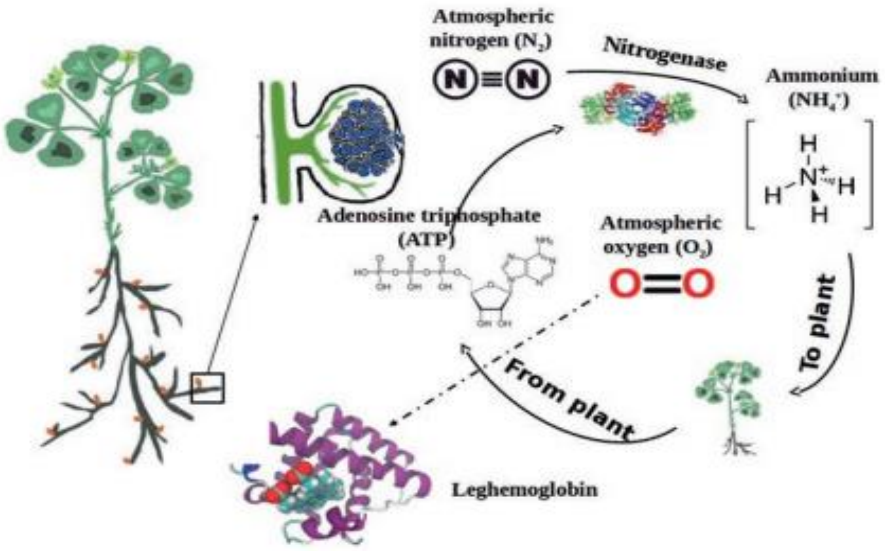

Figure 2.2: Schematic representation of symbiotic nitrogen fixing legume plants through the activities of nitrogenase enzyme (Klenert, Thuysma, Magadlela, Benedito & Valentine, 2017).

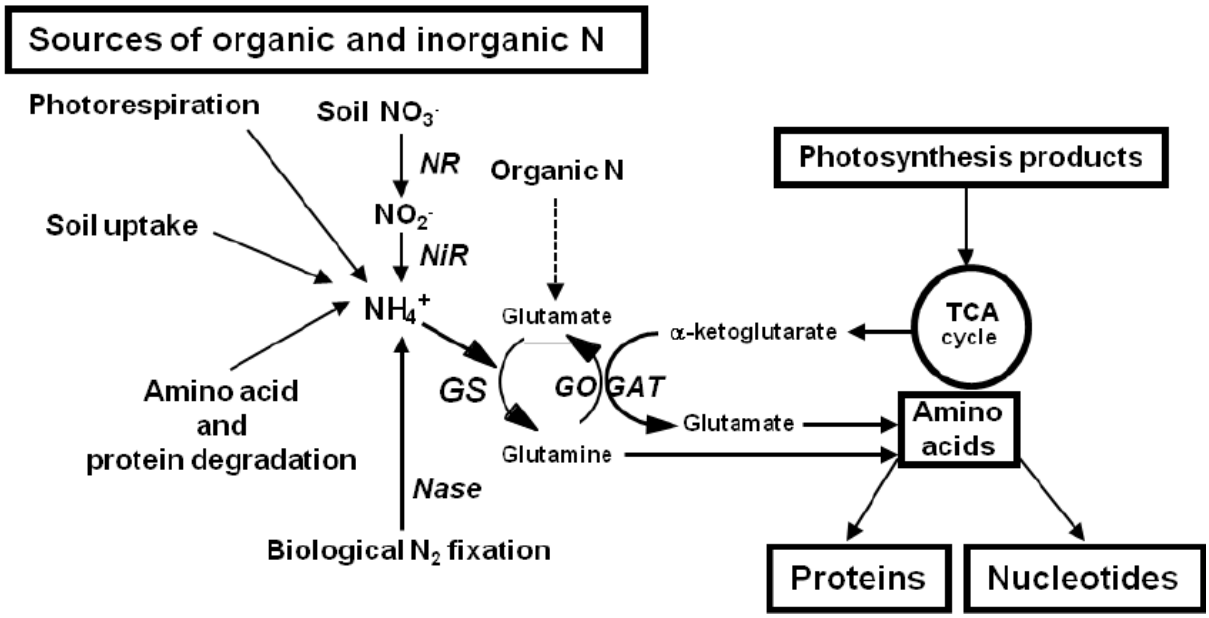

Figure 2.3: Nitrogen assimilation pathway in plants from soil uptake to photosynthetic products (Hirel, Tetu, Lea & Dubois, 2011).

Biological nitrogen fixation allows plants to supply all or part of their requirements through interactions with endo-symbiotic, associative, and endophytic symbionts, thereby offering a competitive advantage over any non-nitrogen-fixing plant. However, BNF it is a very sensitive process influenced by many factors such as the environmental and nutritional conditions, for example it is susceptible to O_2 (Mabrouk *et al*., 2018). The nitrogenase becomes inactive or destroyed when exposed to O_2 (Mo-nitrogenase is slightly sensitive while the Fe and V-nitrogenase are extremely susceptible to O_2) (Soumare *et al*., 2020). Hence, the fixation of nitrogen is achieved during darkness in the absence of O_2 production. However, there are bacteria species that require O_2 to function, such as chemotrophs and phototrophs (Soumare *et al*., 2020). Bacteria species in these two groups, chemo and phototrophs have developed strategies that can enable them to fix N_2 while avoiding the inhibitory effect and protect the nitrogenase. This includes the development of the heterocyst that has thick walls to protect the enzyme against O_2 or some they separate the O_2 they produce in their system from the enzyme.

Some bacteria (*Azotobacter*) maintain a very low O_2 level inside their cell by expressing high rate of respiration. Diazotrophs are able to fix N_2 under anaerobic or microaerophilic conditions (Soumare *et al*., 2020).

Biological nitrogen fixation is an efficient source of nitrogen, as most of N added into the soil is from biological fixation (Mabrouk *et al*., 2018). The usage of micro-organisms that can biologically fix nitrogen for plant uptake and use, provides a great practical significance as this makes is possible to bridge the restrictions to chemical fertilizer use that has resulted in unacceptable levels of water pollution and destroyed soil and terrestrial ecosystems (Mabrouk *et al*., 2018).

Importance of biological nitrogen fixation (BNF) for soil fertility

Nitrogen is a very important element required by plants for the development, plants need it for the synthesis of macromolecules such as amino acids, nucleic acids and for chlorophyll, important compound in the development and survival of plants (Mekonnen & Kibret, 2021). However, approximately 80% of this valuable nutrient is floating in the air and plants cannot assimilate it in this form unless it is reduced (Bhat *et al*., 2015). Soil microorganisms assist in the conversion process, and these include groups of free-living nitrogen fixer, associative nitrogen fixers and symbiotic nitrogen fixers (Prasad *et al*., 2020). The last two groups of PGPRs are associated with legume species while that free-living occurs in non-legume plants as well. The most-studied and longest-exploited PGPRs are the rhizobia (including *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) for their ability to fix N_2 in their legume hosts (Tailor & Joshi, 2014). Plants belonging in the Fabaceae, Poaceae, Asteraceae, Brassicaceae, Asteraceae, Crassulaceae and Solanaceae families are associated with PGPRs, with the Fabaceae family being the most studied and their symbiotic association with nitrogen-fixing endophytic bacteria have well been

characterized (Ramakrishna, Yadav & Li, 2019). The root nodule symbiosis is one of the most studied mutualistic relationships of plants and nitrogen-fixing organisms and is the most superior among all other nitrogen-fixing systems because of its fixing potential (Mus *et al*., 2016). The symbiosis is responsible for maximum global nitrogen fixation, contributing 60% of N added into the soil (Mabrouk *et al*., 2018).

Nodule initiation and formation process during N-fixation

Plants symbiosis with rhizobia is a complex process that involves several steps to be initiated (Mabrouk *et al*., 2018). This relationship occurs through chemical signalling, the plant secretes biomolecules such as flavonoids and isoflavioids into the rhizosphere region which are then taken by the bacteria and signals, then bind the transcriptional regulator *Nod*D which activates the bacteria nodulation genes (Mabrouk *et al*., 2018) (Figure 2.4). These genes are responsible for the production of lipochitooligosaccharides (LCOs) also referred to as Nod factors which are responsible for nodule organogenesis that occurs later during root hair infection by the bacteria (Mabrouk *et al*., 2018). After Nod factors have been produced, the bacteria are entrapped by root hair curling which result in the formation of infection thread that facilitate the penetration of root hair and adjacent cortical cells (Mahmud *et al*., 2020). During the division of these cortical and pericycles, nodule primordium is formed (Mahmud *et al*., 2020).

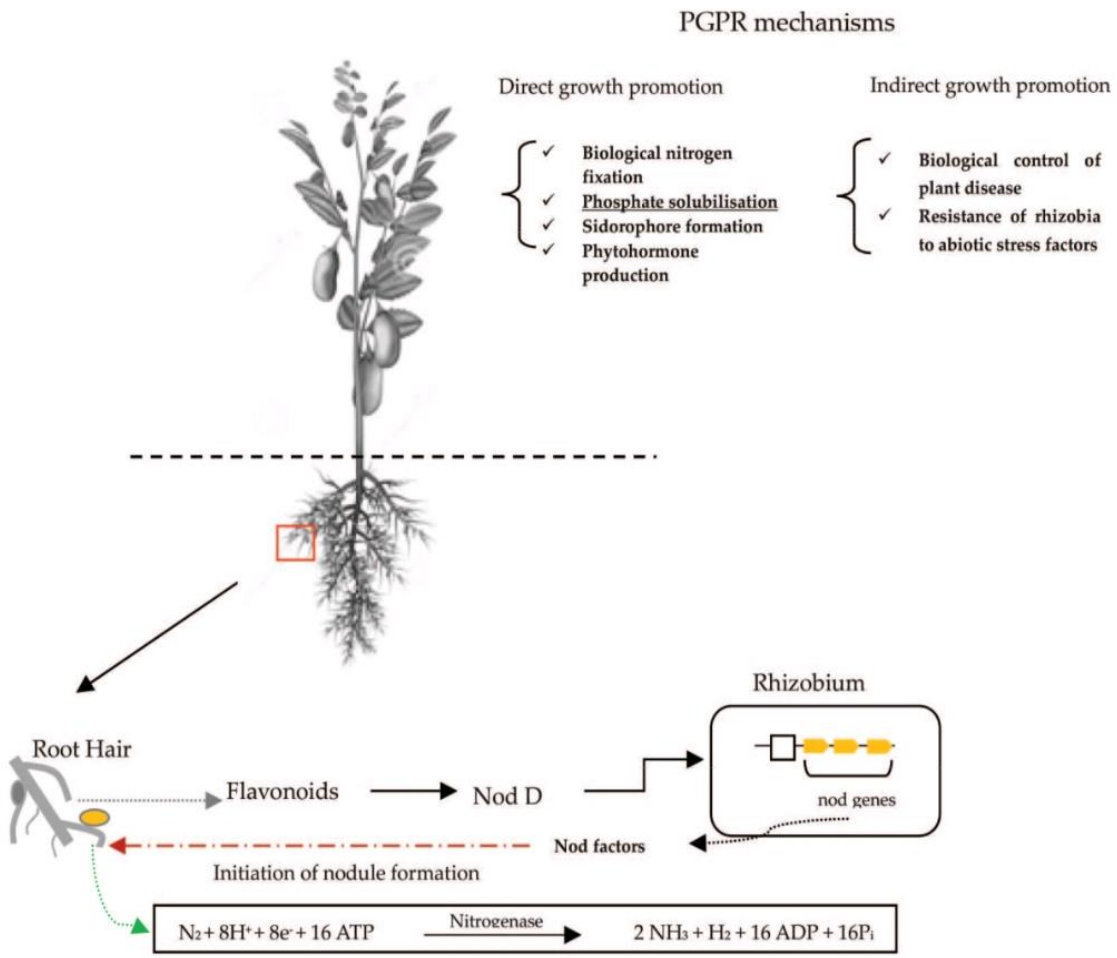

Figure 2.4: Root nodule initiation, formation and plant growth promotion by PGPR (Mabrouk *et al*., 2018).

Role of non-rhizobia bacteria in BNF

Several studies have proven that non-rhizobial bacteria (Figure 2.5) have the capability to contribute to legume-rhizobia symbiosis and plant nitrogen through various action mechanisms shown Figure 2.6 below (Etesami, 2022; Martínez-Hidalgo & Hirsch, 2017; Xu, Zhang, Wang, Chen & Wei, 2014). These organisms have been found to produce *nif*H and *nod* genes within them which are important for nitrogen fixation and nodulation. Species that have been identified include but not limited to *Bacillus, Frankia*, *Burkholderia*, that also possess the same functions, but they are not as dominant as organisms belonging to the rhizobia groups (Thomas & Singh, 2019). *Bacillus* species that have been characterized include *Bacillus subtilis*, *Bacillus*

megaterium, *Bacillus thuringiensis*, *Bacillus firmus* and *Bacillus cereus* (Sumbul *et al*., 2020; Singh *et al*., 2015; Solanki *et al*., 2011). Species such as *Azotobacter chroococcum* (Garcia, 2016; Shi *et al*., 2011), *Brevibacillus agribacterium*, *Klebsiella pneumonia*, *Pseudomonas putida*, *Alcaligenes* sp., *Enterobacter* sp., *Pseudomonas rhizosphaerae*, *Flexibacter* sp., and *Cronobacter sakazakii* have also been identified (Sumbul *et al*., 2020; Singh *et al*., 2015; Solanki *et al*., 2011).

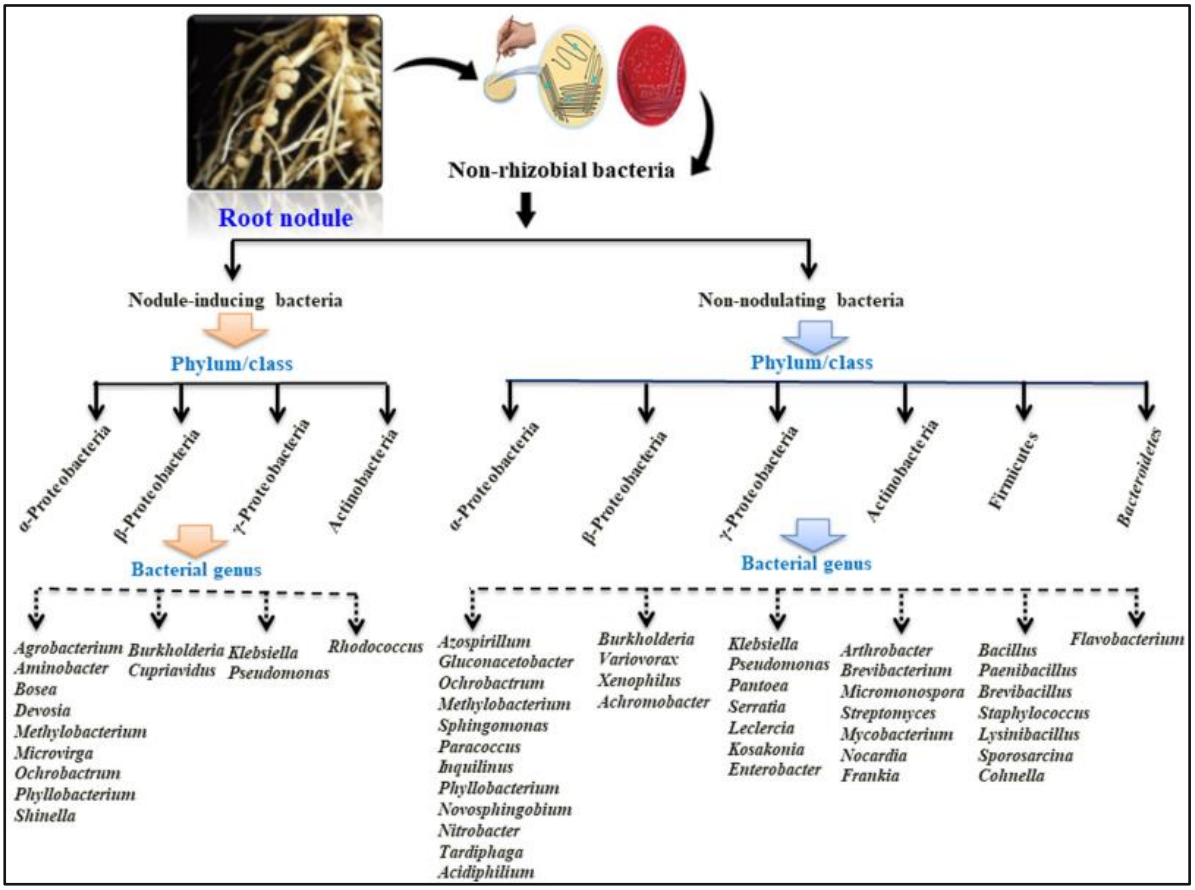

Figure 2.5: Nodule nodulating and non-nodulating non-rhizobia bacteria associated with various legume plants (Etesami, 2022).

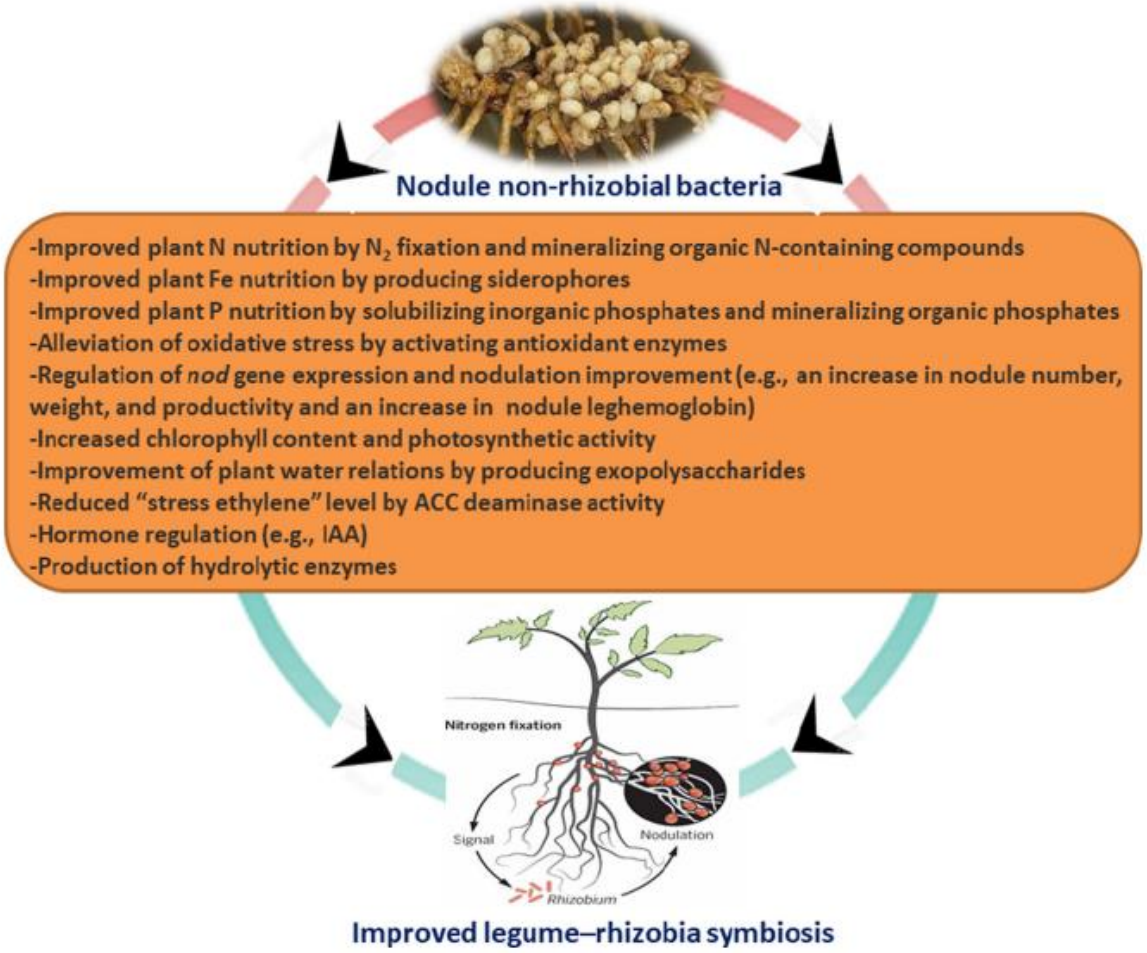

Figure 2.6: Action mechanisms of rhizobial bacteria in improving legume-rhizobia symbiosis and plant nitrogen (Etesami, 2022).

Effects of soil acidity on BNF

Majority of the soil worldwide is acidic, approximately 40% of the arable agricultural lands are considered acidic (Makaure, 2022). Such conditions lead to the retention of essential nutrients, most importantly P and a resultant increase in ions like Mn^{2+}, Al^{3+} and Fe^{3+} which cause poor productivity (Ferguson & Gresshoff, 2015). The Al^{3+} is mostly dominant in highly acidic (pH < 5.5) soil and this may hinder cation uptake thereby impairing root and plant development (Kopittke *et al*., 2015). Moreover, symbiotic N-fixation and nodulation is greatly affected by soil acidity which may reduce overall legume production. Subsequently, *Rhizobium*

survival and persistence in the soils because of their symbiotic relationship with legumes is affected as a result of soil acidity (Jaiswal, Naamala & Dakora, 2018). Soil acidity associated with high Al^{3+}, Mn^{2+} and Fe^{3+} may disturb the functioning of rhizobia as a result reducing their competitive ability in the soil. Moreover, *nod*A gene expression is also reduced under acidic conditions. According to Ferguson and Gresshoff (2015), this may lead to reduced biosynthesis of Nod factor signal which are is a major component involved in the exchange of signals and in facilitating recognition of the symbiotic partners. Ferguson and Gresshoff (2015) further highlights that soil acidity effect on rhizobia vary depending on the strain and this eventually affects the BNF efficiency of the strains, with the fast-growing rhizobia strains generally having lower ability to withstand acidic conditions than slow growing rhizobia strains such as some *Bradyrhizobium.*

Impact of phosphorus deficiency on BNF

Biological nitrogen fixation (BNF) in legume plants is a process that is induced by N-fixing rhizobia in root nodules (Mitran, Lal, Meena & Layek, 2018). This symbiosis relationship is greatly influenced by several environmental stressors which among them is P (Mitran *et al*., 2018). Lopez-Arredondo *et al*. (2014) reported that legume production and the BNF is influenced by low availability of P in the soil. Zhang *et al*. (2014) stated that P supply and availability are very important components of N transformation and regulating the activities of enzymes for improved fixation in plants. Legumes have a great demand for P, for optimal nitrogen fixation compared to non- nodulating plants as P plays a significant role in nodule transformation. Metabolic processes such as dinitrogen fixation and assimilation of nutrients (NH_4^+) into amino acids and ureides occurring in the plant cell fraction of nodules demand a large P amount (it is an energy demanding process) and depend on nodule energy status to function (Sulieman & Tran, 2015). Hence, P deficit inhibit nodule growth, and a result

symbiotic nitrogen fixation is reduced. Sulieman and Tran (2015) further explained that if P supply is not optimal, legume growth might be retarded as there will be insufficient nodules to support the requirements for growth and development. Magadlela, Kleinert, Dreyer and Valentine (2014) shown that low concentrations of P led to a decline of approximately 70% in nodule dry weight in cape lilac (*Virgilia oroboides*). This reduction in the concentration of P in *V. oroboides* as a result caused a reduction in the percentage of nitrogen derived from atmosphere (% NDFA) (Magadlela *et al*., 2014). This according to Magadlela *et al*. (2014) is indicates a decreased biological nitrogen fixation rate. Efficient P allocation and usage of the available P in the nodules during P stressful conditions is very important in maintaining optimal symbiotic interaction between the rhizobial-partner and its host plant (Meena *et al*., 2016). At critical low P levels in soil, majority of plants tend to allocate more of their resources towards increasing belowground biomass which might increase the C cost.

Adaptive strategies of plants to overcome P deficiency for better N-Fixation and legume productivity

During P starvation/ P deficiency in soil, is it important that the increased concentrations of P are conserved in the nodules to maintain growth and high rates of N-fixation (Sulieman & Tran, 2015). The adaptive response of nodule metabolism to P deficiency is very important for the improvement of symbiotic efficiency under P-deficient conditions (Mitran *et al*., 2018). Several adaptive strategies such as P homeostasis in nodules, increased P acquisition, upgrading N-fixation per unit of nodule masses well as the consumption per unit nodule mass which compensate the reduction in the number of nodules (Sulieman & Tran, 2015; Lopez-Arredondo *et al*., 2014). However, according to Sulieman and Tran (2015), the main adaptive strategy for P-deficient soil is the maintenance of the P-homeostasis in nodules for rhizobia legume symbiosis. This main emphasis of this strategy is to conserve more P in the nodule in

order to maintain a high N-fixating rate (Dhakal, Meena & Kumar, 2016). The symbiotic tissues has several ways of stabilizing phosphorus and these include higher P allocation to nodules, formation of a strong P sink in nodules, direct P acquisition through nodule surface and P remobilization from organic-P containing products (Sulieman & Tran, 2015). Literature report that symbiotic N-fixation can take place without any disturbances if an allocation of up to 20% of the total P is made towards the nodules (Jebara, Aouani, Payre & Drevon, 2005). Nodules represent a preferential strong sink for P incorporation during P starvation among the other plant parts (Le Roux, Kahn & Valentine, 2008). The ability of plants to form cluster root and mycorrhizas also plays a major role in N-fixation by improving root surface area and exudation of an organic acid and hence enhanced P acquisition during low P supply (Mitran *et al*., 2018). Another vital biochemical and physiological adaptive strategy to P deficiency include the remobilization of organic P within the plant by encoding acid phosphatase (Zhang *et al*., 2014).

2.3.2 Phosphorus-solubilizing microbes

The soil is rich in phosphorus, however, most of it is present in forms that the plants cannot use, which is an inorganic form (apatite). Plants can only absorb P as monobasic (HPO_4^-) and dibasic ($H_2PO_4^{2-}$) ions which are soluble forms (Mekonnen & Kibret, 2021). Given this large P reservoir in the soil, only 0.1% is available for plant use, most of it is in insoluble forms that cannot be assimilated by plants (Alori, Glick & Babalola, 2017). This makes phosphorus the second most important and limiting element in the soil after nitrogen (N_2) (Thomas & Singh, 2019).

Soil microorganisms in the soil can increase plant nutrient acquisition through biological processes that can transform insoluble nutrients into soluble forms that the plant can use. For example, certain bacteria species can dissolute phosphorus that is bound to soil rocks by (1) secreting organic acids that reduce the pH or chelate ions to release P, thereby increasing the

bioavailability of P in the soil and (2) release of extracellular enzymes/mineralization (Alori *et al*., 2017) as shown in Figure 2.7. These organisms are called phosphorus-solubilizing bacteria (PSB), and may include members of *Bacillus*, *Pseudomonas, Azotobacter, Rhizobium, Serratia*, *Bradyrhizobium*, *Xanthomonas*, *Rhodococcus*, *Burkholderia*, *Enterobacter*, *Actinobacteria* (Mekonnen & Kibret, 2021) and some Cynobacteria and Actinomyccetes (Sharma *et al*., 2013). Specific fungal species called phosphorus-solubilizing fungi (PSF) such as *Penicillium*, *Aspergillus*, *Trichoderma*, *Alternaria*, *Mycorrhizia* can also solubilize phosphorus. The application of rock phosphorus with *Bacillus megaterium* var. phosphaticum was able to increase sugar cane yield and juice quality by 12.6% while reducing the phosphorus requirement by 25%, thus further causing a 50% reduction of the costly superphosphate usage (Thomas & Singh, 2019).

Phosphate-solubilizing mechanisms used by phosphorus-solubilizing microorganisms

Lowering pH and ion chelation: In soil with high pH, the phosphate is precipitated into Tri Calcium Phosphate [Ca_3 (PO_4)$_2$] and rock phosphate (fluorapatite and francolite), and plants cannot utilize these forms (Yousefi, Khavaz, Moezi, Rejali & Nadian, 2011). A decrease in rhizosphere pH renders them soluble. Soil microorganisms called phosphate-solubilizing microorganisms (PSM) can convert the insoluble form of P by releasing several organic acids such as acetic, citric, lactic, oxalic, succinic, tartaric, gluconic, ketogluconic acid which through their carboxyl and hydroxyl group lower the pH or chelate the cations that are bound to phosphate and ultimately converting it into soluble phosphate thus making it available to plants (Mekonnen & Kibret, 2021). The secretion of the organic acids causes a drop in pH leading to the acidification of the microbial cells to release P by exchanging H^+ for Ca^{2+} (Alori *et al*., 2017). Briefly, acidification by H^+, means H^+ released is associated with the assimilation of cations which together brings P solubilization. Phosphorus solubilization efficiency by PSM is

dependent on the strength and nature of acids produced, and different organisms can release different type and quantity of acid (Walpola & Yoon, 2012). The most effective acid in solubilizing P include the one with tri- or di-carboxylic group compared to the monobasic and aromatic acids (Kalayu, 2019). Aliphatic acids also have more solubilizing effects as compared to phenolic acids and citric acids. Ketogluconic acid among all the organic acids that PSM produced, is a powerful chelator of calcium (Zaidi, Ahemad, Oves, Ahmad & Khan, 2017). Nitrifying bacteria release some inorganic acids (nitric and sulphuric acids) that may react with calcium phosphate and converting them to soluble forms of P that can be assimilated by plants (Walpola & Yoon, 2012).

Mineralization and mobilization: Durng mineralization, P covertion occurs through the production of phosphatase (i.e. phytase) by the PSM, that catalyze the hydrolysis of phytic acid (indigestible organic form of P present in plant tissues) to release organic form of P that the plant can immobilize (Santana *et al*., 2016). Some fungal species and some bacteria species such as *Bacillus* and *Streptomyces* species have these phytases (Kalayu, 2019). Contrary to the mineralization process where the converted P becomes directly available to the plant, on the immobilization P is made available to the plant indirectly whereby the microbes convert inorganic P and consume it preventing it from being accessible to plant immediately. Overtime, due to unfavourable environmental conditions (starvation) that may cause death of the microbe makes P available to plants as P will be released from the microbial cells into soil (Sharma *et al*., 2013).

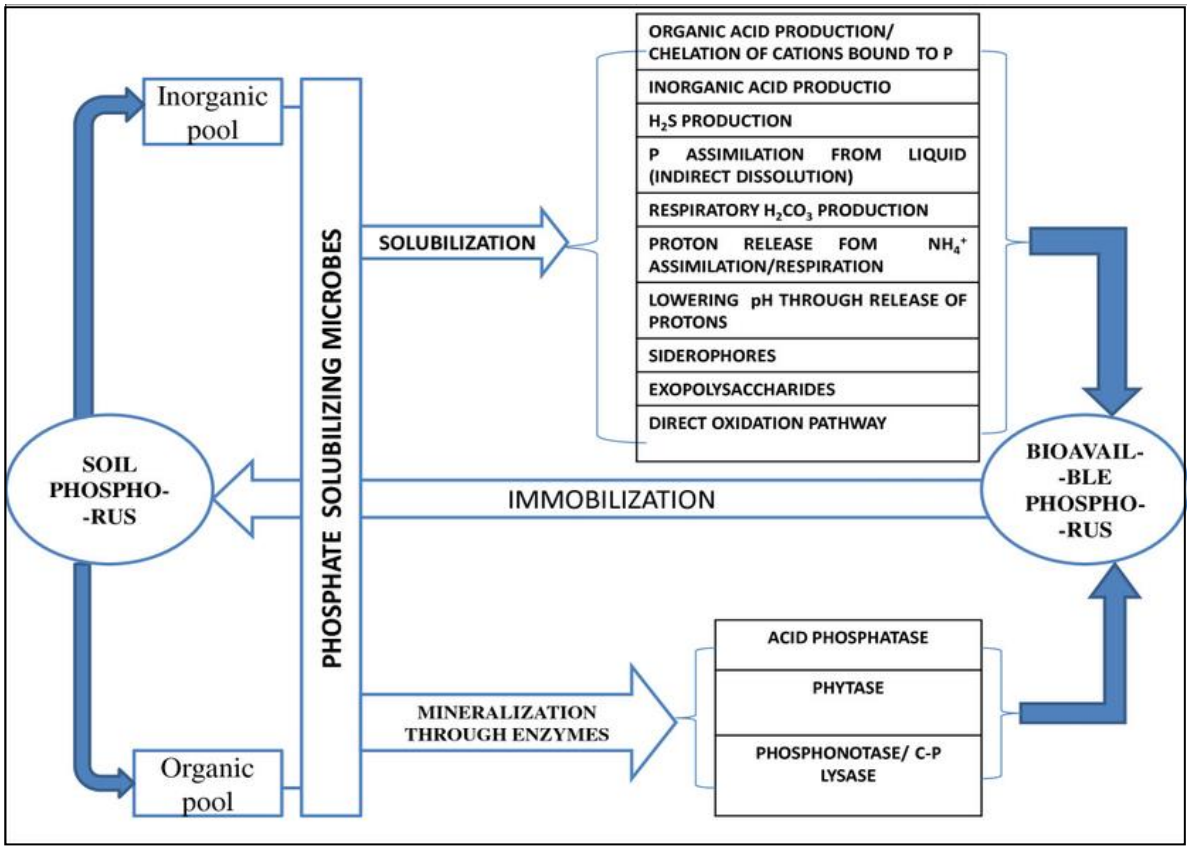

Figure 2.7: Schematic representation of phosphorus solubilization in soil by P solubilizing microorganisms and molecules produced to facilitate the process (Sharma *et al*., 2013).

2.3.3 Carbon cycling

The major two ways carbon cycling occurs (1) Bacterial CO_2 fixation by photo-chemoautotrophic microbes and (2) through photosynthesis by autotrophic organisms (mainly the photosynthesising plants) (Gougoulias, Clark & Shaw, 2014). During the two processes, carbon is made available to plants. The recycled carbon is returned to the air through animal and plant respiration, microbial respiration during decomposition and as well as methanogenesis. The schematic representation in Figure 2.8 is a brief summary of the whole carbon cycle.

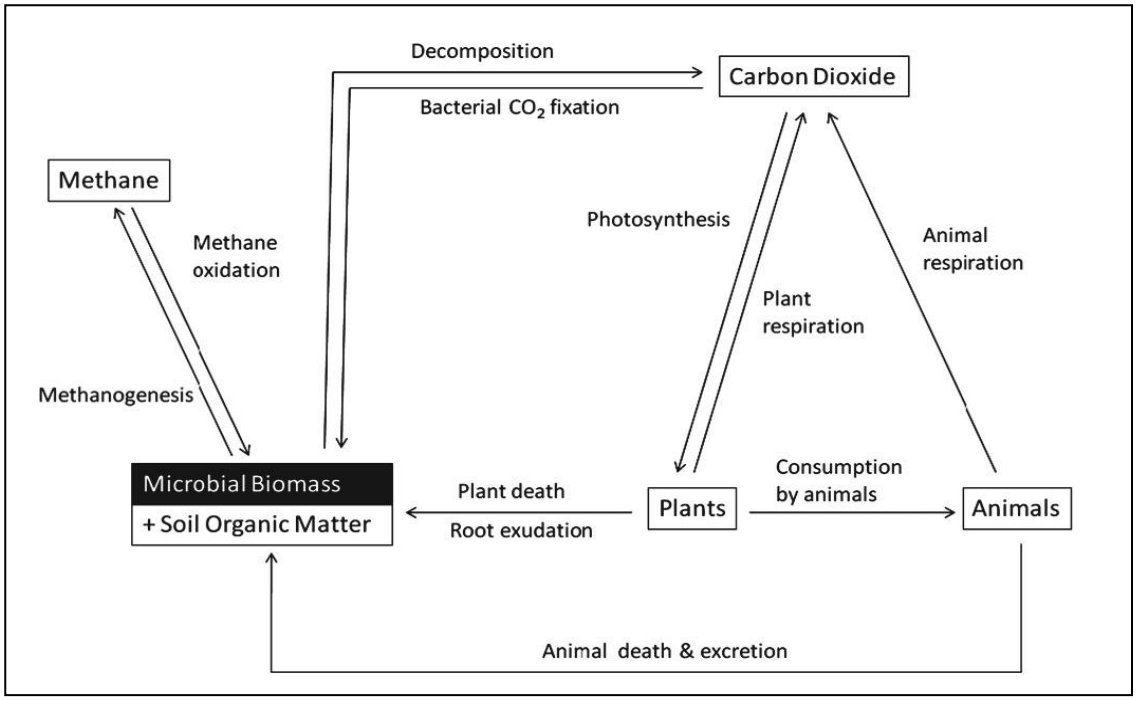

Figure 2.8: Soil microorganism-mediated carbon cycling (Gougoulias *et al*., 2014).

Soil is the largest reservoir of organic carbon containing at least three times what is floating in the air (Malik *et al*., 2016; Lange *et al*., 2015). Topsoil is the major contributor to this pool/reservoir with nearly half the amount (Malik *et al*., 2018). Topsoil is mostly dominated by soil organic matter (SOM) or litter of plant, animals, and microorganisms), root exudates and microbial biomass (Figure 2.8), carbon is the backbone therein (Malik, Dannert, Griffiths, Thomson & Gleixner, 2015; Gleixner, 2013). Plant-derived organic carbon from root or shoot litter is the largest (Kogel-Knabner, 2001).

Soil organic matter (SOM) decomposition and soil organic carbon (SOC) input to soil

Soil organic carbon (SOC) enters the soil through decomposition of SOM. During decomposition, small organic fractions of this SOC such as cellulose, hemicellulose, lignin, chitin, and lipids are decomposed/depolymerized by extracellular enzymes released by microbes to release C and other nutrients (Gougoulias *et al*., 2014; Gleixner, 2013). Soil organic matter decomposition is a microbes-mediated process, which may also be referred to as carbon mineralization, as it releases nutrients (N, P and other inorganic ions) to the soil. Soil

organic matter decomposition is not an independent process, external factors such as essential nutrient availability (used as electron doors (NO_3^-) or acceptors (NH_4^+) for microbial metabolism), environmental factors such as pH, soil texture, temperature, moisture and mineralogy have an influence. Carbon immobilization is when the released nutrients are then made available for plants uptake and to microbes. Carbon consuming heterotrophic microorganisms help plant access this unavailable form of carbon through microbial decomposition (Malik *et al*., 2015). Microbes utilize the carbon of either plant, animal or microbial origin as a substrate for metabolism to create their own biomass (energy source) and releasing the rest as metabolites or as CO_2 back to the atmosphere (Malik *et al*., 2015). Soil organic carbon is the main energy source that microbes use, it is also the source and sink of nutrients and contribute greatly to fertility.

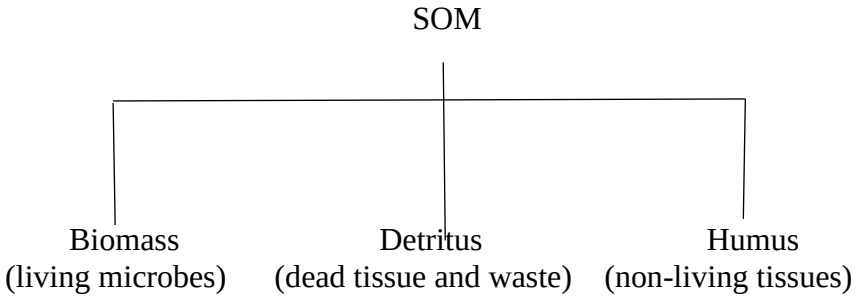

Figure 2.9: Composition of soil organic matter (SOM) (adopted from Gleixner, 2013).

Microbial decomposition of plant-derived carbon and persistence of organic matter

There are pathways through which SOC can enter soil during the decomposition: (1) is the above-ground leaching of dissolved plant material (or organic carbon- from leaf and woody tissue litter) into soil by infiltration and (2) through the below-ground pathway called rhizodeposition, whereby carbon containing simple molecules (i.e. sugar, amino acids, sugar alcohols, organic acids) found in plant roots moves to soil (Berhongaray, Cotrufo, Janssens & Ceulemans, 2016). The below ground method is defined by root mortality and their microbial decomposition occurring over a specific period (Lange *et al*., 2015). The below ground

translocation of plant photosynthates and their consequent decomposition by microbes is the major contributor to the terrestrial ecosystem C budget. ‘Between 30 to 60% of net photosynthesized carbon is allocated to roots, and as much as 40 to 90% of this fraction enters soil in the forms of root exudates, sloughed-off cells, and decaying roots’ (Lu & Conrad, 2005).

Carbon cycling microbes and their role in nutrient cycling

Microbes are involved in a wide range of processes that are responsible for the largest flows of C in soil systems and a one example being SOM decomposition and C storage (Schimel & Schaeffer, 2012). These organisms can decompose a wide range of plant-derived compounds to use it as their energy source and release some back to the air (Kallenbach, Frey & Grandy, 2016). Bacteria and Fungi are the major contributor to C cycling and SOC input to soil. About 90% of the fixed carbon is through bacterial and fungal decomposition (Song *et al*., 2020). However, it is important to recognize the role of mycorrhizal fungi. About 80% of plants on land establish a symbiotic relationship with mycorrhizal fungi which benefit the plant growth and fitness (Miozzi *et al*., 2019). There two groups of mycorrhizal fungi are involved in carbon cycling, obligate symbionts (*Arbuscular mycorrhizal* fungi, AMF) and the facultative symbionts (*Ectomycorrhizal* fungi, ECM). The facultative symbionts (*Ectomycorrhizal* fungi, ECM) are involved in organic carbon mineralisation. The AMF is associated with the rhizo-deposit translocation of plant carbon. *Arbuscular mycorrhizal* fungi enhance below-ground C allocation and assimilation by forming mycelial networks that connect plant roots and soil particles, these networks help host plants absorb mineral nutrients from the soil (Nakano-Hylander & Olsson, 2007). The fungi increase below-ground allocation of carbon, nearly 20% of the photo-assimilates are utilized by the AMF which make mycorrhizal turnover a substantial process for carbon input into SOM (Malik *et al*., 2015; Johnson, Leake & Read, 2002). Part of the plant transferred carbon to the mycelia (fungi) is rapidly returned to the

atmosphere, which is a short route of the soil carbon cycle. Bacteria species mostly from the *Burkholderiaceae* and *Pseudomonadaceae* families, make a considerable contribution to root exudates decomposition (Philippot *et al*., 2013).

Benefits of soil organic matter (SOM)

Soil organism matter is known to be associated with improved soil properties such as ion exchange capacity, water-retention, improved soil aggregation that reduces its erosion and it is the reservoir of greenhouse gases (GHGs) such as CO_2 and CH_4 and essential nutrients such as P, C, N (Finn *et al*., 2017). Although, these nutrients remain unavailable to plants until they are converted into forms that are accessible to plants.

2.4 Role of soil microbes and extracellular enzyme activities in nutrient cycling and acquisition by plants

Soil microorganisms play a prime role in maintaining terrestrial ecosystems. Soil enzymes released by microbes are involved in biogeochemical cycling of nutrients (P, C and N) in the soil. (Liu *et al*., 2021; Li *et al*., 2018). These enzymes are called extracellular enzymes. Extracellular enzymes are the major means microbes use to access the biological unavailable nutrients such as C, P, N in SOM as demonstrated in Figure 2.10 below (Blonska *et al*., 2020). Some of the most abundant organic soil compounds that are enzymatically degraded are lignin, cellulose, hemicellulose, chitin, starch, proteins (Blonska *et al*., 2020). Microbes utilized these enzymes to catalyze processes that convert the insoluble macromolecules comprised in SOM matrix into simpler substrates that can be assimilated by plants, which are decomposition, degradation or depolymerization (Wallenstein & Weintraub, 2008). Bacteria and fungi make up more than 90% of soil microbial biomass and are the primary agents in organic matter decomposition in soil (Song *et al*., 2020).

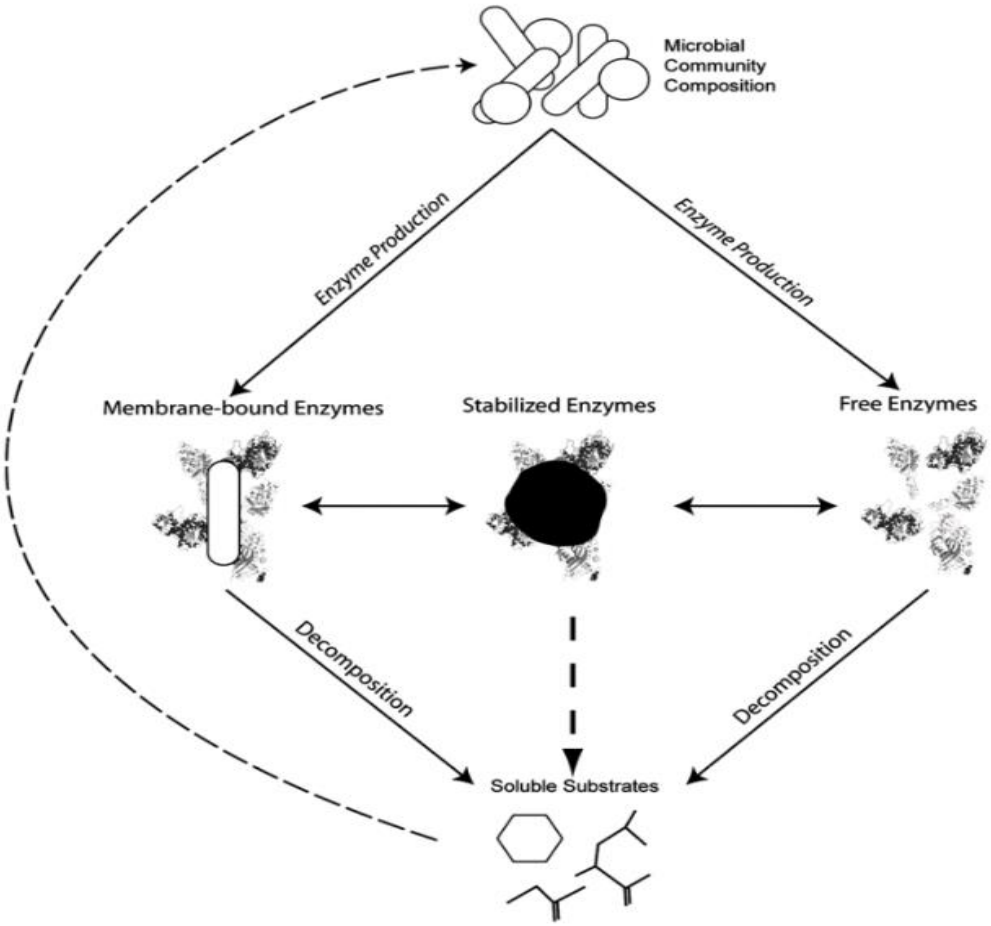

Figure 2.10: Depolymerization and degradation process of insoluble macromolecules comprised in soil organic matter and extracellular enzyme activities released by microbes (Wallenstein & Weintraub, 2008).

These enzymes are found in cell's plasma-membrane, periplasmic membrane, cell walls of organisms (animals, plants, and microbes) and may be released into the cell's environment (Talyor, Wilson, Mills & Burns, 2002). Once released into the soil, they then associate with target substrates molecules, and either hydrolases or oxidize them to release inorganic molecules that can be assimilated by plants as carbon and other nutrients (Ghosh *et al*., 2020). These include phosphatase, β-glucosidase, urease, β-glucosaminidase, cellulobidase and are indices of P, C and N. According to Song *et al*. (2019), soil invertase, β-glucosidase, urease and

acid phosphatase are highly associated with soil total carbon, total nitrogen and dissolved organic carbon (DOC) contents (Liu *et al*., 2021). Soil microbes tend to secrete more acid phosphatase, since microbial P solubilization process require a low pH environment for the enzymes to function optimal and to also meet soil P demand (Zhao, Ren, Han, Yang, Wang & Doughty, 2018).

Fungi and bacteria are responsible for the secretion of some of the extracellular enzymes, such as phosphatase and β-glucosidase, which makes an important part of the soil matrix as abiotic enzymes (Kotroczo *et al*., 2014). The β-glucosidase and phosphatases enzymes contribute a prime role mineralization of organic matter in the soil. Among other enzymes, phosphatase can convert the unavailable and organically bound form of P to smaller soluble molecules that can be assimilated by plants and microorganisms (Santana *et al*., 2016). The activities of phosphatase enzyme are associated with soil and vegetation conditions, response to changes in management, as well as seasonal changes in soil moisture and temperature (Kotroczo *et al*., 2014). Furthermore, the β-glucosidase enzyme is responsible for hydrolysing cellobiose fragments to glucose, the main component of plant polysaccharides. It is active in the first phases of organic compounds degradation, that reduce the molecular size of organic structures, thus facilitating future microbe enzyme activity. This enzyme is mostly produced group of fungi, including the wood-rotting basidiomycetes (both white and brown-rot) (Kotroczo *et al*., 2014).

2.5 Factors that influence enzyme activity

Enzymes can be damaged or denatured, however, some may survive in solution. A great diversity of compounds comprises of soil organic matter; hence a diversity of enzymes is required to degrade those compounds. Soil enzymes activities are regarded as the major indicator of soil health, fertility since they are sensitive to environmental changes (i.e., such

nutrient availability and pH) and respond rapidly to both natural and anthropogenic factors is rapider than other soil variables (Liu *et al*., 2021). They are also an exceptional indicator in predicting soil nutrient supply to plants as they can catalyze processes that convert unavailable nutrients to easily accessible nutrients by plants (Song *et al*., 2019).

Several factors directly affect the activities of extracellular enzyme, and these factors include temperature, moisture, pH, nutrient availability, and chemical properties of the soil. Soil pH affects soil enzyme activity by controlling the production of their microbial enzymes, through ionization-induced conformational changes of enzymes, and availability of substrates and enzymatic co-factors (Kotroczo *et al*., 2014). The major drivers of enzyme activities in the soil is C, N and P cycling substrate availability and nutrient limitation (Blonska *et al*., 2020). In addition, the quality and quantity of applied manure and plant species, all these factors greatly impact enzyme activities and their functionally diversity by changing the soil organic carbon pools and microbial substrate availability (Gosh *et al*., 2020).

2.6 Abiotic stressors that influence the performance and efficiency of PGPR

Soil microorganisms are known to be the engineers of soil, involved in many biological processes that benefit plant growth counting in N-fixation, solubilization of nutrients, biocontrol activities etc. However, their development, structure, composition, performance is influenced by several soil and environmental factors predominant in many degraded ecosystems: soil pH, temperature, and salt (Musarrat & Khan, 2014). This means that any alteration to the normal environment can poor growth and survival of organisms thus affecting the plants. These conditions vary with species of microbe as some survive under extreme temperatures and others under normal conditions. Phosphate solubilizing microorganisms exhibit very high solubilizing effects under extreme conditions such as saline-alkaline soil (with a pH range of 0-9% and 0-5% salt concentration), extreme temperatures than normal (30

- 45°C) and low nutrient availability, and these conditions improve efficiency of the symbiosis and plant growth promoting effects (Mehta, Walia, Chauhan, Kulshresth & Shirkot, 2013; Zhu *et al*., 2011).

Opposite to PSMs, extreme conditions prevent the development and efficiency of BNF symbiosis (Lebrazi & Benbrahim, 2014). For instance, the survival and performance of organisms like rhizobia is reduced under high temperatures. Moreover, temperature does not only affect persistency, but molecular signalling between the symbiotic partners is also affected. The optimum temperature range for nitrogen fixing symbiosis and the nitrogen fixers is between 28-31°C, temperatures beyond 38°C affect the growth and development of the organism (Lebrazi & Benbrahim, 2014). Biological nitrogen fixation symbiosis requires neutral or slightly acidic soils with a pH ranging from 6 to 7 for maximum production (Lebrazi & Benbrahim, 2014).

In addition to all these factors, climate conditions and competition between micro-organisms (for resources, phosphate solubilisation/plant nodulation host, partner fidelity and specificity mediated by genetic and molecular mechanisms) are also success-limiting factors that may affect the performance and efficiency of an organism in the soil (Soumare *et al*., 2020). For instance, phosphorus solubilisation is much quicker in warm climates and very low in cooler to dry climates, and rapider in well-aerated soils than saturated wet soils (Alori *et al*., 2017). The indigenous community of rhizobia is much competitive compared to introduced nitrogen fixing strains as the indigenous community can make better use of low concentrations of the organic compounds (Al-Falih, 2002).

2.7 Work not done on the problem

Information on the symbiotic microorganisms associated with cancer bush and their efficiency of nutrient cycling is scanty. These symbiotic microorganisms (PGPRs) in the rhizosphere of

medicinal plants are known to provide a wide range of services that benefit the plants while in return the plants provide reduced carbon and other metabolites that the organisms use as energy sources. Backer *et al*. (2018) reported that rhizosphere microbes contribute a major role in plant nutrient acquisition and assimilation, soil texture improvement, and secretion of modulating extracellular molecules such as secondary metabolites, hormones, antibiotics, and various signal compounds that altogether stimulate the overall plant growth and improve it tolerance to stressors (drought, heat, and salinity). Plant growth promoting rhizobacteria are involved in activities of biological nitrogen fixation, P-solubilization, phytohormone production and as biocontrol agents (Jaiswal *et al*., 2021). Hence, a better understanding of rhizosphere microbe interactions of cancer bush, characterizing of its symbionts and the role they played in plant growth promotion is very crucial and requires an investigation as it can be useful in the development of effective local bacteria strains. The development of a potential artificial inoculation is a best strategy for enhancing plant growth and maximizing productivity as these are considered significant to the overall plant health and growth.

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CHAPTER 3

BIODIVERSITY OF PLANT GROWTH PROMOTING RHIZOBACTERIA ASSOCIATED WITH CANCER BUSH (*SUTHERLANDIA FRUTESCENS* (L.) R. Br) ROOT NODULES IN LIMPOPO PROVINCE

3.1 Introduction

The rhizosphere of plants is known to contain many beneficial microorganisms for plant growth also called the plant growth promoting rhizobacteria (PGPR) (Sezen, Ozdal, Koc, & Algur, 2016). These are found in both managed and natural ecosystems, in very low portions, approximately 2-5% of the total rhizobacteria community (Islam *et al*., 2020). Plant growth promoting rhizobia are heterogenous group of microorganisms that have the ability to colonize rhizosphere (rhizobacteria), phyllosphere (epiphytes) or living tissues of plants (endophytes) (Dhole, Shelat, Vyas, Jhala & Bhange, 2016). Through indirect and direct mechanisms, PGPRs are able to enhance plant growth and development (Marakana, Sharma & Sangani, 2018; Malleswari & Bayanarayana, 2013). The indirect mechanisms through which PGPRs enhance plant health is by suppression of phytopathogens (such as *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium solani* and*Rhizoctonia solani*) using various approaches (Ghodsalavi, Ahmadzadeh, Sleimani, Madloo & Taghizad-Farid, 2013). One of these approaches is the ability to synthesize fungal cell wall-lysing enzymes (protease) or hydrogen cyanide (HCN) which suppress the growth of fungal pathogens and increase competition with the pathogens for nutrients or specific niches on the root surface (Ghodsalavi *et al*., 2013). The direct mechanisms through which PGPRs promote plant growth and health is through their ability to fix atmospheric nitrogen, solubilize phosphorus and decomposition of organic matter to the release of other minerals making them available to plants (Yarte, Gisodi, Llente & Larraburu, 2022). Lastly, through the production of plant growth hormones (like indole-3-

acetic acid-IAA) and siderophore production (Kumar, Singh, Singh, Singh, Singh & Pandey, 2016).

The major groups of PGPRs include those belonging to Proteobacteria and Fermicutes (Rojas-Tapias, Moreno-Galvan, Pardo-Diaz, Obando, Rivera & Bonilla, 2012; Chen *et al*., 2010; Jiang, Sheng, Qian & Wang, 2008). Within the Fermicutes phylum, *Bacillus* spp. are the dominant group with growth promoting abilities. Within the Proteobacteria, the class Gammaproteobacteria is predominant containing the genera: *Enterobacter*, *Pseudomonas*, *Acinetobacter*, *Pantoea*, *Serratia*, *Psychrobacter* and *Rahnella* and lastly the free-living bacteria (*Burkholderia* and *Achromobacter* sp. which belong to Betaproteobacteria) (Batista *et al*., 2018). Plant growth promoting rhozobacteria are associated with plants within the Fabaceae, Asteraceae, Brassicaceae, Poaceae, Crassulaceae and Solanaceae family and these microbes help with cycling and acquisition of most important nutrients and other essential elements (Ramakrishna *et al*., 2019).

Phosphorus is one of the most important element in the soil after nitrogen to carry out important metabolic processes such as macro-molecular biosynthesis, energy transfer, cellular respiration, photosynthesis, and signal transduction (Yarte *et al*., 2022). Although it is abundant in soil, the availability of its organic and inorganic forms is restricted for plant use as it occurs in insoluble forms that plant cannot utilize (Sharma *et al*., 2013). Numerous microorganisms in the soil called phosphorus solubilizing bacteria (PSB) are able to dissolve these inorganic unavailable nutrients into bioavailable forms that can be assimilated by plants (Pan & Cai, 2023). Phosphorus solubilizing microbes are everywhere and vary in density and mineral phosphate solubilizing ability from soil to soil or from one production system to another (Sharma *et al*., 2013). Phosphorus solubilizing bacteria make up 1 to 50% of the whole microbial population in soil while other microorganisms in the soil such as phosphorus

solubilizing fungi (PSF) only make up the lowest percentage of 1 to 0.5% in P solubilization potential (Khan, Jilani, Akhtar, Saqlan & Rasheed, 2009).

Biological nitrogen fixation is a process channelled by either symbiotic or non-symbiotic microbes operating in the presence of nitrogenize enzyme activities in the soil (Kumar *at al*., 2016). Several PGPRs that have been reported to work an important role as growth promoters (N-fixation etc.) include but not limited to *Rhizobium* reported in pea (*Pisum sativum* L.) (Shahzad *et al*., 2019), *Bacillus* and *Pseudomonas* in garden heliotrope (*Valeriana officinalis* L.) (Thakur, Kaur & Mishra, 2016; Ghodsalavi *et al*., 2013) *Serratia* and *Enterobacter* in lupin or lupine (*Lupinus albescens*) (Giongo *et al*., 2010) and *Sinorhizobium* in velvet bean (*Mucuna pruriens* (L.) DC. var. utilis) (Kumar, Kumar, Annapurna & Maheshwari, 2006). The genera within the Rhizobaceae family such as *Rhizobium*, *Bradyrhizobium, Sinorhizobium, Mesorhizobium*, *Azorhizobium, Allorhizobium* are the only groups that are connected to symbiotic N-fixation (Ndusha, 2011). Other non-rhizobia species: *Aerobacter*, *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Chryseomonas*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Flavimonas,* and *Sphingomonas*, *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Azotobacter*, *Alcaligenes*, *Flexibacter*, *Cronobacter*, *Brevibacillus* and *Klebsiella* have been isolated from tissues of legumes as plant growth promotors involved in nitrogen fixation (Sumbul *et al*., 2020; Dubnath *et al*., 2016; Singh, 2015; Rajendran, Patel & Joshi, 2012). Biological N-fixation is a process that occurs between leguminous plants and their rhizobia bacteria, whereby the bacteria is able to convert the inert N_2 into forms that can be assimilated by plant (Islam *et al*., 2020). It is by virtue of this association with microbes that plants are able to acquire nutrients such as nitrogen. The *Rhizobium*-legume symbiosis is one of the well-known and most studied association (Teixeira & Rodríguez-Echeverría, 2015). Nitrogen fixing legumes not only improve plant growth through this important nitrogen element in the soil but also can support soil nitrogen status and growth of other associated plants through plant residues

accumulation and decomposition (Islam *et al*., 2020). The symbionts of cancer bush and the interaction existing between the organisms have not been investigated. The study seeks to investigate the PGPR species around the root nodules of cancer bush plants growing in the two study sites. The study hypothesized that there will be high diversity of PGPRs associated with roots nodules of cancer bush growing in different sites in the Limpopo Province.

3.2 Material and methods

3.2.1 Study location and sample collection

Cancer bush nodulated roots (Figure 3.1), were collected from the wild in two locations, Tubatse (24°63′52.5″S; 30°16′ 4.28″E) and Magkupheng (23°88′ 92.5″S. 29° 17′ 8.38″E) in the Limpopo Province during winter of 2022 and summer of 2023. The roots were transported to the laboratory in zip lock plastic bags placed inside a cooler box with ice cubes, labelled according to the location name, coordinates and sampling date. Bacterial isolation from cancer bush roots was conducted at the Research Laboratory 203, University of Mpumalanga (25º27′06.18″S, 30º58′5.21″E), Mbombela South Africa.

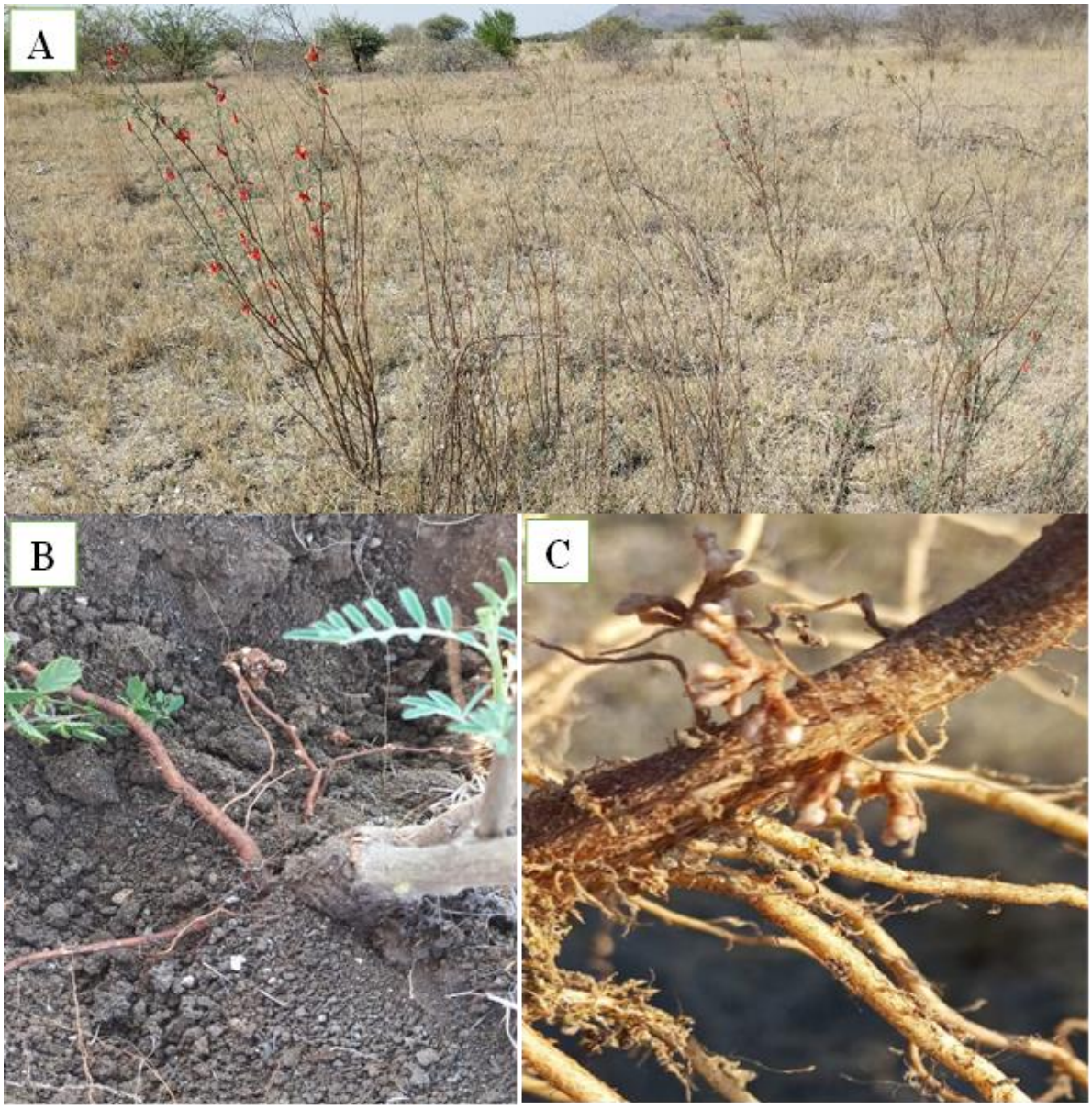


Figure 3.1: Cancer bush in the wild (A); sampled rhizosphere (B) and nodulated roots (C) (Photos by Thobile Mkhwanazi, 2022).

3.2.2 Nodule sterilization

The roots were first washed in running tap water to remove soil particles or debris. Nodules were then detached from the roots and placed in a beaker with 1% laboratory grade sodium hypochlorite (NaOCl) surface sterilized for 3 min and subsequently washed in seven rounds of sterilized distilled water to remove any traces of chemicals (Muthini *et al*., 2014). The surface sterilization process was done to eliminate any surface biological contamination. Sterilized

nodules were dried using a sterile absorbent paper towel and then preserved in a refrigerator at 4°C until required. This was done to prevent any physico-chemical changes on the nodules (Wagh, Shermale & Mahure, 2015).

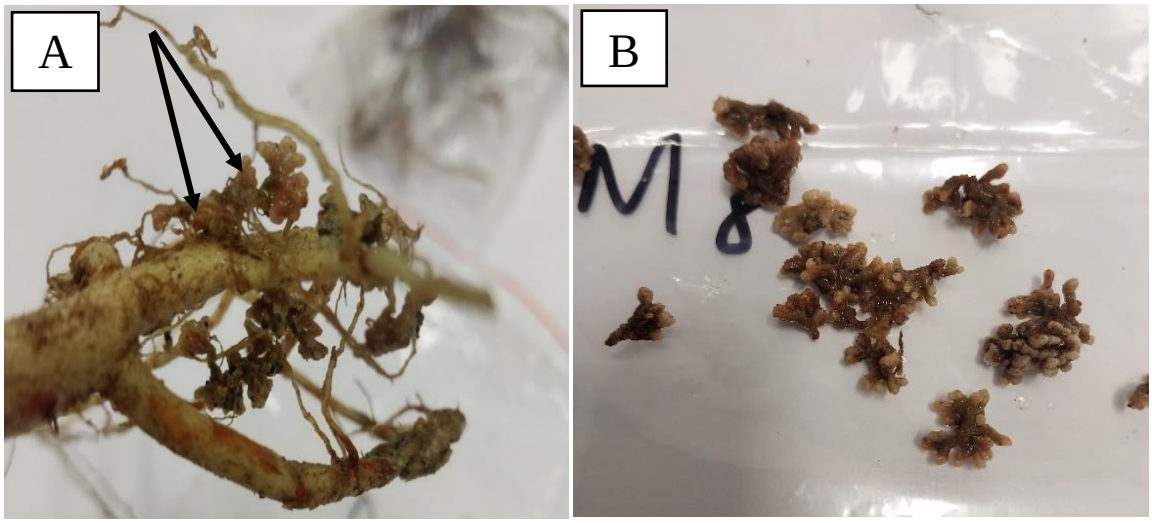


Figure 3.2: Cancer bush nodules (arrows indicate active nodules - pink pigmentation) (Photo by Thobile Mkhwanazi, 2022).

3.2.3 Media preparation

Nutrient agar (NA) with composition: 5.0 g peptone, 3.0 g beef extract, 8.0 g sodium chloride and 12.0 g Agar was used for isolation. Briefly, 28 g of the NA was weighed and 1 L of distilled water was added into it, stirred gently before autoclaving the mixture at 121°C for 15 min. After, autoclaving the media was allowed to cool down between 45 and 50°C. The media was then poured into 9 cm diameter Petri dishes under aseptic conditions in the laminar flow.

3.2.4 Inoculum preparation and inoculation

Previously detached nodules were crushed in a drop of sterile distilled water using a sterilized mortar and pestle to obtain a milky suspension of bacteroid. Thereafter, a loopful of bacteroid was streaked onto media plates, replicated three times and incubated for 3-5 days (fast growers)

and (5-7 days slow growers) at 30°C until maximum recovery of bacteria colony was observed (Koskey *et al*., 2018).

Pure cultures were made by three subculturing of single colonies that grew from the media (Figure 3.3) for further identification using morphological and molecular analysis. Colonies with different colors and shapes (more than one colony type) on the culture plates (Figure 3.3) were indicative of variants of the same strains or occupancy of more different strains in the same nodule and were re-streaked separately on a fresh media (Ouyabe, Kikuno, Tanaka, Babil & Shiwachi, 2019). A control plate without sample was used to check the purity of the media. Absence of growth on the control plate represent absence of any epiphytic contamination.

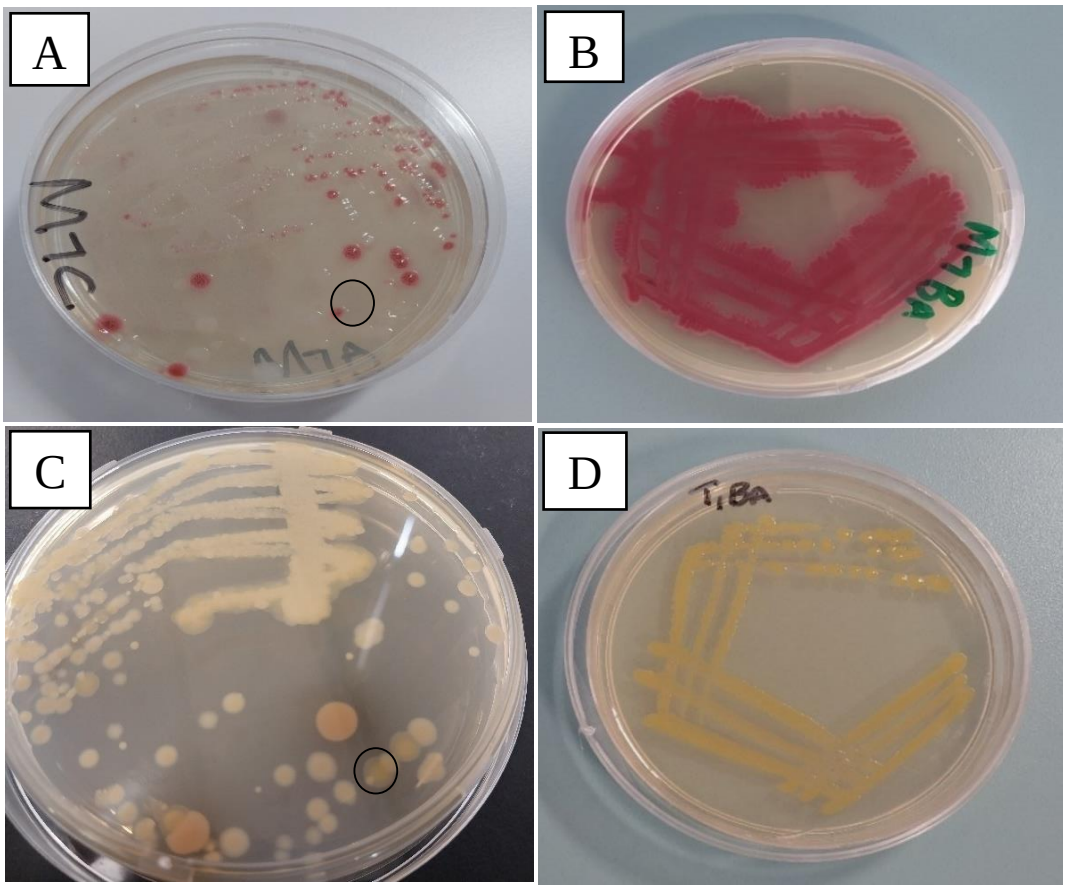


Figure 3.3: Mixed cultures (A & C) and pure cultures (B & D) (Photo by Thobile Mkhwanazi).

3.2.5 Characterization of the bacteria isolates

Morphological identification

The pure bacteria isolates were observed on NA agar media and characterized according to colony morphology such as color, shape, elevation, surface and margin (Hamza, Hussein, Mitku, Ayalew & Belayneh, 2017; Woomwer, Karanja, Kisamuli, Murwira & Bala, 2011).

Genomic DNA (gDNA) extraction, PCR amplification, and sequencing

The DNA extraction, amplification and sequencing were done at Inqaba Biotechnical Industries (Pty) Ltd. Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). PCR amplification of the extracted bacterial DNA was performed by Inqaba Biotechnical Industries (Pty) Ltd using the 16S universal primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) forward primer (10 µM) and 1492R (5′-CGGTTACCTTGTTACGACTT- 3′) reverse primer (10 µM) with an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 68°C for 1 min and a final elongation at 68°C for 10 min. The primers were used to amplify the 16S rRNA region of the bacterial DNA. The hold temperature was at 4°C. The integrity of the PCR amplicon products was visualised on 1% Agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA dye (Figure 3.4). The NEB Fast Ladder was used on all gels (N3238) as size standard.

Molecular and phylogenetic analysis of the isolated bacteria

The chromatograph files of the forward and reverse sequences obtained from Inqaba Biotechnical Industries were edited on Chromas software v.2.6.6.0, then assembled and aligned in BioEdit sequence alignment editor v.7.2.0 software to obtain the consensus sequence. After consensus sequences were created, the BLAST program at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was used to analyse the molecular and genomic data (Duhan *et al*., 2020). Phylogenetic analysis was carried out to verify the identified species. The

closest matched sequences obtained from BLAST were compared against the query sequence (consensus sequence) obtained through BioEdit and used to construct the phylogenetic trees. The sequences retrieved from Blast were first aligned (multiple alignment) together with the generated DNA sequence using clustalW software with the tree drawn using neighbour-joining (NJ) method on MEGA v.5.2. The general time reversible model and maximum likelihood method using MEGA v.5.2 software was used (Tamura *et al*., 2011) with 1 000 bootstrap replicates (Tamura, Nei & Kumar, 2004; Saitou & Nei, 1987).

3.2.6 Characterization of bacteria for plant growth promoting properties

N-fixation assays

Nitrogen fixation ability of bacteria isolates was tested on Simmons citrate agar containing citrate as the source of carbon and inorganic ammonium salts as source of N, at 30°C for 5-7 days (Rodrigues, Forzani, Soares, Sibov & Vieira, 2016). Successful growth of bacteria on plate and colour changes of media from green to blue represented a positive (+) test and qualitative evidence of atmospheric nitrogen fixation.

Phosphate solubilising ability

Bacterial isolates were tested for phosphate solubilisation activity by growing them on the Pikovskaya's agar containing insoluble tricalcium phosphate (TCP) as source of P, incubated at 30°C for 3-5 days (Singh, Pandey, Kuma & Singh, 2017). The formation of a halo or clear zone around the bacterial colony indicates phosphate solubilisation potential of bacteria.

3.2.7 Leaf nutrient composition and percentage nitrogen derived from atmosphere (% NDFA)

Leaf N and P concentrations were considered to determine the role of N-fixing and P solubilizing bacteria on leaf nutrition following the procedure by Motsomane, Suinyuy, Perez-Fernandez and Magadadlela (2023). Leaves of cancer bush plants were sampled as per location.

The sampled leaves were sun dried for two weeks till constant weight, ground into fine powder and sent to Central Analytical Facilities at the University of Stellenbosch (South Africa) for P and N analysis through Inductively Coupled Mass Spectrometry (ICP-MS) and N isotope analysis at the Archeometry Department at the University of Cape Town (South Africa). Samples of 2.10 to 2.20 mg of powdered leaves were weighed using the Sartorius microbalance (Goettingen, Germany) into 8 mm x 5 mm tin capsules (Elemental Micro-analysis, Devon, UK). The samples were analysed using the Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy) through the combustion process. Thereafter, Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) which was connected to a CHN analyser by a Finnigan MAT Conflo control unit was used to determine the N isotope values for N gas released. Five standards were used to correct the samples for machine drift, namely, two inhouse standards (Merck Gel and Nasturtium) and the IAEA (International Atomic Energy Agency) standard $(NH_4)2SO_4$. The isotopic N ratio was calculated as δ = 1000 (Rsample/ Rstandard) with R representing the molar ration of heavier isotope of the sample and standards to lighter isotopes. The % NDFA was calculated using the formula below:

$$\% \text{ NDFA} = \frac{(\delta \text{ 15N reference plant} - \delta \text{ 15N cycad})}{(\delta \text{ 15 N reference plant} - \beta)} \text{ x } 100$$

3.2.7 Data analysis

Shannon-Wiener diversity index (H) by Shannon and Weaver (1964) was used to measure the degree of microbial diversity and richness found in cancer bush rhizosphere among different localities. Shannon-Weiner index assumes that all species are represented in the sample, and therefore are randomly sampled from an independent large population.

The formula below was used to calculate species diversity:

$$H' = -\sum_{\infty}^{s}(pi)(\ln pi)$$

H’ = Shannon-Wiener index of species diversity

S = number of PGPR species (richness)

In = natural logarithm

p_i = proportion of total abundance represented by i^{th} species

Values range from 0 to 1. Increased values indicate increased diversity. When H’ equals to 0, the population has only 1 species represented.

Equitability index / evenness of Pielou’s evenness index (*J*) (1966) was used to get a measure of equitability among species in a community:

$$J = \frac{H'}{Hmax}$$

E = Evenness = H'/H_{max}

H’ = calculated Shannon-Weiner diversity

H_{max} = In(S) = maximum diversity possible & s = number of species

The scale ranges from 0 to 1. The higher the *J’* value, the less variation in communities. The closer to 1 the more even the populations that form the community.

Simpson diversity index (1949) assumes that randomly selected individuals in a population with belong to the same species (most common species).

$$\text{Ds} = 1 - \left(\frac{\sum n\,(n-1)}{N\,(N-1)}\right)$$

n = number of individuals of a particular bacteria species

N = total number of bacteria species in the various groups (n_1, n_2, $n_3 \ldots n_z$ = N)

The leaf analysis data were subjected to Sample-Two test on Statistix 10 software to determine the difference in means at five probability level. Before the T-test, data were subjected to Shapiro-Wilk normality test, and any found to be not normally distributed were transformed. Transformation was done using the $\log_{10}$ (x +1) for normal data values and arcsine for

percentage data $\sqrt{x \div 100}$ (Gomez & Gomez, 1985). A simple linear correlation analysis was performed to determine if there is a relationship between carbon and nitrogen percentage on the leaves at 5% probability level.

3.3 Results

3.3.1 Morphological (macroscopic) characteristics

Based on morphological analysis, the study isolated a total of 30 bacteria species in winter and 70 in summer which were further classified into 16 and 13 different morphological taxa, respectively (Appendix 3.1). Due to financial constraints, some isolates were only characterized to morphological level (Appendix 3.1) and similarities with molecular characterised isolates was used to group them. The morphologically identified species were further divided into identified (most likely species based on morphological characteristics) and unidentified group (Appendix 3.1). The isolates identified during the winter season could be placed into 10 genera (Appendix 3.1). Makgupheng had the highest number of species isolated and characterized morphologically. In Makgupheng the bacteria were identified as five *Leucobacter* and *Stenotrophomonus* spp. (31.25%) the most dominant genera in Makgupheng during the winter season, followed by two *Enterobacter* spp. (12.50%) and one of each species from *Serratia* sp. (6.25%), *Celullosimicrobium* sp. (6.25%), *Sphingobacterium* sp. (6.25%), and *Kosakonia cowanii* (6.25%) (Appendix 3.1; Figure 3.5). In Tubatse, the bacteria were identified as six *Stenotrophomonus* spp. (42.85%) the most dominant genera in Tubatse followed by two *Rhizobium* spp. (14.29%) and each species from *Bacillus* sp. (7.14%), *Kosakonia* sp. (7.14%), *Alcaligenes* sp. (7.14%) and an unidentified group (21.43%) (Appendix 3.1; Figure 3.5). During the summer season, Tubatse had the highest number of species isolated based on morphological characteristics (Appendix 3.1). The isolated species were placed into 6 different genera: *Bacillus*, *Serratia*, *Stenotrophomonas*, *Enterobacter*,

Micrococcus and *Lysinibacillus*. In Tubatse there were four *Bacillus* and *Celullosimicrobium* spp. (7.27%), three *Lysinibacillus* spp. (5.45%), and one of each species from *Enterobacter* and *Micrococcus* sp. (1.82%) as well as an unidentified group (42.00%). In Makgupheng, there were three *Serratia* spp. (20.00%) and one of each species from *Stenotrophomonas* and *Lysinibacillus* sp. (6.67%) as well as an unidentified group (66.67%) (Appendix 3.1; Figure 3.6). The species *Stenotrophomonas maltophilia* and *Serratia marcescens* found in winter were also detected in summer. Across the two seasons, *Serratia* spp. were only found in Makgupheng, while *Stenotrophomonas* sp. was found in both sites. The species *Enterobacter bugandensis*, *Micrococcus yunnanensis*, and *Lysinibacillus sphaericus* were new species found in summer which were not observed in winter (Appendix 3.1). In addition, the genera *Stenotrophomonas*, *Kosakonia*, *Lysinibacillus*, *Cellulosimicrobium* and *Enterobacter* were shared among the two sites (Figure 3.7).

Overall, *Stenotrophomonas* was the predominant species among all the bacterial isolates and was found in both seasons and sites. *Stenotrophomonas* and *Leucobacter* spp. emerged as the most dominant genera in winter for Makgupheng while *Stenotrophomonas* spp. was dominant in Tubatse in the same season. In summer, *Bacillus* and *Celullosimicrobium* emerged as the dominant groups in Tubatse while *Serratia* was dominant in Makgupheng in the same season.

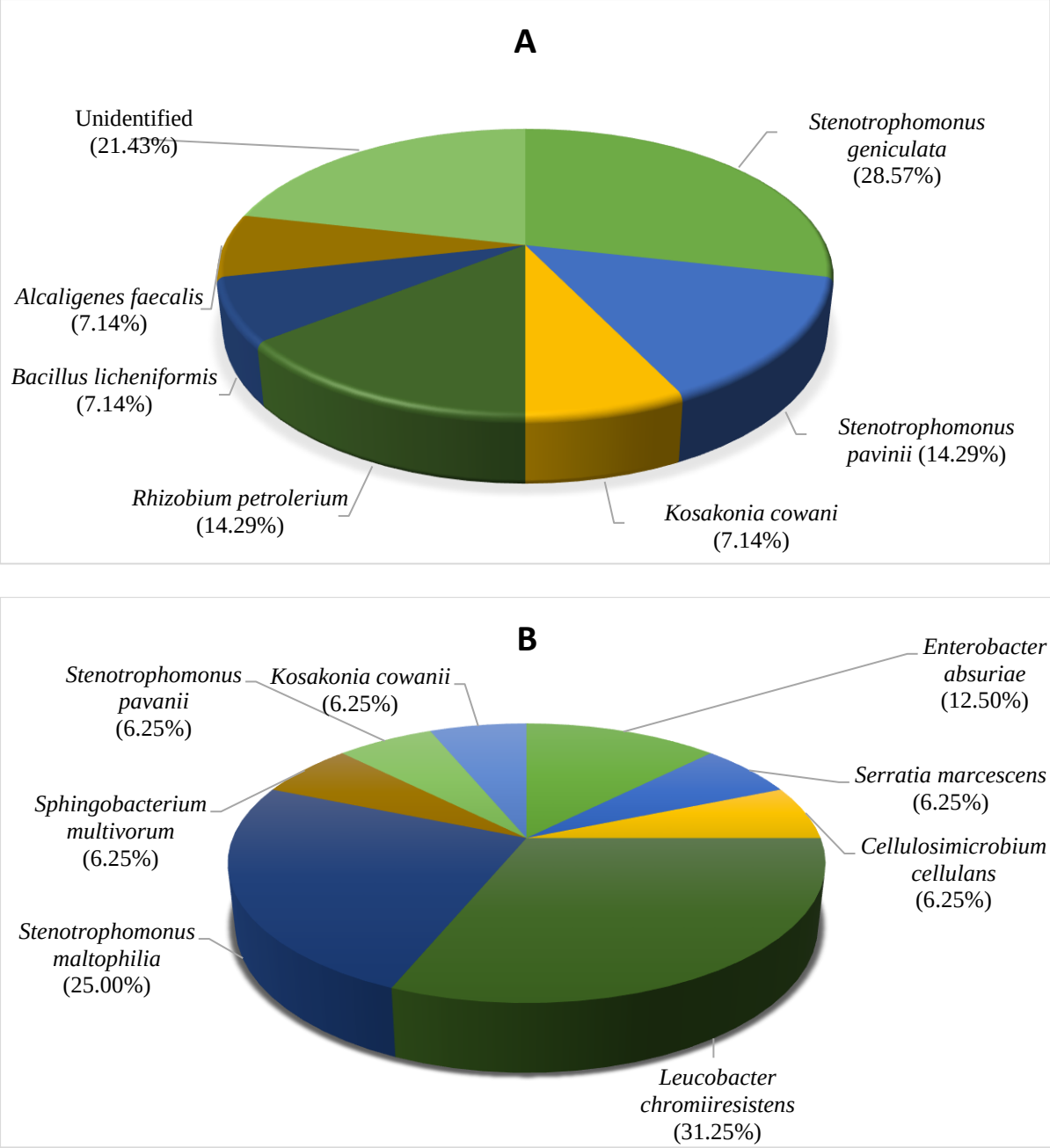


Figure 3.4: Species distribution and percentage abundance of identified and unidentified PGPR species from Tubatse (A) and Makgupheng (B) during winter.

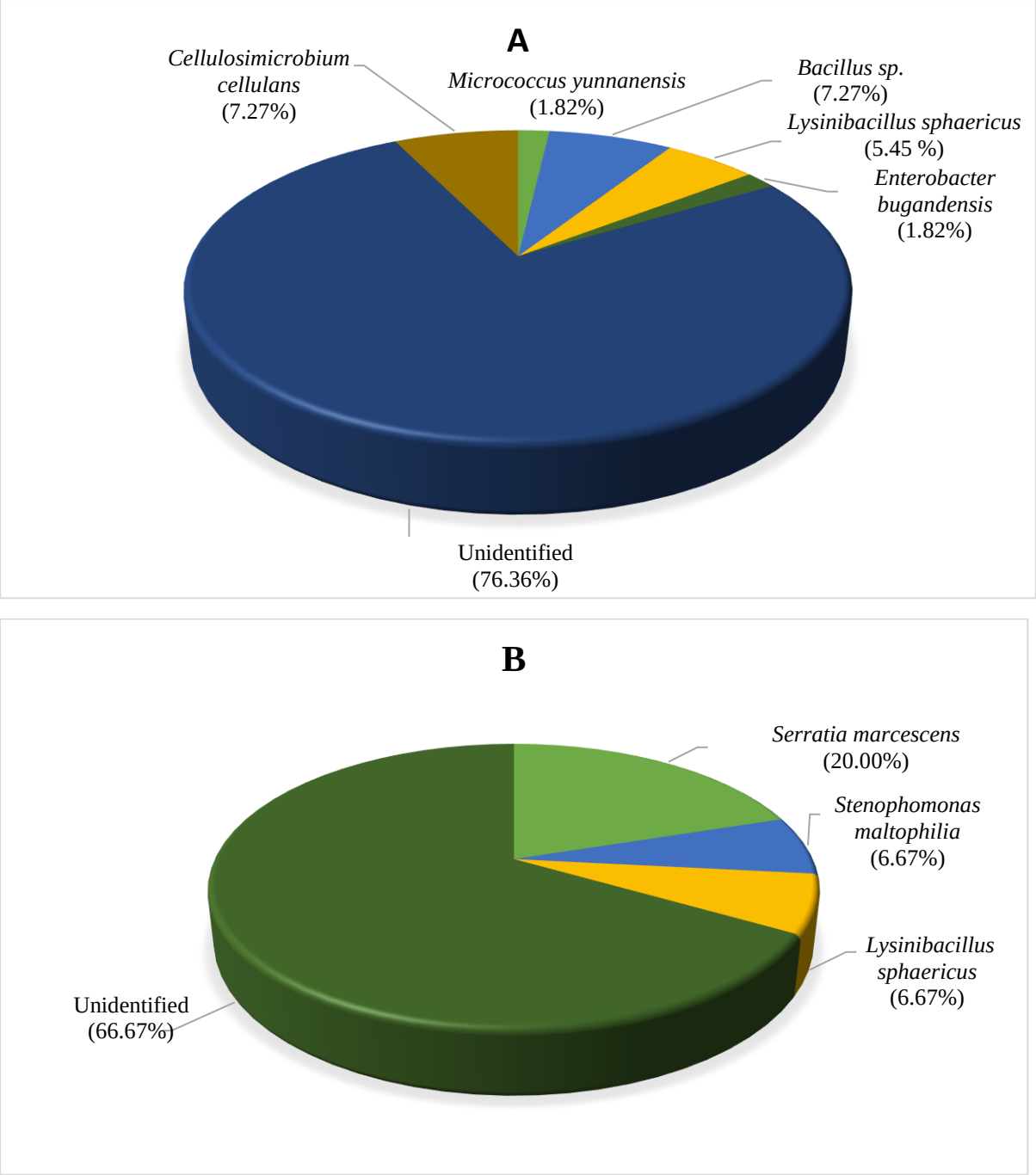


Figure 3.5: Species distribution and percentage abundance of identified and unidentified PGPR species from Tubatse (A) and Makgupheng (B) during summer.

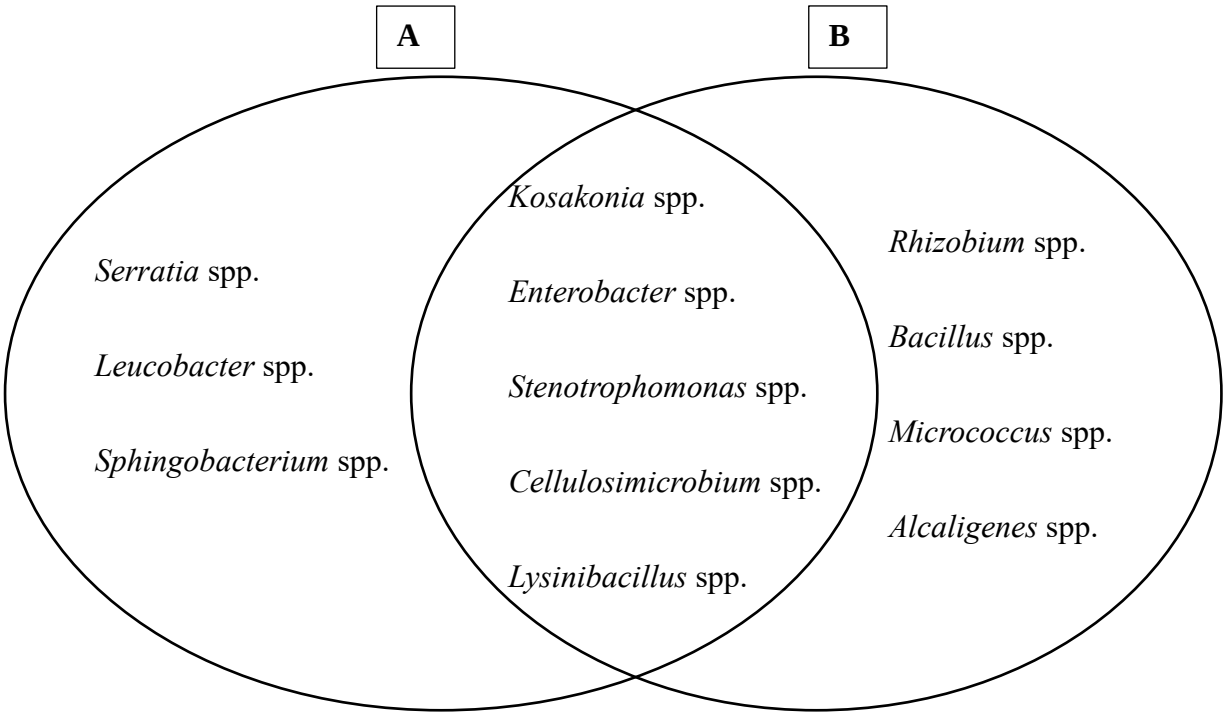


Figure 3.6: Venn diagram of species found in the two sites and shared species among the Makgupheng (A) and Tubatse (B).

3.3.2 Molecular characteristics

The use of 16S rRNA gene sequence analysis enabled the successful determination of the phylogeny of the isolates (Table 3.1). From the morphologically identified species, only nineteen (19) unique isolates: T2CB, T1AA, M4BA, M7BA, T1BA, M4CA, M7CA, M6CA, M8AB, T1CB, T2BA, T2CA, M8AA, M5B2, M3A, T1A, T2B1, T5A2 and T4B41 were submitted to Inqaba Biotechnical Industries for DNA sequencing and characterized as *Rhizobium petrolearium*, *Bacillus licheniformis*, *Enterobacter absuriae*, *Serratia marcescens*, *Kosakonia cowanii*, *Cellulosimicrobium cullulans*, *Leucobacter chromiiresistens*, *Sphingobacterium multivorum*, *Stenotrophomonas maltophilia*, *Stenotrophomonas geniculata*, *Stenotrophomonas pavanii*, *Alcaligenes faecalis*, *Stenotrophomonas maltophilia*, *Stenotrophomonas maltophilia*, *Serratia marcescens*, *Enterobacter bugandensis*, *Micrococcus yunnanensis*, *Bacillus* sp. and *Lysinibacillus sphaericus*, respectively (Table 3.1). The BLAST

search on the NCBI database (Table 3.2) showed a high homology between the isolates and the DNA sequences obtained from the NCBI database with highest homology of 100% with *Serratia marcescens* (CP055161.1) and lowest homology of 78% with *Lysinibacillus sphaericus* (FJ528593.1). All isolates had very low (stronger alignment) E-values (Table 3.1). Subsequent comparisons using the neighbour-joining tree and maximum likelihood model with high bootstrap (1 000 replicates) showed a strong association/homology between the isolates and those obtained from the NCBI database. The bacteria isolates (winter) separated into 6 clusters with the sequences obtained from the NCBI database (Figure 3.8). Cluster 1 (*Stenotrophomonas* group) showed a strong homology between the isolates M8AA and M8AB with the *Stenotrophomonas* with a confidence probability of 89%. Cluster 2 (*Sphingobacterium* group) represents a strong homology (82%) between M6CA with the *Sphingobacterium* group. Cluster 3 (*Rhizobium* group) represents a strong homology between isolate T2CB and *Rhizobium* group with a strong confidence probability of 99%. Cluster 4 (*Bacillus* group) also had a strong homology (99% confidence level) between isolate T1AA and *Bacillus* group. Cluster 5 (*Cellulosimicrobium* and *Leucobacter* groups) shows a high homology between the isolates M4CA with *Cellulosimicrobium* group (96% confidence level) and M7CA with *Leucobacter* species (56% confidence level). Lastly, cluster 6 (*Alcaligenes* group) shows a high homology between T2CA (100% confidence level) with *Alcaligenes* spp. (Figure 3.8). Furthermore, there was a strong homology between GeneBank sequences from NCBI and bacteria isolates from cancer bush root nodules collected during summer. The isolates were separated into 6 group: *Enterobacter*, *Lysinibacillus*, *Bacillus*, *Micrococcus*, *Stenotrophomonas* and *Serratia* species. A strong homology was observed between isolate T1A and *Enterobacter* species with a strong confidence level (100%) (Group 1) (Figure 3.9). Group 2 represent a strong homology between isolate T4B41 and *Lysinibacillus* species with a strong confidence level (90%) (Figure 3.10). Group 3 represent a strong homology between

isolate T5A2 and *Bacillus* species with a strong confidence level (97%) (Figure 3.11). Group 4 represent a strong homology between isolate T2B1 and *Micrococcus* species with a strong confidence level (92%) (Figure 3.12). Group 5 represent a homology between isolate M5B2 and *Stenotrophomonas* species (Figure 3.13) while Group 6 represent a strong homology between isolate M3A and *Serratia* species with a strong confidence level (100%) (Figure 3.14).

Table 3.1: Homology of isolates with NCBI GenBank sequences

Isolate name	Nearest BLAST search	Accession number	% Similarities	[y]E-value
T2CB	*Rhizobium petrolearium*	JX042461.1	84.86	0.000
T1AA	*Bacillus licheniformis*	MN013952.1	81.38	0.000
M4BA	*Enterobacter absuriae*	CP134636.1	83.13	0.000
M7BA	*Serratia marcescens*	CP055161.1	99.66	0.000
T1BA	*Kosakonia cowanii*	CP035129.1	80.58	2e-143
M4CA	*Cellulosimicrobium cullulans*	OP990691.1	80.77	6e-109
M7CA	*Leucobacter chromiiresistens*	MT533900.1	87.37	1e-174
M6CA	*Sphingobacterium multivorum*	CP068088.1	82.65	2e-153
M8AB	*Stenotrophomonas maltophilia*	OQ940482.1	83.32	0.000
T1CB	*Stenotrophomonas geniculata*	OR117356.1	93.97	0.000
T2BA	*Stenotrophomonas pavanii*	MN030333.1	91.26	0.000
T2CA	*Alcaligenes faecalis*	OQ028682.1	85.78	4e-115
M8AA	*Stenotrophomonas maltophilia*	MN09019.1	86.01	0.000
M5B2	*Stenotrophomonas maltophilia*	CP040439.1	89.61	0.000
M3A	*Serratia marcescens*	CP055161.1	99.66	0.000
T1A	*Enterobacter bugandensis*	CP097255.1	82.51	0.000
T2B1	*Micrococcus yunnanensis*	KT44390.1	87.60	0.000
T5A2	*Bacillus* sp.	MW272534.1	86.74	0.000
T4B41	*Lysinibacillus sphaericus*	FJ528593.1	78.33	2e-151

[y]E-values = Lower (stronger) E- value (≤ 0) = Significant alignments; Higher (weaker) E-value (> 0) = Alignment might be a random event.

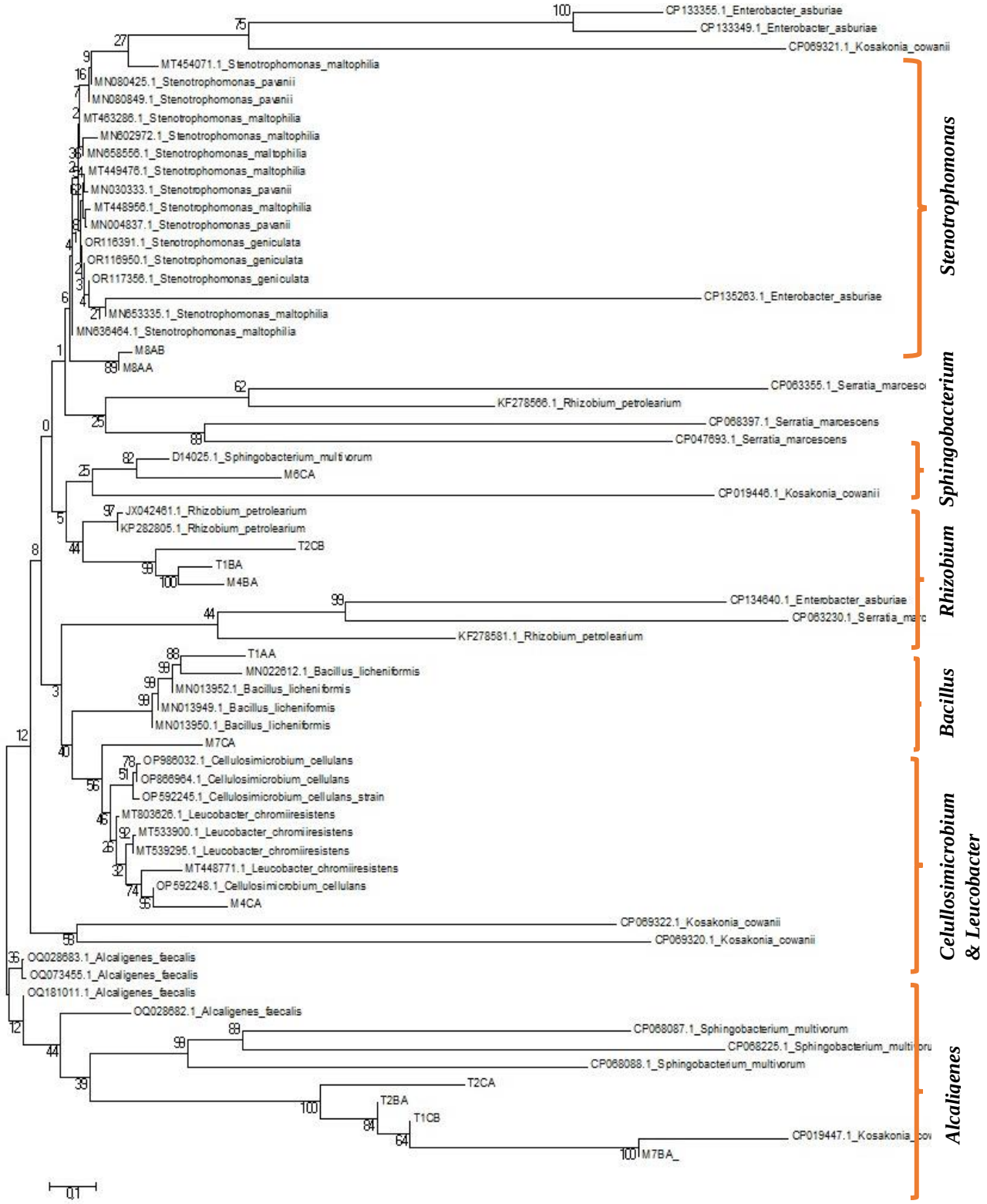


Figure 3.7: Neighbour-joining phylogenetic tree constructed from 13 16S rRNA gene sequence obtained from root nodules of cancer bush collected from two sites in Limpopo Province during the winter season.

Figure 3.8: Neighbour-joining phylogenetic tree constructed from identified bacteria isolate of *Enterobacter bugadensis* isolated from root nodules of cancer bush in summer and NCBI GenBank sequences.

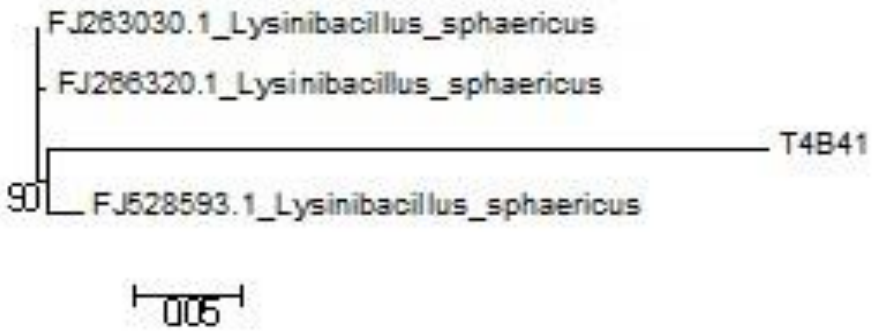


Figure 3.4: Neighbour-joining phylogenetic tree constructed from identified bacteria isolate of *Lysinibacillus sphaericus* isolated from root nodules of cancer bush in summer and NCBI GenBank sequences.

Figure 3.10: Neighbour-joining phylogenetic tree constructed from identified bacteria isolate of *Bacillus* sp. isolated from roots nodules of cancer bush in summer and NCBI GenBank sequences.

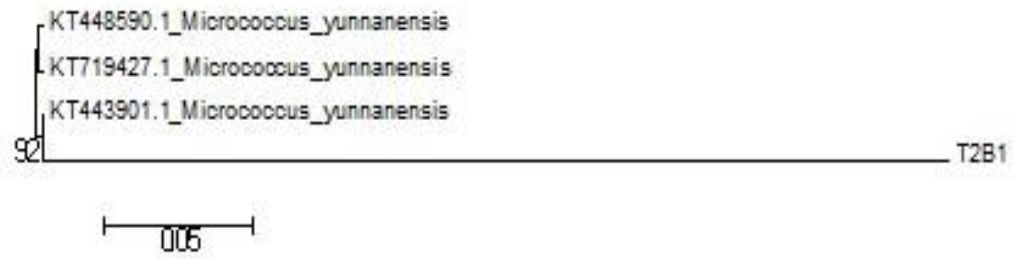


Figure 3.11: Neighbour-joining tree constructed from identified bacteria isolate of *Micrococcus yunnanensis* isolated from root nodules of cancer bush in summer and NCBI GeneBank sequences.

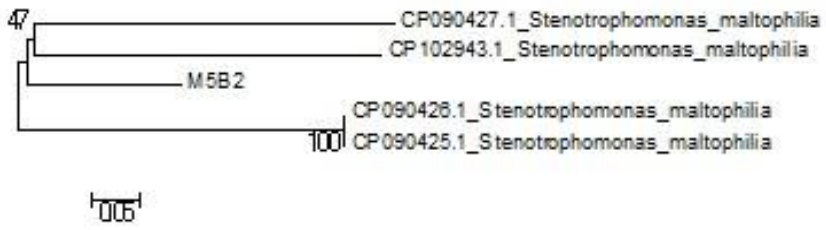


Figure 3.12: Neighbour- joining phylogenetic tree constructed from identified bacteria isolate of *Stenotrophomonas maltophilia* isolated from root nodules of cancer bush in summer and NCBI GeneBank sequences.

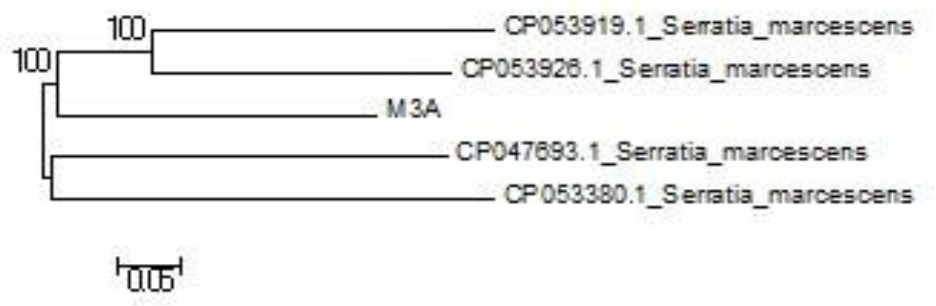


Figure 3.53: Neighbour-joining phylogenetic tree constructed from identified bacteria isolate of *Serratia marcescens* isolated root nodules of cancer bush in summer and NCBI GeneBank sequences.

3.3.3 Microbial diversity index, abundance and evenness

Although Tubatse had higher species incidence (richness) (S) of 20 in both seasons combined when compared with Makgupheng with combined 16 species, if seasons were observed separately, Tubatse had lower number of species (richness) in winter and higher in summer and vice versa for Makgupheng. Both localities had diversity indices greater than 1. Makgupheng had slightly lowest Shannon- Wiener index observed of 1.61 in summer, while Tubatse was lower in winter. (Table 3.2). The highest Sampson indices were 0.94 (winter) and 0.93 (summer) from Tubatse, and the lowest being 0.91 (winter) and 0.85 (summer) from Makgupheng. Tubatse was more diverse than Makgupheng. The population were evenly distributed in both locations as explained by Pielou's evenness (*J*) value that is closer to 1 (Table 3.2). Highest evenness was observed in winter than in summer.

Table 3.2: Microbial diversity index, abundance and species richness

Location		Winter	Summer
Makgupheng	S	11	5
	H'	2.40	1.61
	Ds	0.91	0.85
	J	0.92	0.82

Tubatse	Ds	0.94	0.93
	S	9	11
	H'	2.04	2.00
	J	0.93	0.83

*H= Shannon Diversity Index (H = -Σpi * ln(pi)); S= number of species found/richness; J = Shannon Equitability Index (J = H / ln(S); Ds= Sampson Index (1- ($\sum\frac{n(n-1)}{N(N-1)}$)).

3.3.4 Analysis of isolates for plant growth promoting capabilities

In Tubatse, the relative percentage abundance of N-fixing bacteria was 90 and 86% greater than non-fixing bacteria in summer and winter, respectively (Table 3.3). In Makgupheng, the relative percentage abundance of N-fixing was 60 and 100% greater than non-cycling bacteria in summer and winter, respectively (Table 3.3). A total of 94 nitrogen fixing bacteria were isolated with 66 from Tubatse and 28 from Makgupheng (Table 3.3). Overall, Tubatse had high nitrogen fixing efficiency compared with Makgupheng, shown by the highest number of efficient (+++) nitrogen fixing bacteria (Appendix 3.16). No bacteria were identified as active solubilizers of phosphorus inside the root nodules of cancer bush (Appendix 3.17).

Table 3.3: Nitrogen fixing efficiency of bacteria

Isolate	Nitrogen fixation ability (+/-)			
	Summer		Winter	
N fixing bacteria	Tubatse	Makgupheng	Tubatse	Makgupheng
Total bacteria	53	12	13	16
% Bacteria	95	80	93	100
Non-fixing bacteria				
Total bacteria	2	3	1	0
% Bacteria	5	20	7	0

% N fixing bacteria (Tubatse summer) = Total N-fixing/ (Total N-fixing +Total non-fixing) *100

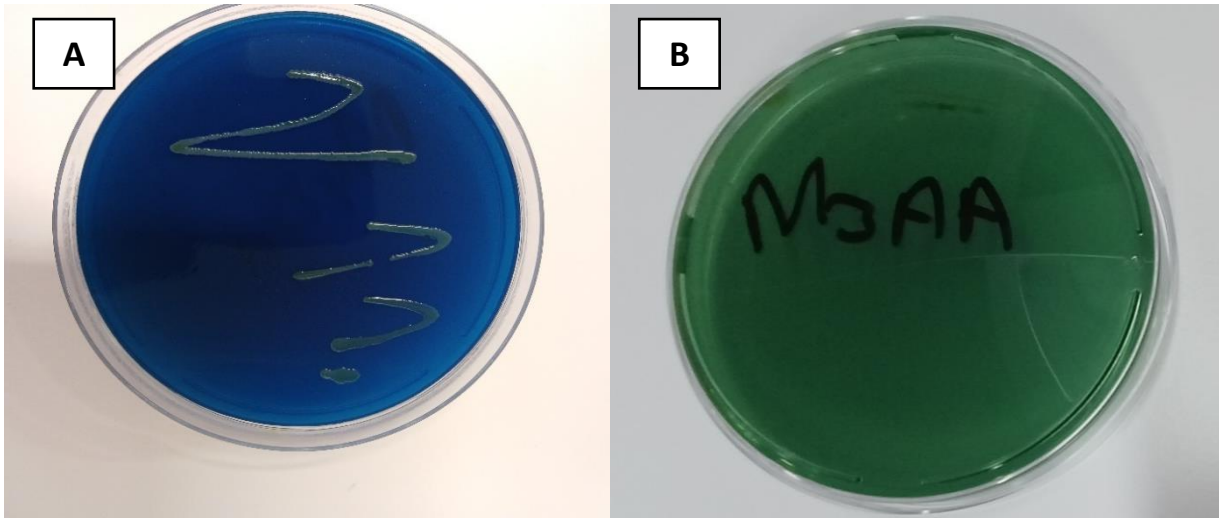


Figure 3.14: A positive test for N cycling of bacteria confirmed by colour change from green to blue (A) and negative test indicated by no colour change in media from green (A).

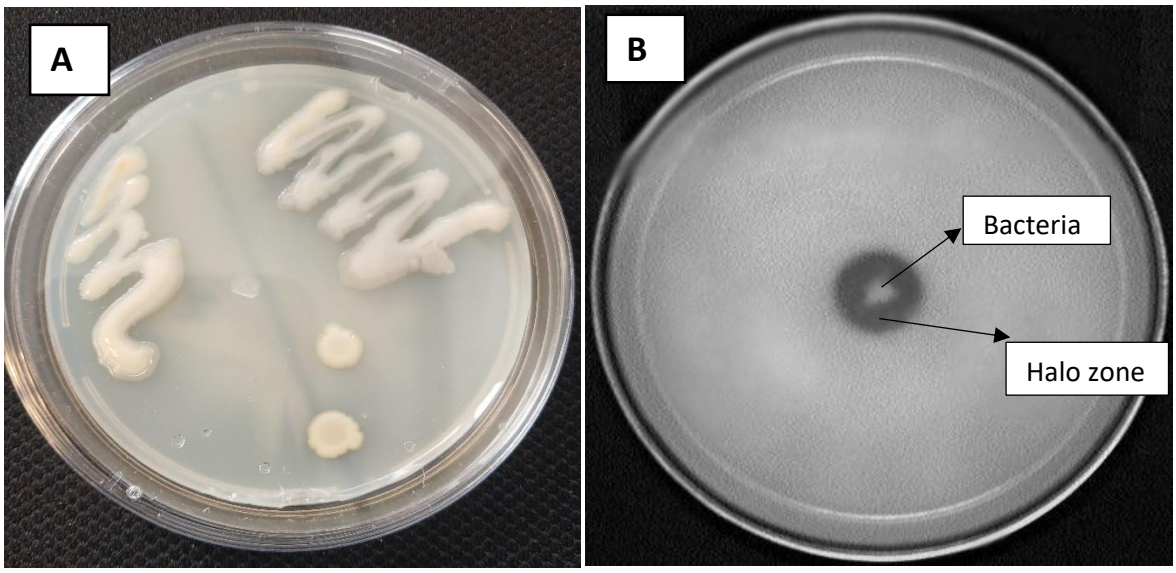


Figure 3.15: A negative test for P solubilization of bacteria confirmed by lack of clear or halo zone (A) and an example of a positive test for P-solubilizing in a bacteria (B) with an arrow showing the halo zone region around the bacteria.

3.3.5 Leaf analysis of nitrogen, C: N, phosphorus and percentage nitrogen derived from atmosphere (% NDFA)

All measured variables were normally distributed at $P \leq 0.05$ according to Shapiro-Wilk normality test (Appendix 3.2). All measured variables were significantly different, except for phosphorus according to null hypothesis (H_0: difference = 0)/ homogeneity of the Folded F test and P-value of pooled variance $\alpha > 0.05$) (Appendices 3.3).

A significant variation ($P \leq 0.05$) in total plant nitrogen (TN), % nitrogen (N), % carbon (C), C: N ratio, nitrogen derived from atmosphere (NDFA) and nitrogen derived from soil (NDFS) between Tubatse and Makgupheng was observed (Table 3.4). Tubatse plants (leaves) had a typically high amount of nitrogen accumulated in the plant (TN), high % carbon and nitrogen (Table 3.4). In particular, averages in TN (mmol), % N and C were 0.04, 0.05 and 1.01 points greater in Tubatse than in Makgupheng, respectively. Furthermore, Makgupheng had high % NDFA levels than soil derived nitrogen while the plants from Tubatse utilized high nitrogen derived from the soil (high NDFS) than nitrogen derived from atmosphere (Table 3.4).

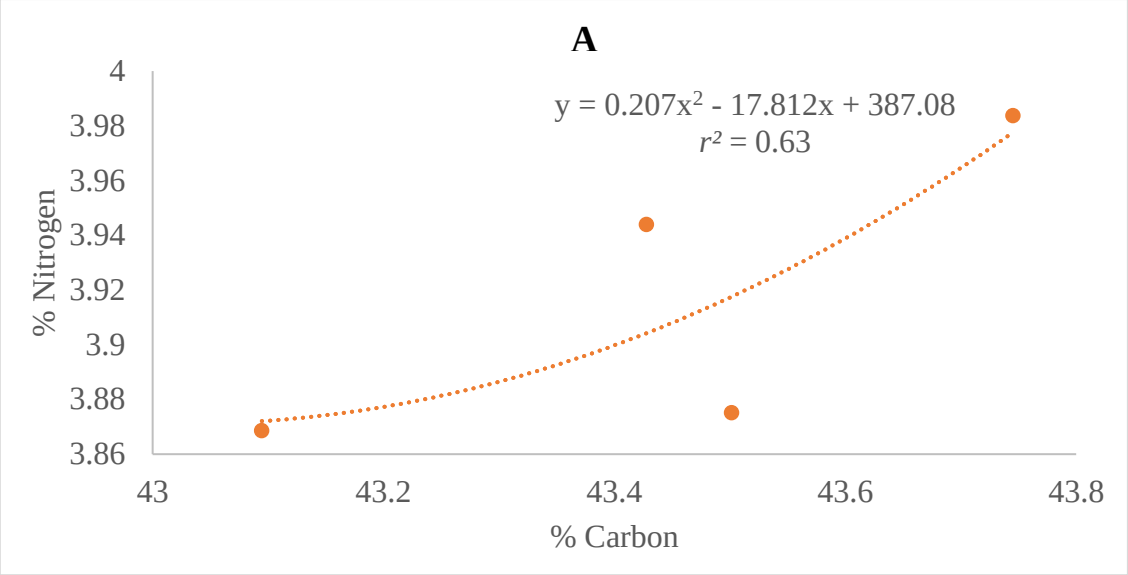
A positive strong correlation in C: N was observed in Makgupheng ($r^2 = 0.99$, $P < 0.05$) and Tubatse ($r^2 = 0.63$, $P < 0.05$) shown by r^2 closer to 1 (Appendix 3.10; Figure 3.12). Graph A demonstrated a slight stimulation effect of nitrogen at lower carbon levels for Tubatse until minimum optimal was reached at 43.02% carbon (Figure 3.12). A sharp increase in % nitrogen was observed with high levels of carbon, beyond the optimum level (Figure 3.12).

In Makgupheng, a high stimulation effect on % nitrogen was observed with a lower carbon level until a maximum optimal was reached at 42.50% carbon (Figure 3.12). Thereafter, a sharp decrease in % nitrogen was observed beyond the optimum level (Table 3.4).

Table 3.4: Mean differences and standard error (SE) in leaf nitrogen, carbon, C: N, % NDFA and NDFS in plants between Tubatse and Makgupheng

Sample	TN (mmol)	% N	% C	C: N	% NDFA	NDFA (mmol)	NDFS (mmol)
Makgupheng	0.24 ± 0.00^{b}	3.38 ± 0.07^{b}	42.43 ± 0.30^{b}	12.57 ± 0.20^{a}	59.32 ± 1.17^{a}	0.14 ± 0.00^{a}	0.04 ± 0.00^{b}
Tubatse	0.28 ± 0.00^{a}	3.92 ± 0.03^{a}	43.44 ± 0.13^{a}	11.09 ± 0.06^{b}	38.94 ± 3.63^{b}	0.12 ± 0.00^{b}	0.07 ± 0.00^{a}
Difference	-0.04	-0.54	-1.01	1.48	20.37	0.03	-0.03

Null Hypothesis (H_0): difference = 0; Alternative Hypothesis (H_1): difference ≠ 0. TN-Total nitrogen in plant, N-Nitrogen, C -Carbon, NDFA-Nitrogen derived from atmosphere, NDFS- Nitrogen derived from soil.



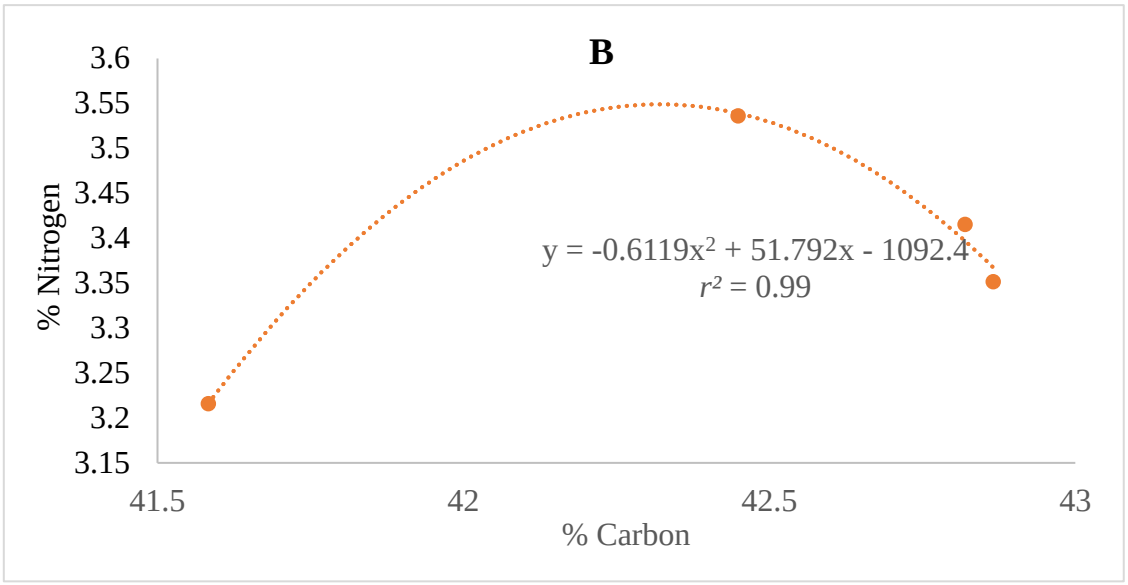


Figure 3.16: Correlation between carbon and nitrogen in plant organ (leaves) between the two locations, Tubatse (A) and Makgupheng (B).

3.4 Discussion

3.4.1 Diversity of PGPRs associated with cancer bush root nodules

Studies on microbial diversity are essential for understanding microbial ecology in soil and other ecosystems (Srivastava, Bhandari & Bhatt, 2014). Microorganisms tend to be very environment specific, hence, the necessity to understanding their diversity, application and

investigation on the occurrence of various groups of microorganisms from different environments (Srivastava *et al*., 2014). The rhizosphere microbial communities have beneficial role of improving plant growth and adaptation to different environments (de la Torre-Hernández *et al*., 2020). These also include plants growing in arid and semi-arid terrains and their relations with microbes (Fonseca-García, Desgarennes, Flores-Núñez & Partida-Martínez, 2018). Generally, arid areas are characterized by nutrient poor soils and long periods of water deficit and these microorganisms play a major role in promoting the exchange of plant nutrients through different growth promoting mechanisms (Malleswari & Bagyanarayana, 2013). Some of these microorganisms (rhizobia and non-rhizobial) live in symbiotic association with plants through their ability to colonize internal tissues of legume and non-legume plants and exist as endophytes while others are only able to colonize the rhizosphere (epiphytic) (Yarte *et al*., 2022). Rhizosphere microbiome and adjacent soil have been reported as most diverse compared to the root microbiome because of the filtering process between the epiphytic and the endophytic compartment of the plants (Wallace, Laforest-Lapointe & Kembel, 2018; Liu *et al*., 2017). Hence, only a few taxa can colonize the internal tissues of the plant (Wallace *et al*., 2018; Liu *et al*., 2017). In the current study, the diverse microbes (PGPRs) associated with root nodules of cancer bush plants growing in the wild were characterized. The species identified were both rhizobia and non-rhizobia (root nodulating and non-nodulating) bacteria with a majority being non-rhizobia species (˜98%). The major classes identified were Gammaproteobacteria followed by Actinobacteria. The Actinobacteria was only detected in Makgupheng. Gammaproteobacteria was detected across location, Tubatse and Makgupheng. Other classes such as *Bacillus*, Betaproteobacteria, Alphaproteobacteria, Actinomycetes and Sphingobacteria were also present, but in small components. The findings of this study show that *Stenotrophomonas* spp. represent the largest group found in cancer bush nodules occurring across the two locations. This is contrary to what most studies have reported that *Bacillus* and

Pseudomonas are the dominant groups isolated in the rhizosphere of wild medicinal plants and other legume plants including the common winged prickly ash (*Zanthoxylum armatum* DC.) (Srivastava *et al*., 2014), garden heliotrope (*Valeriana officinalis* L.) (Ghodsalavi *et al*., 2013), pink trumpet tree (*Handroanthos impetiginus* Mart. ex DC.) (Yarte *et al*., 2022) and holy basil (*Ocimum sanctum* L.) (Sumbul *et al*., 2020; Singh *et al*., 2015; Solanki *et al*., 2011). *Bacillus* is considered as the major component of the microbial flora living in close association with diverse group of plants due to its ability to efficiently use the nutrients provided by the plant through exudates (Srivastava *et al*., 2014). It also has an ability to inhibit growth of other strains through the release of growth inhibitors. *Stenotrophomonas* species were earlier reported as opportunistic and disease causing-pathogen in humans and animals since early 1980s (Adamek, Overhage, Bathe, Winter, Fischer & Schawartz, 2011). Hu *et al*. (2021) reported the phytopathogenic character of different strains of *Stenotrophomonas maltophilia* on soft rot clivia decayed leaves. However, several studies have also reported on the plant growth promoting ability of these species, mainly as effective nitrogen fixer and solubilizer of phosphorus around the rhizosphere and root tissues of non-legume plants such as maize (*Zea mays* L.) (Perez-Perez, Oudot, Hernandez, Napoles, Martinez & Castillo, 2020) and tomato (*Solanum lycopersicum* L.) (Helal, El-khawas & Elsayed, 2022). Species within the *Stenotrophomonas* genus have been reported to have a great potential for growth promotion, especially under stress conditions through their ability to improve plant tolerance to abiotic stress such as salinity and drought (Ulrich, Kube, Becker, Schneck & Ulrich, 2021). Ali and Osman (2022) isolated *Stenotrophomonas maltophilia* species from the root nodules of clover plants and were able to promote their growth under saline conditions. Similar to the findings of the current study, *Stenotrophomonas* species (*S. maltophilia, S. geniculata* and *S. pavanaii*) was found to be an efficient nitrogen fixer which is the most element required by plants for growth and development.

Other plant growth promoting rhizobia such as *Serratia*, *Enterobacter*, *Sphingobacterium*, *Rhizobium*, *Alcaligenes* and many others that the study isolated have also been found commonly in some medicinal plants (Aeron, Chauhan, Dubey, Maheshwari & Bajpai, 2015; Srivastava *et al*., 2014; Ghodsalavi *et al*., 2013; Giongo *et al*., 2010). Despite the high specificity between *Rhizobium* and legumes, the presence of non-rhizobia species in legume tissues has been reported in many leguminous crops (Dhole *et al*., 2016). The species within the genus *Serratia*, *Enterobacter*, *Kosakonia*, *Bacillus*, *Sphingobacterium*, *Micrococcus*, *Stenotrophomonas*, *Cellulisimicrobium* and other species isolated in this study except *Rhizobium* are among the non-nodulating and non-rhizobial bacteria (Etesami, 2022). The occurrence of *Bacillus* group as a root nodule endophyte has been reported in several plants such as soybean (*Glycine max* L.), pigeon pea (*Cajanus cajan* (L.) Huth), kudzu (*Pueraria montana* var. lobata (Wild)) and wheat (*Triticum aestivum* L.) to have beneficial role for their host by promoting nodulation and growth (Zhao, Xu, Sun, Deng, Yang & Wei, 2011). Tariq, Hameed, Yasmeen and Ali (2012) reported that endophytic non-rhizobia bacteria were able to co-exist with rhizobia and as result enhance nodulation and growth of mung bean plant [*Vigna radiata* (L.) Wilczek] through the various mechanisms of growth promotion (P solubilization, N fixation IAA production etc.). Additionally, a study by Stajkovic, Meyer, Milicic, Willems and Delic (2009) isolated non-rhizobial endophytes from root nodules of alfalfa (*Medicago sativa* L.) belonging to *Bacillus megaterium*, *Brevibacillus chosinensis* and *Microbacterium trichothecenolyticum.* None of these species were able to nodulate the same host when re-inoculated in gnotobiotic culture. However, when the same non-rhizobia strains were co-inoculated with *Sonorhizobium meliloti* were able to influence nodulation of the plant. The interactions of the co-existence of these non-rhizobia and *Rhizobium* bacteria in cancer bush has not been determined, hence there is need to further investigate and understand this occurrence.

Saidi, Chebil, Gtari and Mhamdi (2013) reported some of the possible reasons for the occurrence of non-nodulating bacteria in root nodules and one of them was connected to the bacteria being a true endophyte. However, Saidi *et al*. (2013) further stated that some of these bacteria are opportunistic and may colonize root nodules of legume plants, also other reason may be surface contamination of nodules if aseptic conditions are not followed which may lead to misidentification. The *Stenotrophomonas* species which in this case is able to fix nitrogen, has previously been identified as an opportunistic human and animal pathogen causing infection (Adamek *et al*., 2011). According to Saidi *et al*. (2013) it is therefore crucial that microscopic observations are done to conclusively confirm the endophytic character of these non-rhizobia bacteria when isolated in plants and clarify their localization inside nodule tissues. *Rhizobium* was found to be the only group of rhizobia found, among the approximately 98% non-rhizobia bacteria isolated. The colonization and presence of *Rhizobium* within nodule tissues of cancer bush was expected as cancer bush is also a legume plant. Although the percentage was low, *Rhizobium* genus is widely known for its established symbiotic association with legume plants through which it is able to fix N and other nutrient cycling abilities. *Rhizobium* sp. has also been found to colonize the internal tissues of other non-legume plants such as pink trumpet tree (*Handroanthus impetiginosus* Mart. ex DC.) (Yarte *et al*., 2022), Eastern cottonwood plant (*Populus deltoides* L.) and silver poplar (*Populus alba* L.) (Garci-Fraile, Rivas & Willems, 2007; Doty, Dosher & Singleton, 2005). In such cases the bacteria do not form nodule but live as an endophyte inside root tissues and is still able to fix N and improve growth through other indirect mechanisms (Yarte *et al*., 2022).

3.4.2 Plant growth promotion abilities of bacteria and nutrient acquisition of cancer bush

Cancer bush is a legume plant that has established a symbiotic relationship with N-fixing bacteria in the soil which also help with nutrient acquisition, especially, immobilizing the

unavailable P to plant usable forms. It was expected that nutrient concentration in cancer bush rhizosphere be high to help the plant adapt to stressful conditions because of this known symbiosis the plant has with these beneficial microbes in the soil. The PGPRs associated with cancer bush were analysed for their plant growth promoting abilities and how they help the plant with nutrient acquisition. One of the ways is biological nitrogen fixation which occurs through symbiotic and non-symbiotic associations between plants and microorganisms (Gouda, Kerry & Das, 2018). The symbiotic association is between legume plants and rhizobia group while non-symbiotic association occurs between non-legume plants and rhizobia species or legume plant and non-nodulating bacteria. Through this symbiotic interactions, plants fix carbon as well as providing a niche to microbes which in turn fix nitrogen enhancing its fertility (Gouda *et al*., 2018). The study isolated a total of 94 nitrogen fixing bacteria from cancer bush root nodules (over 94% average abundance in Tubatse and 80% in Makgupheng). This was confirmed by their ability to grow in a nitrogen free media (Nfm) (Simmons citrate media), suggesting the recognised and important role of cancer bush symbionts in nitrogen fixation and other nutrient recycling abilities. This also shows the ability of the bacteria to utilize carbon source and release ammonium (NH_4^+) for plant use. Several studies have characterized these high nitrogen-fixing root nodulating bacteria. *Rhizobium* sp. was isolated from several roots of indigenous legume plants such as pink trumpet tree (Yarte *et al*., 2020) faba bean (*Vicia faba* L.) (Saidi *et al*., 2013), pea plant (*Pisum sativum* L.) (Shahza *et al*., 2019) and water mimosa plant (*Neptunia oleracea* L.) (Kumar *et al*., 2016). *Bacillus* sp. have been identified as high nitrogen fixing strain from *Thymus vulgaris* L. (Abdel-Hamid, Fouda, El-Ela, El-Ghamry & Hassan, 2021). In a study conducted by Srivastava *et al*. (2014) in a legume medicinal plant, the isolated *Serratia* strains tested negative for ammonium production, which is contrary to the findings of this study, as the *Serratia* spp. were able to grow in a nitrogen free media (Nfm). Most isolates belonging to Betaproteobacteria (*Alcaligenes*), Gammaproteobacteria (among

these are *Serratia*, *Stenotrophomonas*, *Kosakonia*, *Enterobacter*) and *Sphingobacteria* have been characterized as nitrogen fixers through their ability to grow in Nfm (Aeron *et al*., 2015). The findings of this study also isolated the nitrogen cycling *Enterobacter* sp. which was also isolated from lupine plant (*Lupinus albencens* H.) and having the ability to utilize C source and release ammonia (Giongo *et al*., 2010). Aeron *et al*. (2014) first characterized the *Sphingobacterium* sp. from root nodules of Asian pigeonwing plant (*Clitoria ternatea* L.), however, the species failed to re-nodulate the plant in the field. In a study by Yarte *et al*. (2022) it was reported that some species are able to nodulate and fix nitrogen in the conditions that they are tested on but fail to re-nodulate in different conditions. Aeron *et al*. (2014) highlighted that some non-rhizobia species are able to colonize root tissues and fix nitrogen, however, they are not true symbionts meaning that they may lack symbiotic genes such as *nif*H and *nod* gene. These genes stimulate symbiotic nodule formation in plants. Others may have *nif*H genes but are not true rhizobia, hence the inability to re-nodulate. Some non-rhizobia endophytes contain *nif*H or *nod* genes (the nitrogenase activity of the rhizobia is confirmed) and can nodulate plants. This ability may occur through lateral transfer of symbiotic genes (i.e. *nif*H) between symbiotic rhizobia and non-rhizobia (endophytic bacteria), which is an ecologically important mechanism that offers the emergence of new symbiotic genera by 1-step evolution (Muresu *et al*., 2008). Zakhia *et al*. (2006) isolated endophytic *Bacillus nif*H gene from root nodules and they matched those of true rhizobia, suggesting that the bacteria occupy a specific niche in the nodules through this horizontal gene transfer. The cooperative interactions between rhizobia and other root-colonizing bacteria are of relevance in the improvement of nodulation and N_2 fixation in legume plants (Barea, Maria, Rosario & Concepcion, 2005). Most of the bacteria that the study isolated from cancer bush root nodules were the non-nodulating (endophytic bacteria) which most of them were able to fix nitrogen. However, their *nif*H primers have not been confirmed with those of true rhizobia.

Phosphorus is another important nutrient in the soil after nitrogen that is involved in improving several important metabolic processes such as macro-molecular biosynthesis, energy transfer, cellular respiration, photosynthesis, and signal transduction (Yarte *et al*., 2022). Although it is abundant in soil, the availability of its organic and inorganic forms is restricted to plants as it occurs in insoluble forms that plant cannot utilize (Sharma *et al*., 2013). Fortunately, there are microbes in the soil called phosphorus solubilizing bacteria (PSB) which help in the synthesis of phosphatases and organic acids of low molecular to solubilize phosphorus thus stimulate plant growth (Gouda *et al*., 2018). Several PGPRs isolated from cancer bush were subjected to series of subculturing using solid media supplemented with calcium phosphate (Pikovskaya's media- TCP) to test their solubilizing ability under *in vitro* conditions. Normally, the ability of a PSB to solubilize P is shown by development of a clear/ halo zone around the bacteria colony after several culturing procedures (Sharma *et al*., 2013). Several media such as Pikovskaya (Pikovskaya, 1948), bromophenol blue dye (Gupta, Singal, Shanker, Kuhad & Saxena, 1994) and National Botanical Research Institute (NBRIP) medium (Nautiyal, 1999) have been selected and used as a source of insoluble phosphate to detect phosphorus solubilizing microbes.

Several studies have reported the *Bacillus* species (*B. safensis*) as an efficient phosphorus solubilizer and improved the growth and yield of wheat (*Triticum aestivum* L.) (Wang *et al*., 2022), also *Bacillus thuringiensis* in root nodules of *Erthrina brucei* (Berza, Sekar, Vaiyapuri, Pagano & Assefa, 2022), *Serratia* and *Enterobacter* in *Mimosa pudica* L. (Sanchez-Cruz *et al*., 2019). Moreover, species belonging in these genera: *Rhizobium, Serratia, Bacillus, Pseudomonas, Azotobacter, Rhizobium, Bradyrhizobium, Xanthomonas, Rhodococcus, Burkholderia, Enterobacter, Actinobacteria* and other bacteria have also been characterized as phosphorus solubilizers (Mekonnen & Kibret, 2021). However, the findings of our study are contrary to what have been reported as the isolated bacteria species: *Rhizobium*, *Bacillus*,

Kosakonia, *Stenotrophomonas*, *Serratia*, *Alcaligenes*, *Enterobacter*, *Micrococcus* and others did not show any growth of halo zone when sub-cultured in TCP media for the ability to solubilize phosphorus. Several reasons or theories have been reported around this failure of known P solubilizers to make phosphate available. Early findings of Kucey (1983) reported that most phosphorus solubilizing bacteria, unlike fungi, tend to lose/lack the ability to retain their P solubilizing ability over many repeated subculturing transfers faster than fungi. The study found that most bacteria lost their solubilizing ability when sub-cultured while others were able to solubilize P faster (within the first three days of culturing/incubation) but later fail to maintain the halo zone around the colony after some time (Kucey, 1983). Similar findings were reported by Sperber (1958a) that majority of the bacteria isolates which are known to make phosphate available to plants can rapidly and irreversibly lose their ability solubilize the apatite (phosphate mineral) when repeatedly sub-cultured on glucose yeast extract (GYA). Bashan, Kamney and de Bashan (2013a) proposed that quantitative tests can be carried out to further assay and confirm P solubilization of isolates rather than relying on qualitative tests (formation of a halo zone) as a sole test for phosphorus solubilization. Phosphorus solubilizing bacteria use different mechanisms to dissolve P. One of the mechanisms is the ability to produce several organic acids (acetic, oxalic, succinic, citric, lactic, ketogluconic, tartaric, gluconic acid) which through their carboxyl and hydroxyl group lower the pH or chelate the cations that are bound to phosphate and ultimately converting it into soluble phosphate thus making it available to plants (Mekonnen & Kibret, 2021). Quantitative test of PSB for production of these organic acid and determination of media pH after inoculation of media with PSB is one of the possibilities the study will further consider to conclusively confirm the ability to of the isolated bacteria to solubilize P. Furthermore, to directly test them on a model plant for direct contribution to P plant nutrition as previous researchers have emphasized that ability of an isolate to solubilize P on a freshly prepared medium do not necessarily prove it ability to

promote growth (Collavino, Sansberro, Mroginski & Aguilar, 2010) and inability of isolate to form a clear/halo zone on media do not necessarily confirm inability to solubilize P (Bashan *et al*., 2013a). Growth promotion in plants, even by PSB can be the outcome of other mechanisms (Bashan *et al*., 2013a).

3.4.3 Leaf analysis of plants for nitrogen (NDFA and NDFS) and C: N ratio in both study sites

Leaf represents the major organ of photosynthesis and physiological activities in plants, and it is on the leaves that the ratio of carbon to nitrogen help one understand and quantify the carbon and nitrogen metabolic status in crops (Xu *et al*., 2018). Carbon to nitrogen ratio is considered as a valuable indicator and a quick way of evaluating the metabolic balance of two elements present in the soil that are both essential for dynamic growth and microbial health (Xu *et al*., 2018). Simply, C: N ratio is the ratio between the nitrogen content in the microbes and in the organic residues and to the carbon content (United States Department of Agriculture, 2011). The carbon to nitrogen ratio in plants is important for dynamic regulations in crop fields to quickly monitor the changes in leaf C: N and help to guide field managements and improve the ultimate formation of yield and quality in crop production (USDA, 2011). The ideal C: N ratio of agricultural soil is 10:1, for microbes it ranges around 4: 1 to 8: 1 (maximum is 9: 1) and legume plants normally have a ratio of 20: 1 (ranging < 25: 1) (USDA, 2011). The 10: 1 ratio is considered as an indication of equilibrium state that should be maintained. Microbes need a ratio of 24: 1 as their diet to stay alive which is used for energy and maintenance (USDA, 2011). If material that contains a ratio beyond this diet is added into the soil, it stimulates microbial population since there is plentiful food. This also mean that there's excess carbon than the perfectly balanced diet microbes require and additional N to supplement the excess C will be required by the microbes so that they will be able to consume this high carbon diet. The excess N required will eventually be absorbed from the soil and immobilized in their tissues. As a result, this deprives the plants growing in the soil the N needed for their immediate growth.

However, as C is being broken down by the microbes, N is released into the soil again and the 10: 1 equilibrium is established again. In this study, we observed a lower leaf C: N ratio in both sites (ranging between 12: 1 in Makgupheng and 11: 1 in Tubatse). This ratio of the two sites is quite lower that the balanced diet of microbes (24:1), implying possibilities of increased N levels in the soil and less/no need for excess N by the microbes to consume this C.

The study further observed a high total plant N in the plants, especially in Tubatse with low nitrogen derived from the atmosphere (% NDFA) and high percentage nitrogen derived from soil (NDFS mmol. In Makgupheng, there was a high % N derived from atmosphere than % N derived from soil. This implies that the plants in Makgupheng mostly depended on NDFA to supplement leaf N. The NDFA is the amount of nitrogen that the plant acquired through atmospheric nitrogen fixation by nitrogen fixing microbes. This high % NDFA emphasises the importance of the symbiotic relations between these wild plants and the beneficial soil microorganisms in promoting nutrient cycling and acquisition by plants. de la Torre-Hernández *et al*. (2020) reported the beneficial role of rhizosphere microbial communities in improving plant growth and adaptation to different environments through nutrient cycling mechanisms and increased assimilation by plants. It is also known that plants growing in arid and semi-arid regions are faced with challenges of nutrient poor soils and prolonged periods of water deficit with negatively influence their growth and development as a result reduced abundance and richness (Fonseca-García *et al*., 2018). The establishment of symbiotic relations with the beneficial microorganisms has a major role in promoting the exchange of nutrients through different growth promoting mechanisms such as nitrogen fixation and improving the survival of these plants in their natural habitat (Malleswari & Bagyanarayana, 2013). Therefore, it is important to evaluate and understand C: N ratios of crop residues and other material applied to the soil and soil cover. This should be the goal of any producers interested in improving soil

health for optimum growth/yield, so that they may be able to provide quality habitat for soil microorganisms because of the benefits the perform in the soil.

3.5 Conclusion and recommendation

3.5.1 Conclusion

The species of bacteria isolated from root-nodules of cancer bush growing in different soils were highly diverse and could be placed into 12 genera. The total number of species isolated in both seasons combined, were greater in Tubatse than in Makgupheng. Tubatse was more diverse in summer and less diverse in winter, and while Makgupheng was less diverse in summer and more diverse in winter. A total of four genera: *Rhizobium*, *Bacillus*, *Micrococcus* and *Alcaligenes* were isolated in Tubatse in both seasons combined. A total of three genera: *Serratia*, *Leucobacter* and *Sphingobacterium* were isolated in both seasons combined for Makgupheng. Species belonging to *Kosakonia*, *Enterobacter*, *Cellulosimicrobium*, *Lysinibacillus* and *Stenotrophomonas* were shared among sites. All the isolated bacteria species showed a great potential for plant growth promotion through their ability to fix nitrogen. Tubatse had a high number of N fixing bacteria compared to Makgupheng. Over 94% N fixing bacteria in average were isolated from Tubatse and 90% in Makgupheng. Majority of the bacteria species the study isolated are categorized as non-rhizobia (non-nodulation and nodulating) species. Based on leaf analysis for nitrogen and C: N ratio in plant across these two locations, both locations had low C: N ratio suggesting a balance between % carbon and nitrogen in the soil. The findings of the study also show that soil from Makgupheng was found to have higher % N derived from atmosphere (% NDFA) compared to Tubatse. This indicates the potential role and ability of these microbes in nutrient (N) cycling and improving nutrient acquisition and thus plant growth.

3.5.2 Recommendation

The study recommends testing for presence of symbiotic genes from the isolated bacteria especially the ones that could fix nitrogen when literature does not report of it and efficiency on growth promotion under field conditions. Microscopic observation to definitively confirm the endophytic character and clarify their localization inside nodule tissues is very important as legumes represent a valuable source for selecting effective microorganisms to be used as microbial inoculant's for improving plant growth. Hence, further testing of PGPRs abilities under field conditions could be an added advantage to the potential use of this bacteria.

3.6 References

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CHAPTER 4

EDAPHIC PROPERTIES OF *SUTHERLANDIA FRUTESCENS* (L.) R. Br RHIZOSPHERE AND THEIR EFFECT ON EXTRACELLULAR ENZYME ACTIVITIES OF PGPR

4.1 Introduction

Enzyme in the soil come from animals, plants and microorganisms and their activities may reflect the metabolic state of soil at a given period of time (Shao, Zhao, Liu, Long & Rengel, 2020). Soil enzyme activity have a major role in nutrient recycling, energy flow and increasing nutrient accessibility for plant and microbial use (Raimi, Ezeokoli & Adeleke, 2023). Moreover, soil enzyme activities are not only important as indicators of soil quality/ fertility and initiating soil biological processes involved in nutrient cycling, but also play a key role in preventing pests and diseases (Kuramae, Yergeau, Wong, Piji, Van Veen, & Kowalchuk, 2011). Enzyme activity of β-glucosaminidase in the soil are involved in biocontrol of plant pathogens, the most characterized being the one from the biocontrol fungus, *Trichoderma harzianum* (Parham & Deng, 2000).

Soil microorganisms play a key role in maintaining terrestrial ecosystems. Enzymes released by microbes are involved in biogeochemical cycling of nutrients in the soil, such as carbon, nitrogen and phosphorus and are called extracellular enzyme activities (Liu *et al*., 2021; Li *et al*., 2018; Mohammad, 2015). Microbes use extracellular enzymes as the major means to access the biological unavailable nutrients such as C, P and N in soil organic matter matric (Blonska *et al*., 2020). Lignin, cellulose, hemicellulose, chitin, starch, proteins are some of the most abundant organic soil compounds that are enzymically degraded (Blonska *et al*., 2020). Enzymes such as phosphatase, for instance, may be used as indicators of the concentration of P biotransformation (Ndlovu *et al*., 2023). Phosphatases are used to convert insoluble cation-bound P (organic phosphorus) in the soil into available form of phosphorus (Shao *et al*., 2020). The increased secretion and activities of enzyme such as phosphatase in the soil is how some

plants and microorganisms respond to soil acidity and P deficiencies (Ndabankulu, Egbewale, Tsvuura & Madadlela, 2022). Phosphatase is divided into three groups based on soil pH: (1) acidic (pH 5.0), (2) neutral (pH 7.0) and (3) alkaline (pH 10.00) (Shao *et al*., 2020). Glucosidases are group of carbon cycling enzymes that catalyse lower weight molecules in the soil, such as carbohydrates and are considered as primary energy sources for soil microbes (Nannipieri, Giagnoni, Landi & Renella, 2011). Glycosidase, a-galactosidase also known as cellobiose is an enzyme hydrolyse dissacharides which are a-D Galatopyrranisidase in the soil (Zhang *et al*., 2020). N-acetyl-β-D glucosamine is catalysed by β-Glucosaminidase enzyme, and this hydrolysis is important for C and N cycling in the soil (Turner, 2010; Acosta-Martinez, Perez-Guzman & Jonson, 2019). Several factors such as substrate (of P, C and N) availability, enzymatic cofactors and nutrient limitation have a direct effect on the activities of the listed enzymes (Blonska *et al*., 2020). Chemical factors also influence enzymes activities in the soil with pH as the major contributing factor. Soil pH affects soil enzyme activity by controlling the production of microbial enzymes, through ionization-induced conformational changes of enzymes and availability (Kotroczo *et al*., 2014). Several studies have investigated the effect of pH on the activity of Laccase (Olajuyigbe & Fatokun, 2017) perodase (Bhuyan *et al*., 2019), N-acetyl-B-D-Glucosaminidase (Acosta-Martinez *et al*., 2019) and B-D-Cellobioside (Delgado-Baquerizo, Grinyer, Reich & Singh, 2016). However, the rhizosphere microbial enzyme activities of cancer bush under the two study regions have not been investigated and documented. Hence the objective of the study was to determine the soil extracellular enzyme activity around the rhizosphere of cancer bush plants and how they are influenced by nutrient availability.

4.2 Materials and methods

4.2.1 Study location

Experiments were conducted at the University of KwaZulu-Natal, Westville Campus (-29.81°78′97″ S; 30.94°27′71″ E), South Africa. For total soil analysis, soil was sent to the Department of Agriculture and Rural Development's Analytical Services Unit, Cedara, South Africa.

4.2.2 Soil sampling and preparation for nutrient analysis and enzyme activities

Twenty soil samples collected with every collection of nodules from each location were used to determine the physico-chemical properties that might have an influence on the species occurrence, diversity, and abundance across the two locations (Table 4.1). The soil was collected from a depth of 0 to 30 cm using an auger and thoroughly mixed and sun dried (Figure 4.1). This depth is regarded as the region that is closer to the roots and associated with maximum microbial enzyme activities. After sun-drying the soil were processed by running it through a sieve mesh of 2 mm to get rid of the gravel and plant debris. The soil sample was apportioned into two, one portion of the soil was used for soil nutrient analysis while the other potion for soil enzyme activities. The soil for enzyme activity was not sun-dried and was stored at low temperatures (4^{o}C) in a refrigerator to avoid moisture loss as well as inhibiting the microbial activities and enzymatic reactions.

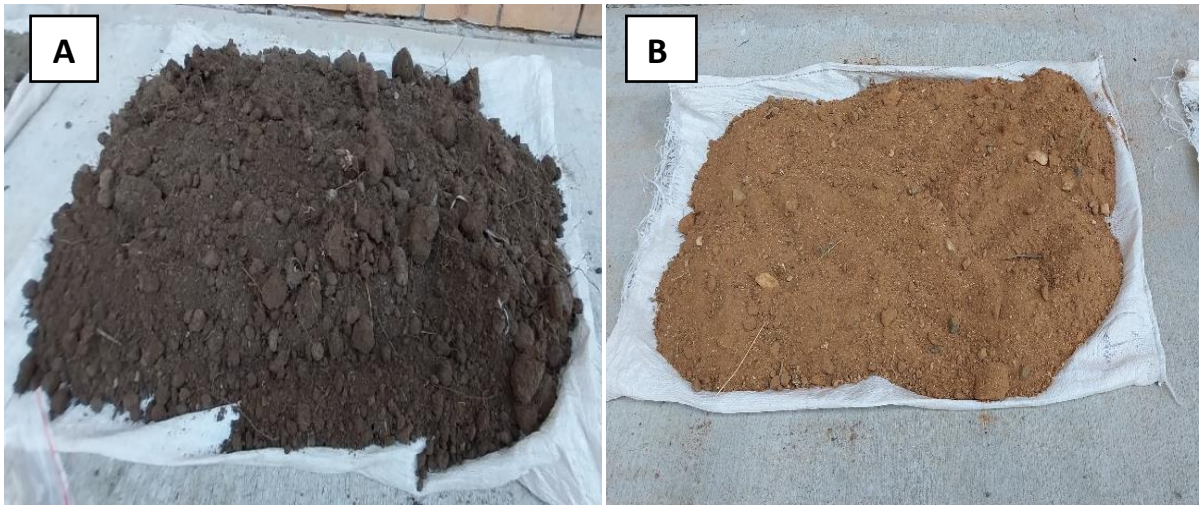

Figure 4.1: Colour variation between soil collected from the two sampling sites, Tubatse (A) and Makgupheng (B).

4.2.3 Quantification of total soil nutrients, pH and cation exchange in *S. frutescens* rhizosphere

All analysis were conducted by the KwaZulu-Natal Department of Agriculture and Rural Development's Analytical Services Unit Cedara College of Agriculture, South Africa following the procedure described below. The textural class was determined by estimation of the three coordinate percentages of clay, silt, and sand on the soil texture chart after sieving (Macvicar & De Villiers, 1991). Density analysis of soil samples was conducted on a volume basis rather than mass basis. To convert the results from volume to mass basis, the mass of a 10 mL scoop of dried and milled sample was measured to calculate the sample density. The pH was measured using potassium chloride (KCl) extraction method (FAO, 2021; Manson & Roberts, 2000). A 50 mL of soil sample was mixed with 25 mL of 1 M KCl. The soil-KCl mixture was carefully swirled using a multiple stirrer at 400 rpm for 5 min until completely homogenized and left to stand for 30 min. After 20 min. a gel-filled combination glass rod was used to measure the pH.

To measure the exchangeable bases (Ca^{2+}, Mg^{2+} and K^{+}), a sample of soil (2.5 mL) was scooped into sample cups containing 25 mL of 1 M KCl. The mixture was carefully swirled using a multiple stirrer at 400 rpm for 10 min. Supernants were filtered using a Whatman No. 1 paper.

A 0.0356 M of $SrCl_2$ was used to dilute 5 ml of the filtrate and Mg and Ca were determined by means of atomic absorption.

To determine the extractable acidity, 10 ml of the de-ionized water containing 2-4 drops of phenolphthalein as a reagent for colour change was used to dilute 10 mL of the filtrate. The mixture was titrated with 0.005 M NaOH (Manson & Roberts, 2000).

To determine the extractable P, K, Zn, Cu and Mn an ambic-2 extractable solution of 0.25 M $NH4CO_3$, 0.01 M Na_2 EDTA, 0.01 M NH_4F and 0.05 g. L^{-1} Superfloc (N100) was first prepared with pH adjusted to 8 using NH3+ solution. Thereafter, a 25 mL of the solution was added into 2.5 mL soil sample and allowed to stir at 400 rpm for 10 min and supernatants were passed through a Whatman No. 1 filter paper. The extractable P was determined using the modified of Murphy and Riley (1962) molybdenum blue method (Manson & Roberts, 2000). A 2 mL of the filtrate was used. Atomic absorption was used to determine the extractable K, Zn, Cu and Mn. For K, 5 mL of filtrate was firstly diluted with 20 mL de-ionized water and the remaining undiluted filtrate for Zn, Cu and Mn.+

The total cations were calculated as the sum of extractable K^+, Ca^{2+} and Mg^{2+} and acidity (Manson & Roberts, 2000).

$$\text{Cation exchange capacity (CEC)} = \text{Base cations (TC)} + \text{Acid cations}$$

$$\text{Acid saturation (\%)} = \frac{\text{Exchangeable acidity}}{\text{Total cations}}\ x\ 100$$

The soil total organic carbon and organic matter were determined using the modified Walkley-Black (1934) method. Oxidizable organic carbon (OC) is oxidized in the presence of concentrated sulfuric acid and potassium dichromate solution at 120ºC (Aregahegn, 2020; FAO, 2019) equation shown below. Titration method with standard ferrous ammonium sulphate solution was then used to determine the excess dichromate acid not reduced by OM while the substance oxidized was calculated from the amount of reduced dichromate. OM was

assumed to contain 58% C and therefore a multiplying factor of 1.72 was used to convert OC to OM (Poudel, 2020) formula shown below:

Formula

OM: Organic Matter (%) = Organic Carbon (%) x 1.72

Equation

$$3C + 2K_2Cr_2O_7 + 8H_2SO_4 \rightarrow 2Cr_2(SO_4)_3 + 3CO_2 + 2K_2SO_4 + 8H_2O$$

Table 4.1: Soil and nodule collection sites in the Limpopo Province

Province	Region	Minimum Temperature (°C)	Maximum Temperature (°C)	Annual rainfall (mm)	GPS Coordinates
Limpopo	Tubatse	7	28	600	24°63′52.5″S; 30°16′ 4.28″E
	Makgupheng	13	30	500	23°88′ 92.5″S. 29° 17′ 8.38″E

4.2.4 Soil enzyme activity assays

Phosphatase (alkaline and acid), glucosaminidase and glucosidase activity in soil

The colorimetric analysis method adopted from Jackson, Tyler and Millar (2013) was used to determine C-cycling and P-cycling activities (β-glucosidase, β-glucosaminidase, acid and alkaline phosphatase, respectively) and the activities were expressed in $nmolh^{-1}g^{-1}$. The enzyme assay involved homogenizing 5 g of soil sample in sterile distilled water using 15 ml centrifuge tubes for 2 h to prepare a slurry. The supernatants were then transferred into 96-well blocks, before adding 5 ml of 50 mM acetate buffer and 10 ml of respective p-nitrophenyl substrate (*p*-NP-β-D-glucopyranoside for C-cycling and *p*-NP-phosphate for P-cycling) into the well. A control without the substrate was added per sample and all the wells containing the mixtures were incubated at a room temperature 22ºC for 1 h. The incubation time was recorded

immediately after adding substrate solution. A 10 µL 1 M NaOH was used to stop/slow down the enzymatic reaction while increasing pH to improves the colour of the released *p*-NP during the reaction. A developed yellow colour was read using UV spectrophotometer with 410 nm wavelength. The amount of p-nitrophenyl released during the enzymatic hydrolysis process was quantified and calculated using the formula below (Adetunji, Ncube, Meyer, Mulidzi & Lewu, 2020).

Enzyme activity = (mean sample fluorescence - mean initial sample fluorescence) / ((mean standard fluorescence / 0.5 mol) x (mean quench control fluorescence / mean standard fluorescence) x (0.2 mL) x (time in hr)).

Nitrate reductase test

Nitrate reductase in soil was determined following a method of Brucker (1995) as described by Ndlovu *et al*. (2023). This method involved a series of steps which was firstly adding 5 g of soil into a 50 mL centrifuge tube containing a solution of 4 ml of 0.9 mM 2.4-dinitrophenol, 1 mL of 25 mM KNO_3 and 5 ml of sterile water. The mixture was mixed vigorously before incubation in a dark room with at room temperature (30ºC) overnight (24 h). Thereafter, 10 mL of 4 M potassium chloride (KCl) solution was added into the solution (after incubation) and mixed briefly. Thereafter, the mixture passed through a Whatman No. 1 filter paper. To initiate the enzyme activities, 2 mL of the filtrate was added into 1.2 mL of 0.19 M ammonium chloride buffer (pH 8.5) and 800 µL of the colour reagent (1% sulphanilamide in 1 N HCl and 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) before incubating for 30 min in the dark at 30ºC. The absorbance was read using UV spectrophotometer with 510 nm wavelength and the amount of nitrite released during the enzymatic hydrolysis process was measured and expressed as 0.1 $\mu molh^{-1}g^{-1}$.

4.2.3 Data analysis

The data on soil physico-chemical properties were subjected to Analysis of Variance (ANOVA) through Statistix 10 software. Before ANOVA, data were subjected to Shapiro-Wilk normality test and any found to be not normally distributed were transformed. Transformation was done using the $\log_{10}$ (x +1) for normal data values and arcsine for percentage data $\sqrt{x \div 100}$ (Gomez & Gomez, 1985). Means were separated using the Fishers Least significant difference at 5% probability level. The enzyme activity data were subjected to Sample-Two test on Statistix 10 software to determine the difference in means between the locations and season at 5% probability level.

4.3 Results

4.3.1 Physicochemical properties of soil collected from Tubatse and Makgupheng

All measured variables were not normally distributed at $P \leq 0.05$ according to Shapiro-Wilk normality test, except for the density, calcium (Ca), exchangeable acidity (EA), zinc (Zn) and manganese (Mn) (Appendix 4.1).

All measured variables were statistically different at $P \leq 0.05$ (Appendix 4.2-4.15, 4.17), except for Zn and nitrogen (N) (Appendix 4.11 and 4.16).

Interactive effect of location and season on P, Ca, ECEC, pH, OC, and OM

The soil analysis report showed that Makgupheng had very low pH (5.43) (acidic) in summer and pH neutral (7.23) in winter. In Tubatse the pH range was between 6.82 - 7.14 (neutral), there were no significant differences in pH values for both seasons (Table 4.2). There were significant differences observed for Ca and ECEC for both sites and seasons with Tubatse having high Ca (> 10 $Cmol_c/kg$) and ECEC (15 - 25 $Cmol_c/kg$) amount in all seasons. Makgupheng had very low Ca (< 4 $Cmol_c/kg$) and ECEC (< 15 $Cmol_c/kg$) in both seasons (Table 4.2). Although, there were no significant differences ($P \geq 0.05$) between seasons

observed for P for Tubatse soil both Tubatse and Makgupheng had very low (0 - 5 mg. Kg^{-1}) amounts (Table 4.2). There were also no significant differences observed in OC and OM for Tubatse, but there were differences for Makgupheng across the seasons (Table 4.2). The organic carbon content and organic matter of both soil collected from Tubatse and Makgupheng were low (< 4%) in both seasons (Table 4.2) despite the differences in Makgupheng.

Effect of location on density, % clay, Mg, Mn, AS and Cu availability in the soil

There were significant differences ($P \leq 0.05$) observed between the two soils, Tubatse and Makgupheng. Soil from Tubatse had a slightly high Mg (> 4 $Cmol_c/kg$), K (0.2 - 0.6 $Cmol_c/kg$) and clay content (> 40%) compared to Makgupheng which had very low Mg (0.5 - 4 $Cmol_c/kg$), K (< 2 $Cmol_c/kg$) and clay (< 15%) (Table 4.3). Makgupheng had high density, Mn, and Cu compared to Tubatse with very low Cu (< 4 mg. Kg^{-1}) and low - moderate Mn (< 10 mg. Kg^{-1}) (Table 4.3).

Effect of season on soil exchangeable acidity

The effect of soil/location on exchangeable acidity was not significant ($P \geq 0.05$), however, season was significant. Winter was found to have a slightly high exchangeable acidity compared to summer (Table 4.4).

Table 4.2: Interactive effect of locality (soil edaphic factors) and season on soil physico-chemical properties in Limpopo Province

Treatment	Season					
	Summer					
	P (mg/kg)	Ca ($Cmol_c/kg$)	ECEC ($Cmol_c/kg$)	Ph	OC (%)	OM (%)
Tubatse	0.26^{c} (0.83)	1.37^{a} (22.24)	1.58^{a} (37.06)	0.89^{b} (6.82)	$5.00E\text{-}03^{b}$ (0.50)	0.01^{b} (0.86)
Makgupheng	0.39^{b} (1.46)	0.54^{d} (2.54)	0.63^{d} (3.53)	0.81^{c} (5.45)	$7.50E\text{-}03^{a}$ (0.75)	0.01^{a} (1.29)
	Winter					
Tubatse	0.26^{c} (0.83)	1.22^{b} (15.65)	1.44^{b} (27.06)	0.91^{ab} (7.14)	$5.00E\text{-}03^{b}$ (0.50)	0.01^{b} (0.86)
Makgupheng	0.8177^{a} (5.60)	0.80^{c} (5.26)	0.89^{c} (6.78)	0.92^{a} (7.23)	$5.00E\text{-}03^{b}$ (0.50)	0.01^{b} (0.86)
F-value	170.88	152.99	35.95	41.27	53.57	53.57
P-value	0.0000**	0.0001**	0.0019**	0.0014**	0.0007**	0.0007**
$LSD_{0.05}$	0.0640	0.0626	0.1279	0.0257	6.637E-04	

Different letters indicate statistically differences ($P \leq 0.05$) among soils for the individual variables based on ANOVA followed by an LSD All-pairwise comparisons. **Highly significant $P \leq 0.01$

Table 4.3: Effect of location on density, Mg, Mn and Cu availability

Treatment	Density	K (mg/kg)	Mg (mg/kg)	Mn (mg/kg)	Cu	A	Clay
Tubatse	3.08[b] (1210.0)	0.11[a] (0.28)	1.12[a] (12.77)	0.81[b] (5.75)	0.19[b] (0.58)	-4.34e-19[b] (-1.11e-16)	0.43[a] (41.33)
Makgupheng	3.15[a] (1400.0)	0.04[b] (0.08)	0.30[b] (1.13)	1.20[a] (14.88)	0.42[a] (1.62)	6.39e-03[a] (0.67)	0.146[b] (14.64)
F-value	103.62	155.31	103.89	34.89	24.24	10.80	126.27
P-value	0.0002**	0.0001**	0.0002**	0.0020**	0.0044**	0.0218*	0.0001**
$LSD_{0.05}$	0.0161	0.0147	0.2055	0.1714	0.1200	4.998E-03	0.0640

Different letters indicate statistically differences ($P \leq 0.05$) among soils for the individual variables based on ANOVA followed by an LSD All-pairwise comparisons. *Significant $P \leq 0.05$. **Highly significant $P \leq 0.01$

Table 4.4: Effect of season on soil exchangeable acidity

Season	EA (Cmol/L)
Summer	0.03[a] (0.07)
Winter	0.01[b] (0.03)
F-value	10.92
P-value	0.0214*
$LSD_{0.05}$	0.0106

Different letters indicate statistically differences ($P \leq 0.05$) among soil for the individual variables based on ANOVA followed by an LSD All-pairwise comparisons. *Significant $P \leq 0.05$.

4.3.2 Spearman's correlation between season and location on edaphic factors

The Spearman's correlation analysis showed a strong significant correlation between location and most of the important edaphic factors, except for exchangeable acidity (EA), pH, nitrogen (N), organic matter (OM) and organic carbon (OC). Location had a strong positive and highly significant correlation with phosphorus (P), manganese (Mn), copper (Cu), density and acid saturation (AS) (Table 4.5). While a strong negative and highly significant correlation was observed with location and potassium (K), calcium (Ca), magnesium (Mg), clay and effective cation exchange capacity (ECEC) (Table 4.5). Season had no significant correlation with all of the edaphic factors, except for exchangeable acidic (EA) and pH (Table 4.5). There was a strong positive and highly significant correlation observed between season and EA, while pH had a strong negative and highly significant correlation.

Table 4.5: Spearman's correlation between season and location on edaphic factors

Treatments	Edaphic factors							
	P	K	Ca	Mg	Density	EA	ECEC	AS
Location	0.85**	-0.85**	-0.85**	-0.85**	0.85**	-0.14^{ns}	-0.85**	0.80**
Season	-0.10^{ns}	-0.03^{ns}	0.17^{ns}	-0.03^{ns}	-0.17^{ns}	0.73**	0.17^{ns}	0.22^{ns}
	pH	Zn	Mn	Cu	OC	OM	N	Clay
Location	-0.21^{ns}	0.43^{ns}	0.85**	0.85**	0.61^{ns}	0.61^{ns}	-0.21^{ns}	-0.86**
Season	-0.73*	-0.31^{ns}	-0.17^{ns}	-0.03^{ns}	0.50^{ns}	0.50^{ns}	0.52^{ns}	0.03^{ns}

^{ns}Not significant $P \geq 0.05$; **Highly significant $P \leq 0.01$. AS represent acid saturation.

4.3.2 Soil enzyme activities

All measured variables were not normally distributed as presented by Shapiro-Wilk normality test (Appendix 4.18).

There were insignificant differences ($P \geq 0.05$) observed in enzyme activities of nitrate reductase, glucosidase, β-glucosaminidase, P alkaline and P acid of both study sites (Appendices 4.19, 4.20, 4.21, 4.22, 4.23, 4.24 and 4.25). Though the differences were insignificant, Tubatse had high activities of nitrate reductase, glucosidase, β-glucosaminidase, P alkaline than Mkagupheng. Makgupheng had high P acidic compared to Tubatse.

In addition, no significant correlation was observed between soil extracellular enzyme activity and edaphic factors, except with pH, potassium (K) and P alkaline phosphatase (Table 4.6). There was a strong positive and highly significant correlation between potassium and pH and alkaline phosphatase (Table 4.6).

Table 4.6: Spearman's correlation between edaphic factors and enzyme activities

Enzyme activity	Edaphic factors							
	P	K	Ca	Mg	Density	EA	ECEC	AS
Glucosidase	-0.12^{ns}	0.60^{ns}	-0.10^{ns}	-0.04^{ns}	-0.47^{ns}	-0.47^{ns}	-0.12^{ns}	-0.11^{ns}
β-Glucosamini-dase	-0.02^{ns}	-0.10^{ns}	-0.32^{ns}	-0.34^{ns}	0.12^{ns}	0.12^{ns}	-0.25^{ns}	0.49^{ns}
P alkaline	-0.35^{ns}	0.71*	0.32^{ns}	0.37^{ns}	-0.53^{ns}	-0.33^{ns}	0.30^{ns}	-0.34^{ns}
P acid	0.49^{ns}	-0.27^{ns}	-0.35^{ns}	-0.19^{ns}	0.29^{ns}	-0.34^{ns}	-0.31^{ns}	0.38^{ns}
Nitrate reductase	0.20^{ns}	0.43^{ns}	0.28^{ns}	0.15^{ns}	0.32^{ns}	0.20^{ns}	0.22^{ns}	-0.11^{ns}
	pH	Zn	Mn	Cu	OC	OM	N	Clay
Glucosidase	0.19^{ns}	0.16^{ns}	0.12^{ns}	-0.20^{ns}	-0.01^{ns}	-0.01^{ns}	0.45^{ns}	0.24^{ns}
β-Glucosamini-dase	0.07^{ns}	-0.19^{ns}	0.20^{ns}	-0.10^{ns}	0.30^{ns}	0.30^{ns}	0.12^{ns}	-0.30^{ns}
P alkaline	0.66*	0.07^{ns}	-0.18^{ns}	-0.36^{ns}	-0.49^{ns}	-0.49^{ns}	0.05^{ns}	0.47^{ns}
P acid	0.62^{ns}	0.04^{ns}	0.40^{ns}	0.23^{ns}	0.07^{ns}	0.07^{ns}	-0.12^{ns}	-0.51^{ns}
Nitrate reductase	0.38^{ns}	0.31^{ns}	0.02^{ns}	-.07^{ns}	-0.28^{ns}	-0.28^{ns}	0.36^{ns}	-0.05^{ns}

^{ns}Not significant $P \geq 0.05$; *Significant $P \leq 0.05$; **Highly significant $P \leq 0.01$. AS represent acid saturation.

4.4 Discussion

4.4.1 Soil physicochemical properties (edaphic factors) and their influence in microbial community

The activities of microbial communities in soil play a major role in soil maintenance through nutrient cycling and availability, hence their diversity is an important index to assess soil health (Chen, Ding, Zhu, He & Hu, 2020). There are a whole range of soil conditions affecting the life of microorganisms and enzyme activities and these are called edaphic factors (soil

moisture, soil type, temperature, soil pH and acidity and mineral salt content/ salinity) (Furtak & Galazka, 2019). Any imbalance in one of them may affect the organism's life. The major findings of the study were that soil physico-chemical properties (edaphic factors) and environmental conditions are not similar between the two study sites. These differences in environmental conditions might have contributed to the diverse microbial communities occurring in different sites due to different selection pressure from the environment. This is observed by having different bacteria species occurring in different location, although some species were shared among localities. One major reason that is linked to this might be the concept of environmental filter theory of microbial communities and its association with the hologenome theory (Hargreaves, Williams & Hofmockel, 2015). The filtering theory state that edaphic factors and abiotic factors are important environmental filters shaping soil microbial communities. The theory further extrapolates that 'to a finer scale, soil pH along with texture, nutrient status, chemistry of root exudates and plant residues have major contribution to the distinct microbial communities occurring in different ecosystems' (Hargreaves *et al*., 2015). On the other hand, hologenome theory hypothesis other factors beyond the abiotic also are significant drivers of microbial communities and these may include host-microbe interactions (Motsomane *et al*., 2023; Jinek, Chylinski & Charpentier, 2005). The hypothesis of the hologenome theory is that the collective genetic material of a host and it symbiotic partner should be considered as a single functional unit or holobiont (Jinek *et al*., 2005). It further states that, the genotype of the host and of its associated symbionts contribute to its ecological fitness and adaptation to specific niches. In this holobiont there are mutualistic and beneficial microbes such as the PGPRs that enhance plant survival in different environmental conditions by helping plants adapt to abiotic stress. These microbes (microbial composition in this holobionts) are selected by the plants host through secretion of organic acids. Motsomane, Suinyuy, Perez-Fernandes and Magadlela (2024) conducted a study on the influence of ecological niches and

hologenome dynamics on growth of *Encephalartos villosus* and the findings were that both theories were at play. Implying that similar (shared) edaphic factors between ecosystems influence a strong degree of similar microbial composition occurring in those ecosystems due to comparable selective pressures. While differences in these edaphic factors will result in different microbial composition in those different areas due to different selection pressures. Moreover, Motsomane *et al*. (2024) highlighted that variation in the holobionts composition (host plants - colloroids roots) might have driven the microbial communities within their vicinity, even in environment with similar abiotic conditions. In the context of our study, the findings are consistent to these two theories, but the filtering theory is more evident. This suggest that environment or edaphic factors could have driven the differences in microbial composition within the two study sites. As per the filtering theory, soil pH and nutrient status contributed more to diverse microbial composition. Soil pH is broadly reported as the predominant factor in determining soil microbial structure (Xue, Carrillo, Pino, Minasny & McBratney, 2018).

In addition, studies have identified a noticeable negative effect of soil pH on microbial community (specifically bacteria and archaea), survival, abundance and diversity, especially in pH extremes (either too low ≤ 3.3 or too high ≥ 9.0) (acidic and alkaline soil) (Furtak & Galazka, 2019). Specific groups such as alkaliphiles (*Bacillus*, *Flavobacterium*, *Methanobacterium*, and *Corynebacter*) and acidophiles (*Acidithiobacillus*, *Thiobacillus*, *Acetobacter*, *Alicyclobacillus* and *Acidobacteria*) can however, tolerate and grow optimally under such extreme conditions (de Gannes, Eudixie, Bekele & Hickey, 2015). Furtak and Galazka (2019) reported that most soil microorganisms prefer pH close to neutral (6-7). This suggest that different groups of microorganisms have distinct optimal soil pH limits, from acidic to alkaline. In this study, Makgupheng in summer season had a very low pH (<5.5, very acidic) and a resultant low microbial composition compared to neutral pH (7.23) in winter. In

Tubatse, the pH was neutral (6.82-7.14) for both seasons hence the increase microbial composition or diversity. Based on these results, the study propose that season might have had an indirect influence on microbial community through alteration of soil edaphic factors, particularly soil pH and nutrient status. According to Muneer *et al*. (2022) and Yan, Dong, Gong, Zhu and Wang, (2021) season produces ephemeral and unique conditions (with respect to temperature, moisture and plant productivity) and shift in nutrient availability as a result alters the structural composition and whole life of soil microbial communities. Moreover, Li *et al*. (2022) and Mouginot *et al*. (2014) reported that high temperatures and precipitation during the summer season accelerate enzyme activity and nutrient turnover in the soil and as result stimulate the growth and metabolism of microorganisms. Contrary, low temperatures, low precipitation (particularly in winter) as well as inactive plants restrict the growth of soil microorganisms (Hawkes, Waring, Rocca & Kivlin, 2017; Neilson *et al*., 2017). Similar findings were also reported by Li *et al*. (2024) that soil fungal Sobs indices were high in summer, supported by elevated temperature, precipitation and increased nutrient turnover and availability in forest and grassland ecosystems which led to microbial growth.

Moreover, the study isolated a very less percentage of rhizobia bacteria compared to the non-rhizobia species with rhizobia species only found in Tubatse which had neutral soil conditions. The *Rhizobium* species were not found in Makgupheng which had acidic soil. Kopittke *et al*. (2015) reported that soil acidity associated with high Al^{3+}, Mn^{2+} and Fe^{3+} may disturb the functioning of rhizobia and as a result reduce their competitive ability in the soil. Highly acidic (pH < 5.5) soils are mostly dominated by the Al^{3+} and this may hinder cation uptake thereby impairing root and plant development (Kopittke *et al*., 2015). The whole symbiotic N-fixation and nodulation is greatly affected by soil acidity which may reduce overall legume production. Jaiswal *et al*. (2018) also reported that *Rhizobium* survival and persistence in the soils and their symbiotic relationship with legumes is affected by soil acidity. Another bacteria group,

Bacillus is generally known as one of the most dominant microbial flora in the rhizosphere of wild medicinal plants and other legume plants (Srivastava *et al*., 2014; Ghodsalavi *et al*., 2013). However, *Bacillus* is most likely to fail under acidic conditions (Furtak & Galazka, 2019). The study made similar observations, none of the *Bacillus* species were found in Makgupheng having acid conditions which the pH might have contributed.

In addition, soil edaphic factors particularly soil pH and nutrient status (ECEC) is dependent on the type of rock/underlying material from which the soil formed (rock weathering) and organic matter, with acidic soil originating from igneous rocks and sands, while alkaline soil forming from carbonate rocks (i.e limestone) (Furtak & Galazka 2019). Soil from Tubatse is most likely to be originating from carbonate rocks (limestone), characterized by grey to black soils, clay (> 40%) and well-structured soil, high accumulation of Ca ($CaCO_3$), Mg ($MgCO_3$) and K and an overall high ECEC. Soil from Tubatse were very low in Cu, Mn, and acid saturation percentage. It has been reported that ECEC in the soil increases heavy metal (high Al, Cu and Zn ions) adsorption while enhancing the release of bioavailability Ca, Mg and K in soil (Campilo-Cora, Gonzalez-Feijoo, Arias-Est'evez & Andez-Calvino, 2022). Campilo-Cora *et al*. (2022) further stated that when ECEC is low, Cu, Zn and other metal adsorption is reduced, toxicity increases which reduce microbial community in the soil. However, when ECEC is high, the bacterial community is not strongly affected by metals since toxicity will be low. This is more similar to results of the study in soil collected from Tubatse, which had was characterized by high ECEC and high sorption of heavy metals and release of Mg, Ca and K into the soil. Makgupheng was characterized by well-drained red to brown subsoil with very low Ca, Mg and K, high acid saturation and very low ECEC amount, hence the high level of Cu, Mg in the soil. This as the result affected the microbial diversity in Makgupheng.

4.4.2 Edaphic factor influence on soil enzyme activities and nutrient availability

Soil enzymes are widely recognized as major drivers of nutrient cycling and bioavailability (Banerjee, Bora, Thrall & Richardson, 2016). Both plants and microbes need available forms of nutrients for uptake, which are largely provided through the conversion of more complex organic substrates to bioavailable products, by breaking down larger polymers through a process catalysed by extracellular enzymes activity (EEA) (Schaaap *et al*., 2023). These enzymes are produced by various microorganisms, including bacteria and fungi, and are responsible for many ecosystem processes, especially those involved in the degradation of organic matter and the cycling of C, N, and P (Li, Ma, LI, Shen & Xia, 2024). In the current study, the soil characteristics and soil enzyme activities of both study sites (Tubatse and Makgupheng) were assayed, together with the effects of edaphic factors on extracellular enzyme activities. The study observed insignificant differences in extracellular enzyme activity (β-glucosaminidase, glucosidase, P alkaline, P acid and N reductase) between both sites (Makgupheng and Tubatse). Although, the activities in Tubatse were slightly higher than Makgupheng. Moreover, the absence of correlation between soil nutrients, particularly the primary nutrients and soil enzymes suggest that the contribution of identified nutrient cycling bacteria of cancer bush and associated enzymes to soil nutrient availability was quite similar between the two sites.

The β-glucosaminidase and glucosidase enzyme are involved in N and C cycling in the soil, with glucosidase involved in C cycling only and glucosaminidase involved in both N and C cycling (Zhang *et al*., 2020; Turner, 2010). Phosphatase enzymes are involved in the conversion and release of cation-bound molecules of P into the soil (Ndabankulu *et al*., 2022). The result of increased activity in soil might have led to the increased mineralization of N contributing to N cycling, hence the high soil N concentration in Tubatse compared to Makgupheng. These findings agree with the findings of Ndabankulu *et al*. (2022) who

reportedthat increased activity of β-glucosaminidase in grassland soil contributed to N cycling in the nutrient deficient grassland soils. Phosphatase enzyme (both alkaline and acid) have been extensively studied and their activity is strongly influenced by P availability and soil pH (Ndlovu *et al*., 2023). In this study, phosphatase alkaline activity was found to be high in Tubatse, while of acid phosphatase enzyme was high in Makgupheng. Makgupheng had very acidic soil to neutral while Tubatse had neutral soil pH levels. The results are consistent to what Ndlovu *et al*. (2023) and other researchers reported that high phosphatase enzyme release in the soil contribute to greater release of P from organic sources for plant uptake and are pH dependent. Makoi, Chimphango and Dakora (2010) who reported a correlation between high P acidic activity in the rhizosphere of cowpea genotypes and greater release of P from organic sources for plant uptake and use. Overall, these nutrient cycling bacteria and their associated enzymes are connected to nutrient bioavailability in these nutrient-limited ecosystems through cycling N and P.

4.5 Conclusion and recommendation

Soil enzymes are abundant in the soil and make a huge contribution to SOM breakdown and nutrient recycling, hence their use as an alternative for improving soil productivity. The study observed no significant correlation between extracellular enzyme activity and soil edaphic factors, except soil pH. This suggest that the contribution of identified nutrient cycling bacteria of cancer bush and associated enzymes to soil nutrient availability was quite similar between the two sites. Even though, Tubatse had soil pH neutral for both seasons high N in the soil and a resultant enzyme activity in glucosidase, β-glucosaminidase. A slight high P and low pH was observed in Makgupheng in both seasons which might have influenced the increased enzyme activity of phosphatase acid. Overall, nutrient in soil, particularly the primary nutrients and soil pH govern the activities of enzymes.

In this study, soil pH and seasonal fluctuation affected nutrient availability. The study observed strong positive and highly significant correlation between location (sites) and edaphic factors including the primary nutrients, except soil pH. However, no correlation was observed between season and edaphic factors, except soil pH and zinc. This suggest that region/location have a sound influence on the soil physicochemical properties and nutrient availability that season do. Different sites or regions are characterized by having different underlying parent material which influence or determine the kind of soil and nutrients available in that soil. Furthermore, the observed variation in soil pH of different seasons, the study then propose that season have an indirect effect on soil nutrient availability simply by altering soil.

Overall, the study highlights the importance of soil edaphic factors and enzyme activity in shaping soil bacteria in cancer bush rhizosphere, with soil pH as a major contributing factor governing all these factors.

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CHAPTER 5

SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH AND CONCLUSION

5.1 Summary

The study investigated the diversity and influence of season on root nodulating bacteria (PGPRs) in cancer bush, along with how edaphic factors influence extracellular enzyme activity of PGPRs. A total of 19 PGPR bacteria species were characterized from the two sites using molecular analysis. The identified bacteria species can be affiliated to 12 genera: *Bacillus, Rhizobium, Stenotrophomonas, Serratia, Kosakonia, Micrococcus, Enterobacter, Alcaligenes, Leucobacter, Cellulosimicrobium, Lysinibacillus* and *Sphingobacterium*. *Stenotrophomonas* was the predominant species common in both sites followed by *Lysinibacillus, Enterobacter* and *Kosakonia*. The *Rhizobium, Micrococcus, Bacillus* and *Alcaligenes* species were only isolated in Tubatse while *Sphingobacterium, Serratia* and *Leucobacter* were isolated in Makgupheng. The microbial populations of both localities were highly diverse, with Tubatse being more diverse than Makgupheng. The population were evenly distributed in both sites supported by Pielou's evenness (*J*) value that is very closer to 1, and with the highest evenness observed in winter than in summer. Majority of the bacteria isolated were non-rhizobia species. Majority of the isolates, including the non-rhizobia species were able to fix nitrogen for the plant through their ability to utilize carbon sources on nitrogen free media (Nfm). Overall, Tubatse had high number of nitrogen fixing compared with Makgupheng, shown by the highest percentage in season combined. The relative percentage abundance of nitrogen fixers in Tubatse during summer was slightly higher (95%) than Makgupheng (80%) and the opposite was observed in winter with Tubatse (93%) and Makgupheng (100%). The isolated bacteria did not show any halo zones when grown on a Pikovskaya's solid media to test for phosphate solubilization.

The study observed no insignificant differences in rhizosphere enzyme activities within the two study sites. Enzyme activities of PGPRs, are connected or govern by nutrient availability, particularly P, N and C. These three elements are the drivers of enzyme activity in the soil. In this study, there were low levels of P and N in both sites. This might have influenced low activities of enzyme in the soil. Although, there were insignificant differences in enzyme activities, a slight increased activity of glucosaminidase and glucosidase in Tubatse was observed compared to Makgupheng. These two enzyme are associated with C and N cycling through organic matter breakdown in the soil. The activities of alkaline phosphatase were high in Tubatse while acid phosphatase was high in Makgupheng. The high release of acid phosphatase in the soil contribute to greater release of P from organic sources for plant uptake and use in such conditions, similar with alkaline phosphatase.

A strong highly significant correlation was observed between location and edaphic factors (P, K, Ca, Mg, density, ECEC, acid saturation, Mn, Cu and Clay), except pH. Season had very weak insignificant negative correlation observed with most of the edaphic factor (P, K, Ca, Mg, density, ECEC, AS, Zn, Mn, Cu, OC, OM, N and clay), however, a strong correlation was observed between season and pH and exchangeable acidity. Based on soil analysis report, Tubatse had neutral pH soil with high ECEC, Mg, Ca, K and clay percentage (> 40%), while Makgupheng had very acidic soil (particularly in summer and neutral winter), low Mg, Ca, K and clay percentage (< 15%).

5.2 Significance of findings

The findings of this study demonstrated that the bacteria isolated within the two sites were diverse, with some species occurring only in Tubatse, some in Makgupheng and others overlapping. The populations were evenly distributed among the two study sites. The major findings of the study were the isolation of non-rhizobia and non-nodulating bacteria from the

root nodules of cancer bush, with nitrogen fixing abilities. The occurrence of such endophytic bacteria with enormous potential for plant growth promotion can be advantageous in sustainable plant improvement through their use as biofertilizer candidate, individually or inoculated in combination with other rhizobia species to improving performance and growth of legumes and other non-legume plants. These results also strengthen the literature on the endophytic bacteria nature of cancer bush.

The study also revealed that season have an indirect effect of microbial abundance and diversity, however, this is site dependant. Tubatse in summer recorded higher microbial diversity and low in winter, while Makgupheng on the other hand had low diversity in summer and high in winter. Lastly, the study also revealed that the edaphic factors of each location influences the microbial communities and their associated enzymes. Soil pH had more influence on the enzyme activities, bacteria communities, their abundance and occurrence. Knowledge of such information may be useful in determining superior combinations of strains for inoculation.

5.3 Future research

The study recommends further testing for the presence of symbiotic genes from the isolated bacteria, to test for their ability to re-nodulate and efficiency on growth promotion under greenhouse conditions followed by field conditions. More research needs to be conducted on the possible occurrence of gene exchanges between the non-rhizobia non nodulating and rhizobia, around rhizosphere soil which might enable them to colonize root nodules of legumes. Moreover, microscopic observation to definitively confirm the endophytic character and clarify their localization inside nodule tissues are essential as legumes represent a valuable source for selecting some of these beneficial microorganisms to be used as microbial inoculants for improving plant growth. Lastly, the study only investigated the diversity of the bacteria only

in Limpopo Province, studying other regions where the plant is found will be essential, especially when considering ways of improving it growth and cultivation.

5.4 Conclusion

Cancer bush root nodules are colonized by diverse beneficial microorganisms known as root nodulating bacteria, important for nutrient cycling, such as nitrogen. Besides being site specific, the abundance and diversity of PGPRs in cancer bush are influenced by seasonal fluctuation. The diversity indices used in this study showed that the bacteria population of Tubatse were more diverse in summer compared to winter, while Makgupheng was more diverse in winter than is in summer. The study further observed a high total plant N in the plants, especially in Tubatse with low nitrogen derived from the atmosphere (% NDFA) and high percentage nitrogen derived from soil (NDFS mmol). In Makgupheng, there was a high % N derived from atmosphere than % N derived from soil. This implies that the plants in Makgupheng mostly depended on NDFA to supplement leaf N. This emphasis the importance of the symbiotic relations between these wild plants and the beneficial soil microorganisms in promoting nutrient cycling and acquisition. The study highlights the importance of evaluating and understanding C: N ratios of crop residues. This should be the goal of any producers interested in improving soil health for optimum growth/yield, so that they may be able to provide quality habitat for soil microorganisms because of the benefits they perform in the soil. The major findings of the study were that edaphic factor had an influence on enzyme activities with the high ECEC, nutrients such as P and N in Tubatse governing the increased enzyme activities of glucosidase and glucosaminidase. Soil pH had an influence on the phosphatase enzyme activity, alkaline and acid phosphatase. Tubatse with high soil pH had high alkaline activity while Makgupheng with acidic soil dominating had more release of acidic phosphate activities in the soil.

Based on the findings of this study, we therefore accept both hypothesis which stated that (i) there will be differences in the species diversity of microbes in the root nodules of the cancer bush growing in different locations (Tubatse and Makgupheng) in Limpopo Province over two seasons and (ii) that the rhizosphere microbial enzyme activities of N-fixation, P- solubilization and C- cycling and soil properties will vary across the two sites.

APPENDICES

Appendix 3.1: Morphological (macroscopic) characteristics of bacteria isolated from cancer bush root nodules							
Season	Isolate	Colony					
		Colour	Shape	Elevation	Surface	Margin	Likely species
Winter	M4BA	Cream	Irregular	Flat	Smooth	lobate	*Enterobacter absuriae*
	M7BA	Reddish	Irregular	Flat	Smooth/shiny	Serrate	*Serratia marcescens*
	M4CA	Bright yellow	Punctiform	Flat	Smooth	Entire	*Cellulosimicrobium cellulans*
	M3AA	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas maltophilia*
	M8AA	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas maltophilia*
	M6AA	Golden yellow	Round	Convex	Smooth	Entire	*Kosakonia cowanii*
	M7CA	Cream-yellow pigmented	Round	Flat	Smooth	Entire	*Leucobacter chromiiresistens*
	M6BA	Cream-yellow pigmented	Round	Flat	Smooth	Entire	*Leucobacter chromiiresistens.*
	M4AB	Cream-yellow pigmented	Round	Flat	Smooth	Entire	*Leucobacter chromiiresistens*
	M3CA	Cream-yellow pigmented	Round	Flat	Smooth	Entire	*Leucobacter chromiiresistens*
	M7CB	Cream-yellow pigmented	Round	Flat	Smooth	Entire	*Leucobacter chromiiresistens*

	M8AB	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas maltophilia*
	M8AA2	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas maltophilia*
	M6CA	Cream	Round	Convex	Smooth	Entire	*Sphingobacterium multivorum*
	M3BA	Cream	Irregular	Flat	Smooth	Lobate	*Enterobacter asburiae*
	M4AA	White	Round	Flat	Smooth	Entire	*Stenotrophomonas pavanii*
	T2BA	White	Round	Flat	Smooth	Entire	*Stenotrophomonas pavinii*
	T2CC	White	Round	Flat	Smooth	Entire	*Stenotrophomonas pavanii*
	T1BA	Golden yellow	Round	Convex	Smooth	Entire	*Kosakonia cowani*
	T2CB	Golden yellow	Round	Flat	Smooth	Entire	*Rhizobium petrolerium*
	T2BB	Golden yellow	Round	Flat	Smooth	Entire	*Rhizobium petrolerium*
	T1AA	Cream-white	Punctiform	Flat	Smooth	Entire	*Bacillus licheniformis*
	T2Bb	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas geniculata*
	T1CA	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas geniculata*
	T1CB	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas geniculata*
	T1AB	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas geniculata*
	T2BB1	White	Irregular	Flat	Rough	Curled	Unidentified
	T2CB1	White	Cratiform	Raised	Smooth/shiny	Entire	Unidentified
	T2CB2	Bright yellow	Round	Convex	Smooth	Entire	Unidentified

	T2CA	Greyish white	Round	Flat	Smooth	Entire	*Alcaligenes faecalis*
Summer	M5B1	Yellow	Filamentous	Flat	Smooth	Filamentous	Unidentified
	M6C4	Cream	Punctiform	Flat	Smooth	Entire	*Lysinibacillus sphaericus*
	M5B2	Cream-yellowish	Round	Flat	Smooth	Entire	*Stenotrophomonas maltophilia*
	M3A	Reddish	Irregular	Flat	Smooth	Serrate	*Serratia marcescens*
	M7CC	Reddish	Irregular	Flat	Smooth	Serrate	*Serratia marcescens*
	M3C1	Reddish	Irregular	Flat	Smooth	Serrate	*Serratia marcescens*
	M6A2	Cream	Filamentous	Flat	Smooth	Filamentous	Unidentified
	M6C2	Cream	Filamentous	Flat	Smooth	Filamentous	Unidentified
	M5B3	Cream	Filamentous	Flat	Smooth	Filamentous	Unidentified
	M5C4	Cream	Filamentous	Flat	Smooth	Filamentous	Unidentified
	M5C1	Cream	Filamentous	Flat	Smooth	Filamentous	Unidentified
	M5B4	Cream	Filamentous	Flat	Smooth	Filamentous	Unidentified
	M6A1	Cream	Round	Flat	Smooth	Entire	Unidentified
	M5C3	Cream	Round	Flat	Smooth	Entire	Unidentified
	M7C4	Cream	Round	Flat	Smooth	Entire	Unidentified
	T6B2	Milky Yellow	Punctiform	Flat	Smooth	Entire	*Cellulosimicrobium cellulans*
	T10B1	Milky Yellow	Punctiform	Flat	Smooth	Entire	*Cellulosimicrobium cellulans*
	T1B1	White	Punctiform	Flat	Smooth	Entire	Unidentified

	T1C1	White	Punctiform	Flat	Smooth	Entire	Unidentified
	T1C2	White	Punctiform	Flat	Smooth	Entire	Unidentified
	T6A3	White	Punctiform	Flat	Smooth	Entire	Unidentified
	T6C2	Yellow	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T6A	Yellow	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T6B3	Yellow	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T6A3	Yellow	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T4C1	Bright Yellow	Punctiform	Flat	Smooth	Entire	*Cellulosimicrobium cellulans*
	T1B2	Bright Yellow	Punctiform	Flat	Smooth	Entire	*Cellulosimicrobium cellulans*
	T4C2	Bright Yellow	Round	Flat	Smooth	Entire	Unidentified
	T4C4	Bright Yellow	Round	Flat	Smooth	Entire	Unidentified
	T5A3	Bright Yellow	Round	Flat	Smooth	Entire	Unidentified
	T4B2	Bright Yellow	Round	Flat	Smooth	Entire	Unidentified
	T10A3	Cream-white	Irregular	Flat	Smooth	Lobate	*Bacillus* sp.
	T2C3	Cream-white	Irregular	Flat	Smooth	Lobate	*Bacillus* sp.
	T1A2	Cream-white	Irregular	Flat	Smooth	Lobate	*Bacillus* sp.
	T5A2	Cream-white	Irregular	Flat	Smooth	Lobate	*Bacillus* sp.
	T4B41	Cream	Punctiform	Flat	Smooth	Entire	*Lysinibacillus sphaericus*
	T2Bb	Cream	Punctiform	Flat	Smooth	Entire	*Lysinibacillus sphaericus*

	T7C	Cream	Punctiform	Flat	Smooth	Entire	*Lysinibacillus sphaericus*
	T1A	Cream	Punctiform	Flat	Smooth	Entire	*Enterobacter bugandensis*
	T10B5	Yellow	Round	Flat	Smooth	Entire	Unidentified
	T1B4	Yellow	Round	Flat	Smooth	Entire	Unidentified
	T4C21	Yellow	Round	Flat	Smooth	Entire	Unidentified
	T2B1	Yellow	Round	Raised	Smooth	Entire	*Micrococcus yunnanensis*
	TB3	White	Round	Flat	Smooth	Entire	Unidentified
	T7B1	White	Round	Flat	Smooth	Entire	Unidentified
	T4A7	Cream	Irregular	Flat	Smooth/shiny	Filamentous/ serrate	Unidentified
	T2C1	Cream-white	Round	Flat	Smooth	Entire	Unidentified
	T6A2	Cream-white	Round	Flat	Smooth	Entire	Unidentified
	T7A1	Cream-white	Round	Flat	Smooth	Entire	Unidentified
	T10C1	Cream-white	Round	Flat	Smooth	Entire	Unidentified
	T10B2	Cream-white	Round	Flat	Smooth	Entire	Unidentified
	T2B3	Cream-white	Round	Flat	Smooth	Entire	Unidentified
	T10C3	Creamy	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T8B1	Creamy	Filamentous	Flat	Smooth	Filamentous	Unidentified

	T10A3	Creamy	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T10B4	Creamy	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T5C3	Creamy	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T8C1	Creamy	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T5B1	Creamy	Filamentous	Flat	Smooth	Filamentous	Unidentified
	T8B2	Cream	Round	Flat	Smooth	Entire	Unidentified
	T5A1	Cream	Round	Flat	Smooth	Entire	Unidentified
	T3A1	Cream	Round	Flat	Smooth	Entire	Unidentified
	T2C2	Cream	Round	Flat	Smooth	Entire	Unidentified
	T3A2	Cream	Round	Flat	Smooth	Entire	Unidentified
	T7B2	Cream	Round	Flat	Smooth	Entire	Unidentified
	T10B3	Cream	Round	Flat	Smooth	Entire	Unidentified
	T10A1	Cream	Round	Flat	Smooth	Entire	Unidentified
	T4C3	Cream	Round	Flat	Smooth	Entire	Unidentified
	T5C2	Cream	Round	Flat	Smooth	Entire	Unidentified
	T8A	Cream	Round	Flat	Smooth	Entire	Unidentified

Appendix 3.2: Shapiro-Wilk normality test for leaf data analysis

Variable	N	W	P
P (mg.kg)	8	0.9119	0.3677
P (µmol)	8	0.9119	0.3677
% N	8	0.8794	0.1859
N (mmol)	8	0.8794	0.1859
% C	8	0.9315	0.5296
C (mmol)	8	0.9315	0.5296
Std corrected d 15N 14/N	8	0.9051	0.3206
% NDFA	8	0.9051	0.3206
Total plant N	8	0.8794	0.1859
NDFA (mmol)	8	0.8032	0.0309
NDFS (mmol)	8	0.7312	0.0050
C/N	8	0.8424	0.0797

Appendix 3.3: Homogeneity of variance of C, N and P by sample

Homogeneity of variance (Folded F Test	DF	F	P
P (mg.kg)	3.3	26.39	0.0117
P (µmol)	3.3	26.39	0.0117
% N	3.3	5.75	0.0923
N (mmol)	3.3	5.75	0.0923

% C	3.3	4.89	0.1124
C (mmol)	3.3	4.89	0.1124
Std corrected d 15N	3.3	9.62	0.0476
14/N			
% NDFA	3.3	9.62	0.0476
Total plant N	3.3	5.75	0.0923
NDFA (mmol)	3.3	12.57	0.0332
NDFS (mmol)	3.3	2.91	0.2021
C/N	3.3	12.81	0.0323

Appendix 3.4: Two sample T test for phosphorus (mg.kg)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-0.40	0.7017	-256.13	183.86
Satterthwaite	Unequal	3.2	-0.40	0.7129	-311.20	238.94

Appendix 3.5: Two sample T test for phosphorus (mmol)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-0.40	0.7017	-8.2702	5.9368
Satterthwaite	Unequal	3.2	-0.40	0.7129	-10.048	7.7151

Appendix 3.6:Two sample T test for % nitrogen (N)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-7.45	0.0003	-0.7148	-0.3614
Satterthwaite	Unequal	4.0	-7.45	0.0017	-0.7384	-0.3378

Appendix 3.7: Two sample T test for nitrogen (mmol)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-7.45	0.0003	-0.0511	-0.0258
Satterthwaite	Unequal	4.0	-7.45	0.0017	-0.0527	-0.0241

Appendix 3.8: Two sample T test for % carbon (C)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-3.11	0.0209	-1.8109	-0.2153
Satterthwaite	Unequal	4.2	-3.11	0.0339	-1.9035	-0.1227

Appendix 3.9: Two sample T test for carbon (C) (mmol)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-3.11	0.0209	-0.1508	-0.0179
Satterthwaite	Unequal	4.2	-3.11	0.0339	-0.1585	-0.0102

Appendix 3.10: Two sample T test for C: N by sample

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	6.97	0.0004	0.9580	1.9938
Satterthwaite	Unequal	3.5	6.97	0.0037	0.8507	2.1011

Appendix 3.11: Two sample T test for standard corrected d 15N /14N

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-5.34	0.0018	-2.0470	-0.7602
Satterthwaite	Unequal	3.6	-5.34	0.0078	-2.1652	-0.6420

Appendix 3.12: Two sample T test for % nitrogen derived from atmosphere (NDFA)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	5.34	0.0018	11.033	29.710
Satterthwaite	Unequal	3.6	5.34	0.0078	9.3175	31.425

Appendix 3.13: Two sample T test for total nitrogen (TN) (mmol) concentration mmol

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-7.45	0.0003	-0.0511	-0.0258
Satterthwaite	Unequal	4.0	-7.45	0.0017	-0.0527	-0.0241

Appendix 3.14: Two sample T test for nitrogen derived from atmosphere (NDFA)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	11.83	0.0000	0.0105	0.0159
Satterthwaite	Unequal	3.5	11.83	0.0006	9.90E-03	0.0165

Appendix 3.15: Two sample T sest for nitrogen derived from soil (NDFS) (mmol)

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	6	-31.32	0.0000	-0.0300	-0.0256
Satterthwaite	Unequal	4.8	-31.32	0.0000	-0.0301	-0.0255

Appendix 3.16: Nitrogen fixing efficiency of PGPR

Isolate	[y]Nitrogen fixation ability (+/-)		
	Summer		Winter
T6B2	+++	T2CC	+++
T10B1	+++	T2BA	+++
T1B1	+++	T1AA	++
T1C1	+++	T1AB	++
T1C2	+++	T1CB	++
T6A3	+++	T2Bb	++
T2Bb	+++	T1CA	++
T4B41	+++		

T7C	+++	T2CB	+++
T1A	+++	T2BB	+++
T10B5	+++	T2CA	++
T1B4	+++	T1BA	++
T4C21	+++	T2CB1	+
T4A7	+++	T2CB2	+
T8B2	+++	T2BB1	-
T5A1	+++	M4BA	+++
T3A1	+++	M7BA	++
T2C2	+++	M4CA	++
T3A2	+++	M3AA	+
T7B2	+++	M6AA	+++
T10B3	+++	M4AB	+++
T10A1	+++	M3CA	+++
T4C3	+++	M6BA	+++
T5C2	+++	M7CA	+++
T8A	+++	M7CB	+++
T4C1	+++	M8AB	+++
T1B2	+++	M8AA2	+++
T2B1	-	M6CA	+++
T4C2	++	M8AA1	++
T4C4	++	M3BA	+++
T5A3	++	M4AA	+++
T4B2	++		
T2C1	+++		

T6A2	+++
T7A1	+++
T10C1	+++
T10B2	+++
T2B3	+++
T10C3	+++
T10B4	+++
T8B1	+++
T10A3	+++
T5C3	+++
T8C1	+++
T5B1	+++
T2B2	+++
T6C2	+
T6A	+
T6B3	+
T6A3	+
TB3	-
T7B1	-
T10A3	+++
T2C3	+++
T1A2	+++
T5A2	+++
M6A2	+++
M6C2	+++

M5B3	+++
M5C4	+++
M5C1	+++
M5B4	+++
M6C4	+++
M5B2	+++
M5B1	+++
M3C1	+++
M7CC	+++
M3A	+++
M6A1	-
M5C3	-
M7C4	-

[y]Isolates with a +++ = 100% N-fixation; ++ 50% N-fixation; + = < 50% N-fixation and - = 0% N-fixation

Appendix 3.17: Phosphorus solubilization activity of PGPRs

Isolate	[y]Phosphorus solubilization ability (+/-)		
	Summer		Winter
T6B2	-	T2CC	-
T10B1	-	T2BA	-
T1B1	-	T1AA	-
T1C1	-	T1AB	-
T1C2	-	T1CB	-

T6A3	-	T2Bb	-
T2Bb	-	T1CA	-
T4B41	-	T2CB	-
T7C	-	T2BB	-
T1A	-	T2CA	-
T10B5	-	T1BA	-
T1B4	-	T2CB1	-
T4C21	-	T2CB2	-
T4A7	-	T2BB1	-
T8B2	-	M4BA	-
T5A1	-	M7BA	-
T3A1	-	M4CA	-
T2C2	-	M3AA	-
T3A2	-	M6AA	-
T7B2	-	M4AB	-
T10B3	-	M3CA	-
T10A1	-	M6BA	-
T4C3	-	M7CA	-
T5C2	-	M7CB	-
T8A	-	M8AB	-
T4C1	-	M8AA2	-
T1B2	-	M6CA	-
T2B1	-	M8AA1	-
T4C2	-	M3BA	-
T4C4	-	M4AA	-

T5A3	-
T4B2	-
T2C1	-
T6A2	-
T7A1	-
T10C1	-
T10B2	-
T2B3	-
T10C3	-
T10B4	-
T8B1	-
T10A3	-
T5C3	-
T8C1	-
T5B1	-
T2B2	-
T6C2	-
T6A	-
T6B3	-
T6A3	-
TB3	-
T7B1	-
T10A3	-
T2C3	-
T1A2	-

T5A2	-
M6A2	-
M6C2	-
M5B3	-
M5C4	-
M5C1	-
M5B4	-
M6C4	-
M5B2	-
M5B1	-
M3C1	-
M7CC	-
M3A	-
M6A1	-
M5C3	-
M7C4	-

Appendix 4.1: Shapiro – Wilk Normality test for total soil nutrients

Variable	N	W	P
Density	11	0.9262	0.3741
P	11	0.6861	0.0003
K	11	0.8266	0.0211
Ca	11	0.8790	0.1009
Mg	11	0.7962	0.0084
EA	11	0.9454	0.5864

ECEC	11	0.8532	0.0470
A	11	0.5724	0.0000
pH	11	0.7138	0.0007
Zn	11	0.8662	0.0693
Mn	11	0.8623	0.0617
Cu	11	0.8874	0.1290
OC	11	0.5118	0.0000
OM	11	0.5118	0.0000
N	11	0.7895	0.0069
Clay	11	0.8278	0.0218

Appendix 4.2: Analysis of variance (ANOVA) for density

Source	DF	SS	MS	F	P
Replication	2	0.00091	0.00045		
Treatment	1	0.01050	0.01050	103.62	0.0002
Season	1	0.00037	0.00037	3.62	0.1155
Treatment*Season	1	0.00007	0.00007	0.65	0.4565
Error	5	0.00051	0.00010		
Total	10				

Appendix 4.3: Analysis of variance (ANOVA) for phosphorus (P)

Source	DF	SS	MS	F	P
Replication	2	0.00235	0.00118		
Treatment	1	0.30166	0.30166	433.22	0.0000

Season	1	0.11547	0.11547	165.83	0.0001
Treatment*Season	1	0.11898	0.11898	170.88	0.0000
Error	5	0.00348	0.00070		
Total	10				

Appendix 4.4: Analysis of variance (ANOVA) for potassium (K)

Source	DF	SS	MS	F	P
Replication	2	5.750E-04	2.875E-04		
Treatment	1	0.01304	0.01304	155.31	0.0001
Season	1	7.074E-07	7.074E-07	0.01	0.9304
Treatment*Season	1	1.913E-04	1.913E-04	2.28	0.1915
Error	5	4.198E-04	8.395E-05		
Total	10				

Appendix 4.5: Analysis of variance (ANOVA) for calcium (Ca)

Source	DF	SS	MS	F	P
Replication	2	0.00162	0.00081		
Treatment	1	1.00057	1.00057	1497.91	0.0000
Season	1	0.00781	0.00781	11.69	0.0188
Treatment*Season	1	0.10219	0.10219	152.99	0.0001
Error	5	0.00334	0.00067		
Total	10				

Appendix 4.6: Analysis of variance (ANOVA) magnesium (Mg)

Source	DF	SS	MS	F	P
Replication	2	0.03918	0.01959		
Treatment	1	1.70723	1.70723	103.89	0.0002
Season	1	0.00019	0.00019	0.01	0.9178
Treatment*Season	1	0.05898	0.05898	3.59	0.1167
Error	5	0.08217	0.01643		
Total	10				

Appendix 4.7: Analysis of variance (ANOVA) for exchangeable acidity

Source	DF	SS	MS	F	P
Replication	2	6.822E-05	3.411E-05		
Treatment	1	1.620E-04	1.620E-04	3.73	0.1113
Season	1	4.745E-04	4.745E-04	10.92	0.0214
Treatment*Season	1	1.571E-04	1.571E-04	3.62	0.1156
Error	5	2.172E-04	4.345E-05		
Total	10				

Appendix 4.8: Analysis of variance (ANOVA) for effective cation exchange capacity

Source	DF	SS	MS	F	P
Replication	2	0.00886	0.00443		
Treatment	1	1.43818	1.43818	516.74	0.0000
Season	1	0.00905	0.00905	3.25	0.1313
Treatment*Season	1	0.10006	0.10006	35.95	0.0019
Error	5	0.01392	0.00278		

Total	10				

Appendix 4.9: Analysis of variance (ANOVA) for acid saturation

Source	DF	SS	MS	F	P
Replication	2	1.806E-05	9.028E-06		
Treatment	1	1.050E-04	1.050E-04	10.80	0.0218
Season	1	2.401E-05	2.401E-05	2.47	0.1769
Treatment*Season	1	2.401E-05	2.401E-05	2.47	0.1769
Error	5	4.861E-05	9.723E-06		

Appendix 4.10: Analysis of variance (ANOVA) for pH

Source	DF	SS	MS	F	P
Replication	2	3.957E-04	1.978E-04		
Treatment	1	3.657E-03	3.657E-03	32.54	0.0023
Season	1	9.466E-03	9.466E-03	84.24	0.0003
Treatment*Season	1	4.637E-03	4.637E-03	41.27	0.0014
Error	5	5.619E-04	1.124E-04		
Total	10				

Appendix 4.11: Analysis of variance (ANOVA) for zinc (Zn)

Source	DF	SS	MS	F	P
Replication	2	0.06832	0.03416		
Treatment	1	0.04191	0.04191	1.38	0.2933
Season	1	0.05529	0.05529	1.82	0.2354

Treatment*Season	1	0.00007	0.00007	0.00	0.9648
Error	5	0.15204	0.03041		
Total	10				

Appendix 4.12: Analysis of variance (ANOVA) for manganese (Mn)

Source	DF	SS	MS	F	P
Replication	2	0.03540	0.01770		
Treatment	1	0.39888	0.39888	34.89	0.0020
Season	1	0.01641	0.01641	1.44	0.2846
Treatment*Season	1	0.01495	0.01495	1.31	0.3046
Error	5	0.05716	0.01143		
Total	10				

Appendix 4.13: Analysis of variance (ANOVA) for copper (Cu)

Source	DF	SS	MS	F	P
Replication	2	0.01586	0.00793		
Treatment	1	0.13577	0.13577	24.24	0.0044
Season	1	0.00122	0.00122	0.22	0.6597
Treatment*Season	1	0.00013	0.00013	0.02	0.8830
Error	5	0.02800	0.00560		
Total	10				

Appendix 4.14: Analysis of variance (ANOVA) for organic carbon

Source	DF	SS	MS	F	P

Replication	2	1.250E-07	6.250E-08		
Treatment	1	4.018E-06	4.018E-06	53.57	0.0007
Season	1	4.018E-06	4.018E-06	53.57	0.0007
Treatment*Season	1	4.018E-06	4.018E-06	53.57	0.0007
Error	5	3.750E-07	7.500E-08		
Total	10				

Appendix 4.15: Analysis of variance (ANOVA) for organic matter

Source	DF	SS	MS	F	P
Replication	2	3.716E-07	1.858E-07		
Treatment	1	1.194E-05	1.194E-05	53.57	0.0007
Season	1	1.194E-05	1.194E-05	53.57	0.0007
Treatment*Season	1	1.194E-05	1.194E-05	53.57	0.0007
Error	5	1.115E-06	2.230E-07		
Total	10				

Appendix 4.16: Analysis of variance (ANOVA) for nitrogen (N)

Source	DF	SS	MS	F	P
Replication	2	1.791E-	8.955E-06		

		05			
Treatment	1	1.301E-06	1.301E-06	0.50	0.5114
Season	1	9.003E-06	9.003E-06	3.45	0.1222
Treatment*Season	1	1.377E-06	1.377E-06	0.53	0.4999
Error	5	1.303E-05	2.606E-06		
Total	10				

Appendix 4.17: Analysis of variance (ANOVA) for clay

Source	DF	SS	MS	F	P
Replication	2	0.00080	0.00040		
Treatment	1	0.20097	0.20097	126.27	0.0001
Season	1	0.00037	0.00037	0.23	0.6512
Treatment*Season	1	0.00593	0.00593	3.72	0.1115
Error	5	0.00796	0.00159		
Total	10				

Appendix 4.18: Shapiro-Wilk Normality Test for enzyme activities

Variable	N	w	P
Glucosidase	84	0.6964	0.0000
Glucosaminidase	84	0.0975	0.0000
P alkaline	84	0.7995	0.0000

P acid	84	0.7123	0.0000
N reductase	14	0.8699	0.0419

Appendix 4.19: Homogeneity of variance of enzyme activity

Homogeneity of variance (Folded F Test	DF	F	P
Glucosidase	41.41	1.01	0.4841
Glucosaminidase	41.41	1622.98	0.0000
P alkaline	41.41	1.20	0.2800
P acid	41.41	1.41	0.1377
N reductase	6.6	2.63	0.1322

Appendix 4.20: Two Sample T Test for nitrate reductase

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	12	-0.40	0.6965	-0.5006	0.3455
Satterthwaite	Unequal	10.0	-0.40	0.6979	-0.5102	0.3551

Appendix 4.21: Two Sample T Test for glucosidase activity

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	82	-1.05	0.2988	-4.00E-04	1.24E-04
Satterthwaite	Unequal	82.0	-1.05	0.2988	-4.00E-04	1.24E-04

Appendix 4.22: Two Sample T Test for glucosaminidase activity

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	82	0.96	0.3390	-4.93E-03	0.0142
Satterthwaite	Unequal	41.1	0.96	0.3418	-5.08E-03	0.0143

Appendix 4.23: Two Sample T Test for P alkaline enzyme activity

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	82	-1.41	0.1625	-5.19E-04	8.86E-05
Satterthwaite	Unequal	81.3	-1.41	0.1626	-5.19E-04	8.86E-05

Appendix 4.24: Two T Test for P acid enzyme activity

					Lower	Upper
Method	Variances	DF	T	P	95% C.I.	95% C.I.
Pooled	Equal	82	0.97	0.3355	-1.40E-04	4.06E-04
Satterthwaite	Unequal	79.7	0.97	0.3356	-1.40E-04	4.06E-04

Appendix 4.25: Mean difference in rhizosphere enzyme activity of Tubatse and Makgupheng

Sample	N reductase	Glucosaminidase	Glucosidase	P acid	P alkaline
Makgupheng	3.8470	5.19E-03	3.19E-04	4.77E-04	4.61E-04
Tubatse	3.9246	5.69E-04	4.57E-04	3.44E-04	6.77E-04

Difference	-0.0776	4.62E-03	-1.38E-04	1.33E-04	-2.15E-04

Null Hypothesis (H_0): difference = 0; Alternative Hypothesis (H_1): difference ≠ 0.