The role of nutritional status of soils from grassland and savanna ecosystems on the biochemical and physiological responses of *Vigna unguiculata* L. (Walp)

By

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Thesis submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy in the Discipline of Biological Sciences, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg

January 2022

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Co-supervisor: Prof Adeyemi Oladapo Aremu

PREFACE

The research contained in this thesis was completed by the candidate based in the Discipline of Biological Science, School of Life Science of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The study was conducted under the supervision of Dr. A. Magadlela and co-supervision of Prof. A. Oladapo Aremu and financially supported by National Research Funding (NRF) Thuthuka grant (113576) and Sustainable and Health Food System (SHEFS).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to the investigations by the candidate.

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As the supervisor I have approved this thesis for submission

Dr. A. Magadlela (Supervisor)

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We hereby declare that we acted as Supervisors for this PhD student:

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Student Number: **218086700**

Thesis Title: **The role of nutritional status of soils from grassland and savanna ecosystems on the biochemical and physiological responses of *Vigna unguiculata* L. (Walp)**

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science, Higher Degrees Office for examination by the University appointed Examiners.

SUPERVISOR _____

DR A. MAGADLELA

CO-SUPERVISOR _____

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Publications From This Thesis

1. B.T. Makaure., A.O. Aremu and A. Magadlela, 2022. Soil nutritional status drives the co-occurrence of nodular bacterial species and arbuscular mycorrhizal fungi modulating plant nutrition and growth of *Vigna unguiculata* L. (Walp) in grassland and savanna ecosystems in KwaZulu-Natal, South Africa. Journal of Soil Science and Plant Nutrition. In Press (Doi**:** 10.1007/s42729-022-00763-6).
2. B.T. Makaure, A.O. Aremu., J. Gruz and A. Magadlela. Phenolic acids and antioxidant regulation in *Vigna unguiculata* L. (Walp) growing in acidic and nutrient deficient grassland and savanna soils. Plant Cell and Environment (Submitted for publication)

College of Agriculture, Engineering and Science Declaration 2 – Publications

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1 contributions: BTM did the experimental work and drafted the manuscript. AM and AOA supervised the whole study and edited the manuscript before submission.

Publication 2 contributions: Experimental work and draft manuscript were done by BTM under the guidance and supervision of AM and AOA. JG assisted with the experimental design and experimental work for the phytochemical analysis. AM and AOA supervised the whole study and edited the manuscript before submission.

Author's abbreviation

BTM	Brenda Tsungai Makaure
AM	Anathi Magadlela
AOA	Adeyemi Oladapo Aremu
JG	Jiri Gruz

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Abstract

Most arable soils in sub–Saharan Africa savanna and grassland ecosystems are acidic and nutrient deficient with nitrogen and phosphorus being the most limiting and this poses a huge threat to agricultural productivity. To overcome soil nutrient deficiency and increase crop yields, farmers have resorted to high inputs of synthetic fertilizers, which are expensive and may cause environmental degradation. Use of legumes is an important alternative as they help enhance soil nutrition through biological nitrogen fixation. *Vigna unguiculata* L. (Walp), a highly nutritious legume crop that could be incorporated in small scale cropping systems to improve soil nutrition. However, there is limited information on the physiological and biochemical strategies enabling the growth of *V. unguiculata* under acidic and nutrient stress conditions. In this study it was hypothesized that symbiotic association between *V. unguiculata* and rhizospheric microbes affects the growth, nutrient assimilation and phytochemicals of the grain legume grown in nutrient stressed soils.

Firstly, this study evaluated the physicochemical properties, microbial composition and soil enzymes activities of soils from four geographically distinct regions of KwaZulu-Natal representing savanna and grassland ecosystems. Secondly, the study investigated how the tripartite symbiosis of *V. unguiculata*, arbuscular mycorrhizal fungi and nodulating bacteria affect phosphorus and nitrogen nutrition, and the growth of *V. unguiculata* grown under acidic and nutrient stress conditions. Then, the study investigated how four *V. unguiculata* varieties regulated their phenolic acids and antioxidants to enhance their growth in acidic and nutrient stressed soils conditions.

The four soil types were acidic with low mineral nutrients, with Bergville being the most acidic. The soils were significantly different in their physicochemical and microbial composition. Most bacterial strains identified in the soils belonged to genera *Lysinibacillus*, and *Bacillus* while the most identified fungal strains belonged to *Fusarium* and *Trichoderma* genera. There were variations in soil lignin degrading, C, N and P cycling enzyme activities. The identified soil enzymes included β-D Phosphatase, L-asparaginase, β-glucosaminidase, β-cellobioside, catalase and lacasse. The availability of this rich pool of soil microbes and soil enzymes is a great opportunity as these can be used to regulate nutrient cycling and enhance nutrient availability for crop production in the savanna and grassland ecosystems.

Four *V. unguiculata* varieties (IT18, Batch white, Brown mix, Dr Saunders) were grown in these acidic and nutrient poor soils. These *V. unguiculata* varieties were nodulated by several bacterial strains including those of genera *Bradyrhizobium*, *Rhizobium*, *Bacillus* and

Paenibacillus. The *V. unguiculata* fixed more than 60% of its total nitrogen from the atmosphere across all soil treatments. Interestingly, *V. unguiculata* plants which were nodulated by non-rhizobial bacteria strains effectively fixed significantly high amounts of atmospheric nitrogen. *Vigna unguiculata* also developed symbiotic association with arbuscular mycorrhizal fungi (AMF) as evidenced by high root mycorrhizal fungi colonization ranging from 58-100%. Variations were observed on growth kinetics, nutrient assimilation and utilization among the four *V. unguiculata* varieties. *Vigna unguiculata* was able to switch N source preferences utilizing both soil and atmospheric nitrogen. These findings revealed that *V. unguiculata* has the capacity to adapt to nutrient poor ecosystems by establishing symbiotic interaction with naturally occurring soil bacteria and AMF and through its ability to switch N source preferences; by using soil N and atmospheric N_2 through biological nitrogen fixation. There were variations in the response of the four *V. unguiculata* varieties to different levels of soil acidity and nutrient stress with regards to phenolic acid concentration and antioxidant capacities. The most abundant phenolic acids were vanillic acid and protocatechuic acid and these constituted 22.59% and 17.22% respectively of the total phenolic acids in the plants. More so, there were differences in correlations between the phenolic acids and plant biomass, plant nutrition, soil nutrition and AMF infection. There was negative correlation between phenolic acids protocatechuic acid and syringic acid, and concentration of plant nutrients N and P. Varieties IT18 and Batch white had relatively lower concentrations of phenolic acids but these had the highest plant biomass. These results confirm that low phenolic acid concentrations have stimulatory effects on growth and nutrient uptake by plants while high concentrations may inhibit plant growth and development. There were variations among the *V. unguiculata* varieties with respect to oxygen radical absorbance capacity (ORAC) across the four soil types. Overall, the study demonstrated that *V. unguiculata* is adaptable to acidic and nutrient poor ecosystems as it has the capacity to regulate its phenolic acids which enhance nutrient uptake, promote legume-microbe symbiosis, and help scavenge radical oxidative species due to their antioxidant properties.

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4CoA	4-Coumaric acid
4HBA	4-Hydroxybenzoic acid
AAPH	2,2'-Azobis (2-amidinopropane) dihydrochloride
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
BNF	Biological nitrogen fixation
CaA	Caffeic acid
FA	Ferulic acid
GA	Gallic acid
GR	Growth rate
IAA	Indoleacetic acid
KZN	KwaZulu-Natal
NDFS	Nitrogen derived from soil
NDFA	Nitrogen derived from atmosphere.
ORAC	Oxygen radical absorbance capacity
PA	Protocatechuic acid
PCA	Principal component analysis
PEP	Phosphoenolpyruvate
RGR	Relative growth rate
ROS	Reactive oxygen species
SA	Salicylic acid
SNAR	Specific nitrogen assimilation rate
SNUR	Specific nitrogen utilization rate
SPAR	Specific phosphorus absorption rate

SPUR	Specific phosphorus utilization rate
SyA	Syringic acid
VA	Vanillic acid

Chapter 1: General Introduction

1.1 Background

In Sub-Saharan Africa, most of the grasslands and savannas are associated with nutrient-poor and largely acidic soils which cannot provide sufficient nutrients to ensure optimal crop growth (Jaiswal et al. 2018, Jaiswal and Dakora 2019). The rapid depreciation of soil fertility, most importantly soil phosphorus (P) and nitrogen (N), in the grasslands and savannas, is ascribed to human activities which include overgrazing, uncontrolled burning and clearing of grasslands for cultivation and urbanization (Sanderson et al. 2013, Blair et al. 2014). Due to depleted soil nutrients, crop productivity has drastically declined especially for resource constrained small-holder farmers in many southern African countries (Oruru et al. 2018, Jaiswal and Dakora 2019). In order to increase crop yields, most farmers have heavily depended on N fertilizers, however most resource constrained small-holder farmers in the Sub-Saharan Africa use limited amounts of chemical fertilizers compared to farmers in other parts of the world (Ruben et al. 2007, Oruru et al. 2018). In addition to being expensive, fertilizers contribute extensively to environmental pollution (Adesemoye and Kloepper 2009).

Legumes can be included in the nutrient poor savanna and grassland cropping systems as they help to promote conversion of atmospheric N_2 to the reduced form (NH_4^+) which is readily accessible to plants, through biological nitrogen fixation (Rodrigues et al. 2013, Oruru et al. 2018, Jaiswal and Dakora 2019). Legumes can fix up to 40-60 million tons of atmospheric N_2 per annum (Group et al. 2013) and this translates to about 65% of the total amount of N that is used in agriculture worldwide (Dupont et al. 2012). *Vigna unguiculata* (cowpea) can be considered as an ideal legume to be incorporated in cropping systems due to its ability to fix N_2 and its resilience to drought stress (Oruru and Njeru 2016).

Vigna unguiculata is an important multipurpose legume which provides nutrients for both humans and animals and it serves as a biofertilizer for maintaining soil productivity (Oruru et al. 2018, Jaiswal and Dakora 2019). Cowpea can establish effective symbiosis with a large array of bacterial species including those belonging to the *Bradyrhizobium* and *Rhizobium* genera help promote nodulation, plant growth and nitrogen fixation (Wahyudi et al. 2011). *V. unguiculata* forms symbiotic associations with non-rhizobial bacteria which promote biological nitrogen fixation and these include bacteria of genera *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Azospirilium* and *Azotobacter* (Kennedy et al. 1997, Wahyudi et al. 2011).

Effective biological nitrogen fixation requires a good supply of soil P because this enhances nodule development and functioning (Vance et al. 2003, Magadlela et al. 2016, Vardien et al. 2016). However, most soils are deficient in P as the mineral forms complexes with Ca^{2+} in calcareous soils while in acidic soils it complexes with Al^{3+} and Fe^{3+} (Khan et al. 2009, Magadlela et al. 2016, Valentine et al. 2018).

Legumes have adopted mechanisms to optimize acquisition of P even in nutrient-poor soils through establishment of symbiotic relationship with microorganisms which solubilize P and these include fungi and bacteria (Khan et al. 2010a, Oruru and Njeru 2016). Some of the notable P solubilizing bacterial genera include *Pseudomonas*, *Bacilli*, *Paenibacillus*, *Rhizobium* and *Enterobacter*, while those of fungi include *Penicillium* and *Aspergillus* (Whitelaw 1999, Govindasamy et al. 2010, Grady et al. 2016). These rhizospheric microorganisms can mineralize or mobilize P through diverse mechanisms among them; the production of acid phosphatases and organic acids (He et al. 2002, Chen et al. 2006, Khan et al. 2010a). Other legume plants may exude phytohormones and phytochemicals (Imas et al. 1997, Makoi and Ndakidemi 2007), which promote nutrient uptake, most importantly P thus ensuring enhanced growth of plants (Hu et al. 2005a, Hu et al. 2005b). Under abiotic stress, especially nutrient stress conditions, legumes produce phenolic acids which contribute immensely to the legume's survival through ensuring enhanced nutrient uptake and though other mechanisms. Phenolic acids such as salicylic acid and ferulic acid contribute to enhanced nutrient uptake by promoting plant rooting (Hayat et al. 2010a, Mandal et al. 2010, Muhal et al. 2014, Zafar-ul-Hye et al. 2020). Furthermore, some phenolic acids promote plant microbe symbiosis as they act as signaling molecules in establishing legume- rhizobia or arbuscular mycorrhizal symbioses (Mandal et al. 2010) and these include caffeic acid (Klein et al. 2015, Zafar-ul-Hye et al. 2020), protocatechuic and p-hydroxybenzoic acids (Mandal et al. 2016). Some phenolic acids can be used as alternative carbon sources by the rhizobial strains thus enhancing the process of biological nitrogen fixation (Irisarri et al. 1996) and these include ferulic acid, p-coumaric acid, protocatechuic acid and vanillic acid (Seneviratne and Jayasinghearachchi 2003). Furthermore, some phenolic acids may have inhibitory or stimulatory effects on microbial growth and proliferation (Zhou et al. 2014, Wang et al. 2018), which affects soil health and nutrient cycling.

Under stressful conditions plants produce reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and these create oxidative stress (Hayat et al. 2010a, Zafar-ul-Hye et al. 2020). ROS cause oxidative damage to biomolecules, nucleic acids and antioxidant systems in plants. To curtail the challenge of

oxidative damage, phenolic acids enhance antioxidant enzymes activities which help scavenge the ROS (Hayat et al. 2010a, Khan et al. 2010b, El-Awadi 2018, Riaz et al. 2019). Phenolic acids such as protocatechuic acid (Marchiosi et al. 2020), salicylic acid (Gunes et al. 2005), p-hydroxybenzoic acid (Ahrabi et al. 2011) caffeic acid, ferulic acid, gallic acid, p-coumaric acid and vanillic acid have such strong antioxidant properties (Kiokias et al. 2020).

Plant acquisition of N through biological nitrogen fixation is energetically expensive for plants (Minchin and Witty 2005, Kaschuk et al. 2010). The cost ranges from 3.3 - 6.6 g $Cg^{-1}N$, and this varies with specific rhizobium-legume species combination. However, the cost reducing NO^{3-} may be less than 2.5 g $Cg^{-1}N$ (Minchin and Witty 2005). In order to save energy, some legumes may utilize alternative N sources found in the rhizosphere (Kaschuk et al. 2010, Mortimer et al. 2013, Magadlela et al. 2016). Such an ability to switch N source preferences has been described as a crucial survival strategy in legume plants (Magadlela et al. 2016).

1.2 Problem statement

Most agricultural systems are low in essential soil micronutrients, most importantly N thus resulting in poor crop production (Valentine et al. 2017, Jaiswal and Dakora 2019, Parihar et al. 2019). Biological nitrogen fixation by legumes could enhance soil fertility in a cheaper and sustainable manner. However, the efficiency of biological nitrogen fixation process is hampered by low soil P which characterizes most tropical and sub-tropical ecosystems (Magadlela et al. 2016, Valentine et al. 2017, Valentine et al. 2018). Through tripartite symbiosis with bacteria and arbuscular mycorrhiza fungi, legumes can enhance P uptake and improve biological nitrogen fixation (Abd-Alla et al. 2014, Hack et al. 2019, Musyoka et al. 2020). Different soil microbes contribute differently to P uptake and biological nitrogen fixation by legumes. Not much is known on the rhizospheric microbes which nodulate legumes including *V. unguiculata* in the natural soils of KwaZulu-Natal. Therefore, there is need to identify the soil microbes which are associated with *V. unguiculata* and understand the role they play in enhancing nutrient acquisition. Biological nitrogen fixation is a costly process therefore there is need to identify the mechanisms adopted by *V. unguiculata* to conserve energy in nutrient acquisition and how this affects plant growth. Currently, limited data exists on the phytochemicals, particularly the phenolic acids produced by cowpea which grows in symbiotic association with soil microbes, as a response to nutrient stress, and how these affect the nutrient acquisition and growth of the legume.

1.3 Rationale of study

The knowledge on how *V. unguiculata* interacts with soil microbes in ensuring enhanced nutrient acquisition and biological nitrogen fixation has the potential for better management and conservation of nutrient depleted savanna and grassland ecosystems of KwaZulu-Natal. Findings from this research will provide useful information in selecting cowpea varieties which effectively fix N_2 in association with these natural soil microbes while saving energy and these identified varieties can then be adopted in intercropping systems. Furthermore, the research will help understand the phenolic acids produced by cowpea and their antioxidant capacities and to relate these to survival strategies of *V. unguiculata* under nutrient stress conditions. In addition, identifying the soil microbes which promote nutrient cycling and enhance N_2-fixation are vital as these symbionts can be isolated and cultured for future re-inoculation experiments in other legumes.

Overall, findings from the research will be useful in developing sustainable agricultural practices to improve food security and for managing N flows in farming systems in KwaZulu-Natal areas and South Africa as a whole. Findings of this study will have relevance to N cycling and amendments in savannas and grassland ecosystems beyond South Africa. Furthermore, the research is in alignment with the United Nations sustainable development goal number 2 which aims to achieve food security, enhance nutrition and encourage sustainable agriculture (https://sustainabledevelopment.un.org/sdg2).

1.4 Aims and objectives

The main goal of the research is to understand the significance of *V. unguiculata* tripartite symbiosis with respect to growth kinetics, nutrient acquisition, and legume N preferences. In addition, the phenolic acids produced by *V. unguiculata* and the antioxidant regulation strategies adopted by cowpea under nutrient stress are evaluated.

The objectives of the study are:

a) To explore the soil microbe diversity and soil enzymes of soils from four regions of KwaZulu-Natal (KZN).

b) To evaluate the effect of *V. unguiculata*-microbe symbiosis on the growth dynamics and N preference (soil N or atmospheric N_2) under nutrient stressed soils of KZN.

c) To identify and quantify the phenolic acids synthesized by *V. unguiculata* when grown in nutrient-stressed soils of KZN.

1.5 Hypothesis

Symbiotic association between *V. unguiculata* and rhizospheric microbes affects the growth, nutrient assimilation and phytochemicals of the grain legume grown in nutrient stressed soils.

1.6 Research questions

1. How does soil microbial composition and soil enzymes relate to the soil nutrient status of KwaZulu-Natal soils
2. How does *V. unguiculata*-microbe symbiosis affect nutrient acquisition, biological N_2 fixation and growth of the legume grown under different nutrient stress conditions?
3. Does differences in soil properties affect N source preferences of *V. unguiculata* varieties grown under nutrient stressed conditions and what are the cost implications on the legume?
4. What are the phenolic acids produced by *V. unguiculata* because of its interaction with soil microbes and how do these contribute to adaptability strategies?

1.7 General overview of chapters in the thesis

The thesis consists of six chapters which are briefly described below:
Chapter 1: outlined the background of challenges of nutrient stress in agriculture and the significance of legumes in enhancing soil nutrition. Furthermore, the adaptation mechanisms exhibited by legumes to ensure efficient acquisition and utilization of soil nutrients under nutrient stress conditions were highlighted. The rationale, hypotheses, aim and objectives of the study were also highlighted.
Chapter 2: focused on literature review which elaborated on how nutrient stress affects growth of crops also highlighting the role of legumes in improving soil fertility. A detailed explanation was given on the effects of P stress on biological nitrogen fixation. Furthermore, an overview of the different mechanisms used by legumes to improve P acquisition were discussed. Nitrogen metabolism strategies adopted in legumes under P deficiency are also discussed.
Chapter 3: focused on identifying and quantifying the different bacteria and fungi microbes found in soils of four selected regions of KZN ecosystems. Furthermore, the extracellular soil enzyme activities were explored. An analysis of the effects of the bacteria, fungi and extracellular enzyme activities on soil nutrient status of KwaZulu-Natal soils was evaluated.

Chapter 4: focused on evaluating the effects of soil microbe interaction with four *V. unguiculata* varieties on the growth, N acquisition and plant nutrition of the legume. The N source preferences adopted by the four *V. unguiculata* varieties were also explored.

Chapter 5: provided a detailed overview of the different phenolic acids produced in the roots and nodules of four *V. unguiculata* varieties under nutrient stress conditions, relating the effects of these phenolic acids to N and P acquisition and biomass accumulation.

Chapter 6: provided a general conclusion and recommendations based on the research findings.

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Chapter 2: Literature Review

2.1 Introduction

It is projected that in 2030, there will be 60% greater demand for agricultural products than in the current time, and of this increased demand, 85% is projected to come from developing countries (Bruinsma 2009, Alexandratos and Bruinsma 2012, Abd-Alla et al. 2014). However, most of the soils under crop production, especially the Sub-Saharan Africa, are nutrient-deficient and acidic which leads to inadequate soil nutrients to ensure optimal crop production (Abd-Alla et al. 2014). As a result of human population increase globally, most savannas and grasslands are being rapidly degraded via practices such as urbanization overgrazing, uncontrolled burning and clearing of grasslands for cultivation (Conant et al. 2001, Vance et al. 2003, Jewell et al. 2007, Sanderson et al. 2013).

Recently, most agricultural systems have extensively depended on synthetic fertilizers in order to provide crops with the essential nutrients most importantly N and P (Valentine et al. 2017, Oruru et al. 2018). However, the high cost of chemical fertilizers remains a challenge to resource constrained small-scale farmers (Oruru et al. 2018). Furthermore, continued use of fertilizers remain a threat to the environment due to pollution associated with their manufacturing and leaching during utilization (Abd-Alla et al. 2014, Valentine et al. 2018). Symbiotic biological nitrogen fixation can be used as an important substitute which is environmentally friendly and can ameliorate the challenge of poor soil fertility in agricultural lands (Valentine et al. 2018, Ferguson et al. 2019). Legumes develop symbiotic association with rhizobia and as a result atmospheric N_2 will be fixed into forms which are assimilable by the legumes thereby enhancing soil mineral nutrition and promoting crop productivity (Gresshoff et al. 2015, Ferguson et al. 2019). Through the mutualistic relationship with *Rhizobium*, legumes fix approximately 40 to 60 million tons of atmospheric N_2 yearly (Boermaa and Curtisb 2004, Group et al. 2013).

2.2 Biological nitrogen fixation

Nitrogen is among the most important nutrients needed by plants for growth and it is the major part of nucleic acids and proteins (Biswas and Gresshoff 2014, Inomura et al. 2017). The nutrient is also present in alkaloids, cytochromes and chlorophyll (Biswas and Gresshoff 2014). Despite its important role in plant growth, N is available in small quantities which are not sufficient for plant growth (Parihar et al. 2019). The atmosphere contains approximately 79%

N but plants cannot use it (Biswas and Gresshoff 2014). The process of biological nitrogen fixation enhances N availability for plant uptake and this contributes nearly 65% of the N used in agriculture globally (Dupont et al. 2012). Biological nitrogen fixation is a process whereby atmospheric N_2 is converted to ammonia. For each N_2 molecule fixed, electron transfer occurs six times and as a result 12 ATPs are required to fix one molecule of N_2 (Biswas and Gresshoff 2014). The net biological reaction is as shown in the following equation:

$$\mathbf{N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ Pi}$$

Symbiotic N_2 fixation is a process comprised of three components viz; nodule formation, symbiotic tissue regulation and the process where atmospheric N_2 is converted into ammonia (Biswas and Gresshoff 2014, Mus et al. 2016). The N_2-fixing bacteria are housed in the nodules which are formed through *Rhizobia*-legume interactions. However, several factors affect the process of nodulation and these include degree of compatibility between host and symbiont, availability of bio-molecules including flavonoids and soil physicochemical conditions (Hayat et al. 2010b). The N_2-fixing bacteria convert N_2 in the atmosphere into ammonia and this is facilitated by nitrogenase enzyme complex and a biochemical machinery (Biswas and Gresshoff 2014, Mus et al. 2016).

In the process, about 16 adenosine triphosphates (ATP) are reduced to adenosine diphosphate in the presence of nitrogenase enzyme. The process of N_2 fixation utilizes about 20% of total ATP produced by the legume (Biswas and Gresshoff 2014). The bacteria are integrated into the infected nodule cells by structures called symbiosome (Biswas and Gresshoff 2014, Mus et al. 2016). The bacteroids and the syndrome space characterizes this symbiosome and is enclosed by the peri bacteroid membrane. During symbiotic N_2 fixation bacteroids are provided with energy by plant cells and the energy will be in the form of malate. Furthermore, the bacteroids are provided with nutrients for both their growth and metabolism (Mus et al. 2016). In this mutualistic relationship, the fixed N_2 is excreted by the bacteroids into the cytoplasm of the surrounding plant cells and it is converted into organic structures (Valentine et al. 2018).

2.3 Types of nodules and nodulation

Plants in Leguminosae family have different nodules whose forms and shapes vary (Sprent 2008). There are four types of nodules which are anatomically different and these include: aeschynomenoid nodules (for example those of *Arachis hypogaea* L., peanut); lupinoid nodules (*Lupinus* sp. L., lupine); nodules with indeterminate meristems (examples are those of the

Trifolieae and Fabeae tribes); and desmodioid nodules and these have determinate meristems (examples are those from Phaseoleae and Loteae tribes) (Sprent 2008, Guinel 2009).

The aeschynomenoid nodule type consists of similar infected tissue however, the lupinoid type is characterized by lateral meristems (Sprent 2008). Nodules can either be determinate or indeterminate depending on their mode of development (Maunoury et al. 2008) and this includes the time taken to termination of the meristem as well as the depth and site of initial cortical cell division. Determinate nodules are globular in shape and they have no permanent meristem (Dupont et al. 2012). Legumes with such nodules include soybean (*Glycine max*), common beans (*Phaseolus vulgaris*) (Dupont et al. 2012). The initiation of determinate nodules occurs sub-epidermically in the external cortex (Dupont et al. 2012). The mature determinate nodules have similar central tissue which consists of infected cells that have bacteroids and uninfected cells (Dupont et al. 2012). The meristematic activities of nodules are completed within a short time resulting in the formation of spherical nodules (Dupont et al., 2012). In the determinate nodules, senescence occurs from the center to the periphery in a radial manner (Dupont et al. 2012). On the other hand, for indeterminate nodules, the cell division firstly occurs in the innermost cortex in an anti-clinal pattern, subsequently, there are periclinal divisions and these result in formation of cylindrical nodules (Rolfe and Gresshoff 1988). *Trifolium* (clover) and *Pisum sativum* (pea) are examples of crops which have indeterminate nodules. Mature indeterminate nodules have many clearly defined zones which depict their successive developmental stages (Dupont et al. 2012) (Figure 2.1). Zone I is the apical meristem and this does not have any bacteria. Zone II is known as the infection zone and here infection threads infiltrate the cells of the plant and as a result the rhizobia is released (Dupont et al. 2012). Zone III is characterized by bacteroids which are able to fix N_2. Zone IV is known as the root proximal senescence zone. This zone is found in older nodules, where there is degradation of bacteroids, as well as the plant cells (Dupont et al. 2012). The zone extends to the apical part where the nodules degenerate as they start aging (Dupont et al. 2012). Zone V, located close to Zone IV, comprises undifferentiated bacteria, and these proliferate in the decaying plant tissue (Timmers et al. 2000).

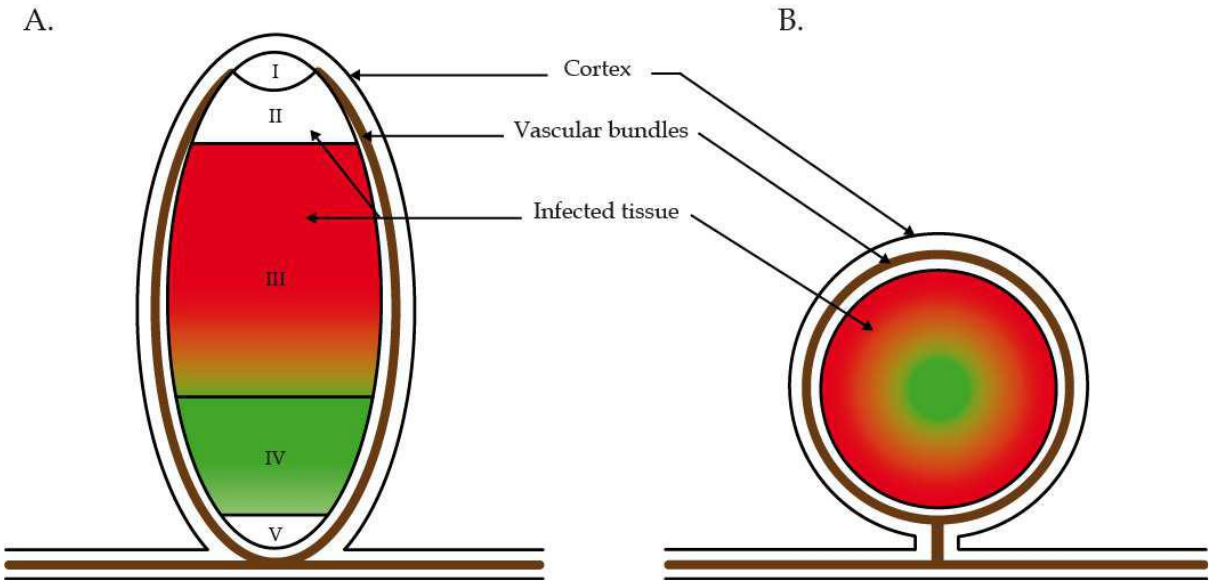

Figure 2.1 Illustration of the indeterminate (A) and determinate mature nodules (B), I = meristemic zone; II = infection zone; III = N_2 fixing zone; IV = senescence zone; V = saprophytic zone. Adapted from Dupont et al. (2012)

The signaling molecule, *Nod* factor plays a pivotal role in nodulation and this is produced by the rhizobia (Dupont et al. 2012). The basic chemical structure of *Nod* factors is similar for all rhizobia species however the different species factors may have varied side groups which are bound to oligosaccharide backbone along with the length and degree of saturation of the lipid moiety (Dupont et al. 2012).

The compatible bacteria in soils can detect flavonoids which are released by plants and this results in the manufacturing of *Nod* factors (NF). As a result, several genes are produced, including the genes involved in early nodulin expression, cation channels, nucleoporin, calcium spiking and cytokinin signaling which lead to pericycle and cortical cell divisions, and bacterial infection (Biswas and Gresshoff 2014).

In the symbiotic interaction, firstly, there is infection process, and this is initiated by signal exchange between the bacteria and plants (Stougaard 2000, Biswas and Gresshoff 2014, Mus et al. 2016). Plants secrete aromatic compounds-mostly flavonoids, into the rhizosphere. This results in the activation of the bacterial *NodD* proteins which then induce expression of the *nod* genes (Long 2001). The signals released activate rhizobia nodulation genes and these release other signals from the bacteria, such as lipochito-oligosaccharide Nodulation or *Nod* Factors (NF). Nodulation is in turn triggered as a result (Dénarié et al. 1996, Stougaard 2000). Thereafter, the *Nod* factor cause root hair curling (Biswas and Gresshoff 2014). In the process, infection thread is formed as the rhizobia are entrapped. Thereafter, rhizobia move through

these infection threads and are released into the nodule primordium (Biswas and Gresshoff 2014). The symbiosome membranes surround the bacteria when the nodule primordium grows into mature nodules (Biswas and Gresshoff 2014). At this stage, the nodules experience lower oxygen concentration, as a result they differentiate into bacteroids, and thus fix atmospheric N_2 with the aid of nitrogenase enzyme complex (Szczyglowski et al. 1998).

2.4 Role of non-rhizobial bacteria in biological nitrogen fixation

Several plant growth promoting bacteria which are non-rhizobial in nature have been identified and isolated from N_2 fixing nodules of legumes and these have been found to contribute directly to biological N_2 fixation (Xu et al. 2014, Martínez-Hidalgo and Hirsch 2017). These isolates have been found to contain the *nif* and *nod* genes which are important for nodulation and they promote N_2 fixation in the same way that α and β rhizobia work (Martínez-Hidalgo and Hirsch 2017, Kawaka et al. 2018), thereby demonstrating that some non-rhizobial strains have the capacity to fix N_2 (Kawaka et al. 2018). N_2 fixation occur in several bacterial strains including *Azotobacter*, *Bacillus*, *Enterobacter* and *Paenibacillus*. N_2 fixing *Bacillus*, which have been identified include *Bacillus cereus*, *B. megaterium*, *B. subtilis* and *B. pulilus* (Martínez-Hidalgo and Hirsch 2017). Several studies have demonstrated that co-inoculation of *Rhizobium* strains with other bacteria which enhance plant growth such as *Bacillus* help increase nodulation, biological nitrogen fixation and promote the functioning of nitrogenase enzyme in most legumes (Govindasamy et al. 2010, Subramanian et al. 2015, Martínez-Hidalgo and Hirsch 2017).

2.5 Carbon costs associated with biological nitrogen fixation

Biological nitrogen fixation is a very crucial process which occurs in legumes as it ensures bioavailability of N for plant uptake, however the plant must contribute a large quantity of energy. The energy will be in the form of photosynthates and some nutritional factors which are required by the arbuscular mycorrhizal fungi and bacteria (Magadlela et al. 2016, Valentine et al. 2018). The process requires about 160 $Kcal.mol^{-1}$ for a molecule of N_2 to be reduced (Postgate 1998, Minchin and Witty 2005). According to Minchin and Witty (2005), the C costs of N_2 fixation range between 3.3 and 6.6 g $Cg^{-1}N$. However, this is dependent on the combination of legume and *Rhizobium*. In this regard, a large amount of photosynthates is required which affects the costs of legume productivity (Minchin and Witty 2005, Valentine et al. 2017). On the other hand, assimilation of mineral N such as NO_3^- is regarded as less

expensive (<2.5 $gCg^{-1}N$) relative to acquisition of atmospheric N_2. As a result, legumes tend to utilize soil N when it is available instead of fixing atmospheric N_2 in order to save energy (Magadlela et al. 2016, Valentine et al. 2017).

2.6 Effects of soil acidity on biological nitrogen fixation

Most of the world's soils are known to be acidic and about 40% of the arable lands of agricultural regions are considered acidic (Ferguson et al. 2013, Ferreira et al. 2016, Valentine et al. 2018). Under acidic conditions, there is retention of essential nutrients especially P and marked increase in such anions such as Mn^{2+}, Al^{3+}, Fe^{3+} and these result in poor crop productivity (Ferguson and Gresshoff 2015, Kopittke et al. 2015). Furthermore, in acidic conditions (pH<5.5), Al^{3+} is predominant and this may inhibit cation uptake thereby resulting in impaired root and overall plant development (Kopittke et al. 2015). Most importantly, soil acidity results in a decrease in symbiotic N_2 fixation as nodulation may be impaired and this affects the legume growth (Zahran 1999, Ferreira et al. 2016). Furthermore, soil acidity may significantly limit *Rhizobium* survival and persistence in the soils as a result their symbiotic association with legumes will be disturbed (Sulieman and Tran 2016, Jaiswal et al. 2018). High levels of Al^{3+}, Fe^{3+} and Mn^{2+} associated with acidic soils may disrupt rhizobia function thereby weakening their competitiveness in the soils (Wood et al. 1984). The expression of nodulation genes such as *nodA* may be reduced under acidic conditions (Richardson et al. 1988). This results in a reduction of biosynthesis of Nod factor signal; an important component which is involved in the exchange of signals and in facilitating recognition of the symbiotic partners (Ferguson and Gresshoff 2015). Under acidic conditions, there is a reduction in the level of events which are induced by Nod factors, and those which occur during nodulation including curling and early *Rhizobium* infection events including the attachment of root hairs (Miransari et al. 2006). The nodulation stages which include cell division, nodule primordia establishment, cell division and nodule growth are also affected by excessively low soil pH (Vassileva et al. 1997). Nevertheless, the effects of soil acidity on rhizobia may vary depending on the strain and this ultimately affects the biological N fixation efficiency of the strains (Zahran 1999, Ferguson and Gresshoff 2015). For example, the fast-growing rhizobia strains are generally known to have a lower capacity to withstand acidic conditions than slow growing rhizobia strains such as some *Bradyrhizobium* (Graham et al. 1994, Zahran 1999, Ferguson and Gresshoff 2015). Under acidic conditions, P may complex with cations most importantly (Al^{3+}) and it will be bound to organic compounds thus making it unavailable to plant growth and this

unavailability has more pronounced negative effects on N_2 fixing legumes compared to other plants (Sulieman and Tran 2016).

2.7 Effects of phosphorus deficiency on biological nitrogen fixation

P deficiency is one of the most important environmental limitations that hinder symbiotic nitrogen fixation (Høgh-Jensen et al. 2002, Vardien et al. 2016, Valentine et al. 2017). Several complex mechanisms may interact at various levels of nodule functional and structural organization under low P environments and these activities may limit symbiotic N_2 fixation (Magadlela et al. 2016, Vardien et al. 2016). The processes of dinitrogen fixation and ammonium assimilation into amino acids and ureides, are energy consuming and they are dependent on the nodule energy status therefore P deficiency limits these processes (Araújo et al. 2008, Magadlela et al. 2016). Under low P, there is inhibition of nodule growth which results in reduction in symbiotic nitrogen fixation (Magadlela et al. 2014, Vardien et al. 2016). As demonstrated by Magadlela et al. (2014), low P concentrations resulted in 70% decline in nodule dry weight of *Virgilia oroboides*. A decrease in P concentration in *V. oroboides* resulted in a reduction in % N derived from atmosphere (%NDFA) which is an indicative of decrease in biological nitrogen fixation rate (Magadlela et al. 2014).

At critically low soil P levels, most plants including legumes allocate more of their resources towards increasing belowground biomass and there will be higher C costs in legume plants (Magadlela et al. 2014, Valentine et al. 2018). Effects of P stress on legumes may differ depending on the duration of exposure to low P conditions (Høgh-Jensen et al. 2002). The authors demonstrated that abrupt removal of P in white clover resulted in marked reduction in the growth of nodules and this caused a decrease in the activity of nitrogenase enzyme per unit root weight. In the same study, the ratio between carbon dioxide fixation and N_2 fixation was maintained on treatments deprived of P for short times, however the ratios were increased on treatments subjected to low P for prolonged time.

2.8 Adaptability mechanisms of legumes to phosphorus deficiency

Leguminous plants have adopted several strategies to cope with P stress conditions and these mechanisms help ensure survival of the legume-microbe symbiosis (Vance et al. 2003). Legume plants use several morphological, physiological and biochemical strategies which enhance the allocation and use of larger proportions of P in nodulating root systems (Sulieman and Tran 2015). Adaptations of legumes to low P conditions may be explained through two

broad mechanisms; i) Conserving P use ii) improved acquisition of P and symbiotic association with soil microbes (Vance et al. 2003). P use may be conserved through processes including increased P use efficiency, reduced growth rate, internal Pi remobilization, alteration in C metabolism that would avoid P requiring steps as well as alternate respiration pathways (Vance et al. 2003). Enhanced uptake of P is facilitated by processes such as increased production of phosphatases, improved root growth and modifications in root architecture, increase in root surface due to the development of root hairs and increased Pi transporter expression (Vance et al. 2003).

2.8.1 Morphological modifications in plant development

Phosphorus is a very important macronutrient for legume-rhizobia symbiosis and as a result different nodulated plants have evolved many responsive and adaptive strategies. Such mechanisms help plants to effectively use and conserve the available P as a result the plants can have high symbiotic activity rates despite the low P levels (Sulieman and Tran 2015). The adaptive strategies constitute some internal reactions which are coordinated at biochemical, molecular and physiological stages (Sulieman and Tran 2015, Valentine et al. 2018). The morphological modifications caused by P stress in plants include reduced shoot mass, reduced specific leaf area and phenotypic plasticity alteration (Vardien et al. 2016). Furthermore, legumes respond to limited P availability through increased P partitioning to root tissue, increased C acquisition through reduced leaf area and increased N uptake by nodule mass maintenance (Sulieman and Tran 2015).

2.8.2 Preferential phosphorus allocation to nodules

Legume-*Rhizobium* symbiosis requires more P than non-nodulating plants and as a result nodulated plants preferentially allocate more P to nodules. Nodules may utilize about 20% of the total plant P and this promotes the plant symbiotic function (Kouas et al. 2005, Valentine et al. 2017). High proportions of P are allocated to the nodules and this helps maintain N_2 fixation under P deficient conditions, however this can be at the expense of total plant growth and other plant organs (Sulieman and Tran 2015) (Figure 2.2). Some studies have shown that the bacteroid fraction of nodule cellular compartments may adapt to P stress by accruing higher levels of P required for nitrogenase activity (Valentine et al. 2018). For an effective symbiotic interaction between host plant and the rhizobia, there is need for an efficient allocation of P and efficient use of available P in nodules in P deficient conditions (Valentine et al. 2018). Consequently, the efficiency of the symbiosis and extent of legume adaptability to P stress, may be determined by the P allocation by the legumes at low P conditions. This is evidenced

by research done by Chaudhary et al. (2008), who demonstrated that soybean was more effective in redirecting greater proportions of P for nitrogenase activity than mung bean thus making soybean better adaptable to P stress.

2.8.3 Induction of strong phosphorus sinks in nodules

Phosphorus concentration in P-stressed plants can be three times higher in nodules than other plant organs and this is evidenced in studies done on white lupin (Schulze et al. 2006). These results are in line with the proposition that nodules represent strong preferential sinks for P incorporation during P stress conditions compared to other plant organs (Sulieman and Tran 2015) (Figure 2.2). Several legume species including *Lupinus luteus* and *Virgilia divaricata* have the capacity to regulate their P reserves to other plant parts under low P environments (Vardien et al. 2016).

2.8.4 Phosphorus acquisition strategies

Most legumes have special mechanisms of ensuring enhanced P extraction and acquisition from soil through the formation of mycorrhizas and cluster roots (Sulieman and Tran 2015). Cluster roots result in enhanced surface area of roots and they promote organic acid exudation, and hence improve symbiotic N_2 fixation. Some legumes have developed special root structures, which enable them to forage for P in the topsoil. Other legumes have developed horizontal root growth and these result in shallow roots, adventitious and lateral roots and enhanced root hair density (Vance et al. 2003). Some legumes have strong associations with mycorrhiza and these help in transporting P from inaccessible areas to the plant (Sulieman and Tran 2015). Legume-mycorrhizal symbiosis results in enhanced P use efficiency and improved total plant N content (Tajini and Drevon 2012).

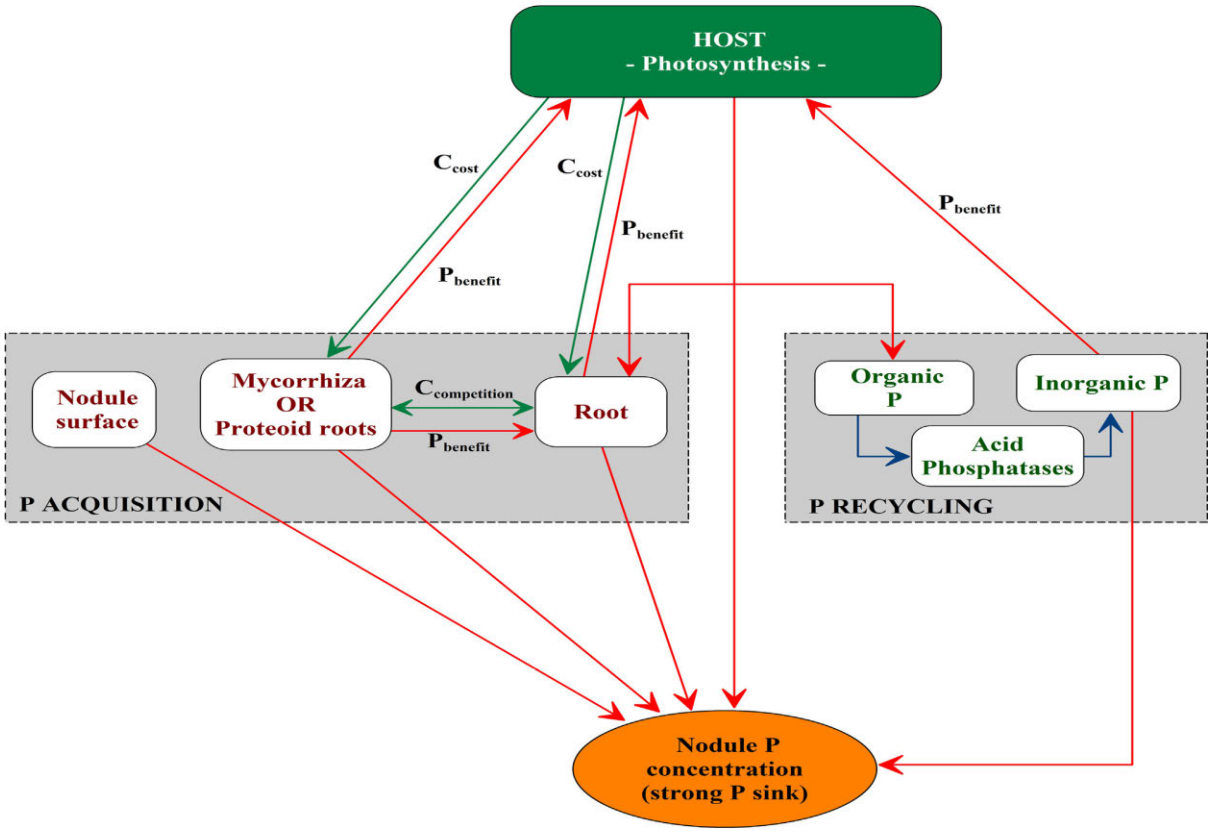

Figure 2.2 Phosphorus (P) resources and the different strategies which lead to P homeostasis maintenance in the nodules of legumes grown in P-stressed environment. Green and red bars indicate the contribution to carbon (C) and P budgets respectively (Sulieman and Tran 2015)

2.8.5 Enzymatic regulation in plants

Nitrogen metabolic pathway in plants is greatly altered by inadequate P (Sulieman and Tran 2015). Roots and nodules exude acid phosphatases and organic acids and these play an important role as they promote solubility of complex P forms making P more available for uptake by plants (Sulieman and Tran 2015). During P stress, there is upregulation of phosphoenolpyruvate (PEP) carboxylase and malate dehydrogenase and these enzymes play an important role in nodule C metabolism (Sulieman and Tran 2015). Investigation of lupine species (*L. albus*) demonstrated that the citrate exuded by cluster roots was equivalent to about 23% of the total dry weight of the plants (Shane et al. 2008). Organic acid exudation improved aluminium toxicity tolerance and enhanced P uptake of *Medicago sativa* plants (Tesfaye et al. 2001). In addition, some legumes exude root malate and this results in improved adaptability of legumes to both P deficiency and aluminium toxicity (Sulieman and Tran 2015).

2.8.6 Internal recycling of phosphorus

Plants are able to mobilize P within them (internal recycling of P) and this is regarded as one of the most important biochemical and physiological responses to P stress (Vardien et al. 2014,

Sulieman and Tran 2016). Phosphates may be translocated from the aerial parts of the plants and this helps support symbiotic N_2 fixation under P stress (Sulieman and Tran 2015).

Organic phosphates play a pivotal role in promoting symbiotic efficiency (Vardien et al. 2014, Sulieman and Tran 2015). Among the organic phosphates, acid phosphatase improves P nutrition in legumes under P stress environments. The intracellular acid phosphatases (EC 3.1.3.2; orthophosphoric monoester phosphohydrolase) play a unique role of increasing P recycling by hydrolyzing and mobilizing inorganic orthophosphate (Pi) from different organic P substances and they also enhance P utilization by internal mechanisms (Høgh-Jensen et al. 2002, Bargaz et al. 2012, Vardien et al. 2014). Furthermore, the enzymes help regulate internal P homeostasis by scavenging P from intracellular phosphorylated compounds (Vardien et al. 2014).

The importance of P recycling as an adaptation mechanism in legumes was clearly demonstrated in the studies by Vardien et al. (2014), whereby under P stress conditions, *V. divaricata* plants had high N_2 fixing efficiency although there were lower levels of Pi in the nodules. This was due to the greater APase activity in the nodules and roots. Other researchers have reported secretion of high amounts of APases for example, white lupin roots and proteoid roots produce large quantities of APases when exposed to Pi stress conditions (Miller et al. 2001). Vardien et al. (2014) confirmed that P deficient roots and nodules showed more pronounced acid phosphatase activity compared to treatments subjected to optimal P. This shows that roots have the capacity to scavenge for P and later channel it to nodules where it is conserved. Further studies on *V. divaricata* confirmed that during low P supply, the legume had a special ability of internally recycling P and this was facilitated by increased nodule extracellular APase activity (Vardien et al. 2016).

2.8.7 Root exudates

As an adaptation to nutrient deficiency in soils, some plants exude chemical substances through their roots which help solubilize otherwise complexed P (Maseko and Dakora 2013). Plant root exudates include simple polysaccharides, fatty acids, organic acids, amino acids, inorganic ions, phenolic compounds and enzymes (Dakora and Phillips 2002, Shane and Lambers 2005, Maseko and Dakora 2013). Some plant roots produce high molecular weight components which include root boarder cells and proteins. Production of root exudates increase with increase in P deficiency (Dakora and Phillips 2002, Balemi and Negisho 2012, Maseko and Dakora 2013). Citric acid, oxalic acid and malic acid are some of the most important organic acid exudates which play a pivotal role in solubilizing inaccessible soil P (Maseko and Dakora

2013). Organic acids have the capacity to form complexes with metal cations that bind phosphate (e.g., Ca, Al, Fe and other trace metals). As a result they displace phosphate from the surrounding soil (Raghothama and Karthikeyan 2005, Hinsinger et al. 2006).

Plant roots may exude phenolics and these solubilize Fe, P and other essential nutrients which may not be available to plants, to forms which are available to plants. The phenolic compounds may enhance Fe and P availability by complexing and chelating with Al and Fe present in insoluble Al and Fe phosphates (Dakora and Phillips 2002). These complexions result in increase in Fe and P solubility and availability for plant uptake (Dakora and Phillips 2002) (Figure 2.3). In addition, plant roots may produce acid phosphatase and phytase enzymes and these can hydrolyze organic P forms thus making the P available for plant uptake under P stress conditions (Maseko and Dakora 2013).

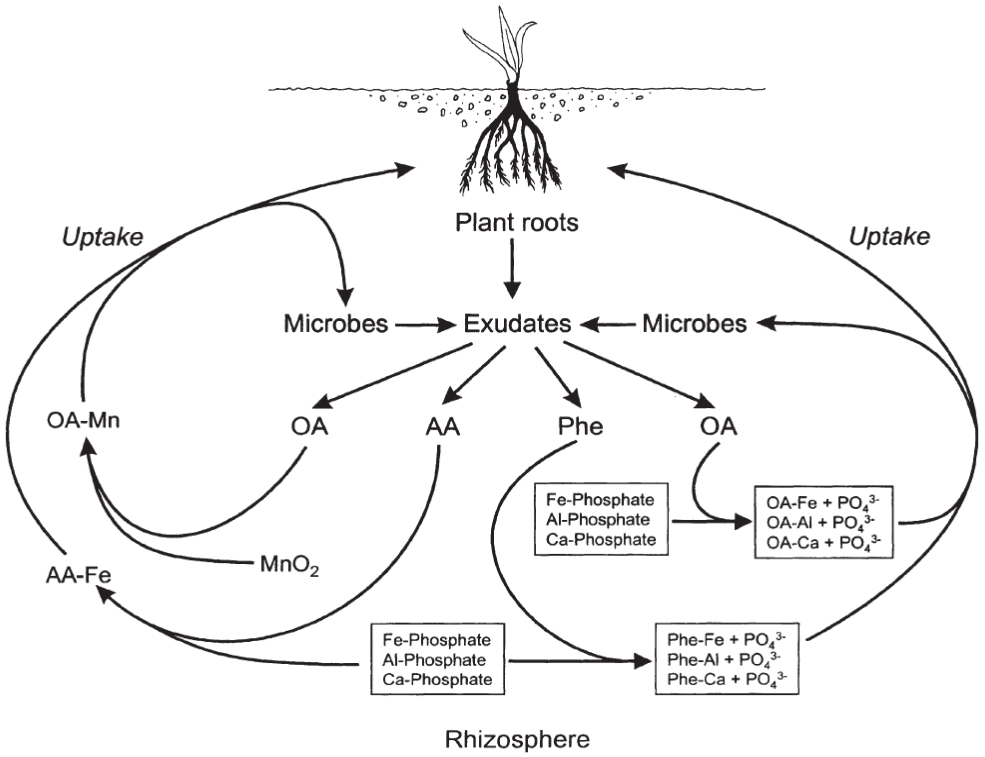

Figure 2.3 Effects of root exudates on nutrient availability and uptake by plants and rhizosphere microbes. OA = organic acids; AA = amino acids including siderophores, Phe = phenolic compounds (Dakora and Phillips 2002).

2.8.8 Role of phenolic acids in plant growth

Phenolic acids are the major polyphenols found in most plants especially in the roots and they play important roles in enhancing plant nutrient uptake in nutrient-stressed environments (Mandal et al. 2010). Enhanced nutrient uptake is made possible through different mechanisms

which include improved root development (Hayat et al. 2010a), enhanced nodule development and establishment of plant-microbe symbiosis (Mandal et al. 2010). Phenolic acids also facilitate mineral nutrient uptake as they are used as alternative carbon sources by microsymbionts (Seneviratne and Jayasinghearachchi 2003) and they are used as antioxidants (El-Awadi 2018, Zafar-ul-Hye et al. 2020) under nutrient stress conditions. Phenolic acids may also alter the proliferation and distribution of rhizospheric microbes thereby affecting soil nutrient cycling (Wang et al. 2018).

Some phenolic acids contribute to enhanced nutrient uptake as they promote root development and this increases surface area for nutrient absorption from deep within the soil profile. Some phenolic acids (e.g., caffeic, ferulic, gallic acid and salicylic acids) enhance root and overall plant growth because of their auxin like properties (Hayat et al. 2010a, De Klerk et al. 2011, El-Awadi 2018). Particularly, gallic acid enhanced rooting and inhibited decarboxylation of IAA in Malus "Jork9" (De Klerk et al. 2011). Cinnamic acid increased activity of IAA oxidase in the roots of *Glycine max* (Salvador et al., 2013). Ferulic acid increase rooting and caused inhibition of decarboxylation of IAA in Malus "Jork 9" (De Klerk et al. 2011). Ferulic acid increased activity of IAA oxidase in the seedlings of *Zea mays* (Devi and Prasad 1996).

Some phenolic acids produced by symbiotic legumes promote plant survival under nutrient stress conditions by enhancing plant-microbe symbiosis. The phenolics act as signaling molecules in establishing legume-rhizobia or arbuscular mycorrhizal symbioses (Mandal et al. 2010). Caffeic acid promotes plant-arbuscular mycorrhizal symbiotic interactions (Zafar-ul-Hye et al. 2020) and legume-*Rhizobium* interaction (Klein et al. 2015). Early stages of arbuscular mycorrhizal establishment in clover and sorghum were shown to be promoted by p-coumaric acid and p-hydroxybenzoic acid (Mandal et al. 2010). The effects of phenolic acids in enhancing legume-microbe symbiosis, however, varies with concentrations, structure of phenolic acids and the microsymbiont strains (Mandal et al. 2010).

Phenolic compounds help enhance nodule development (Mandal et al. 2009, Mandal et al. 2016). More so, phenolic acids help in regulating *nod* gene expression of the *Rhizobium* and they modify the legume-*rhizobium* symbiosis (Seneviratne and Jayasinghearachchi 2003). *Nod* gene expression may be inhibited or stimulated by phenolic acids. However, this depends on the phenolic acid chemical structure, concentration, and it may be strain specific (Seneviratne and Jayasinghearachchi 2003). Protocatechuic and p-hydroxybenzoic acids were shown to be effective in inducing *nod* gene in root nodules on *Mimosa pudica* (Mandal et al. 2016).

Phenolic acids may have inhibitory or stimulatory effects on microbial growth and proliferation (Kuiters 1990, Zhou et al. 2014, Wang et al. 2018) and this affects soil health and nutrient

cycling. Phenolic acids enhance communication between plants and soil microbes. Most soil microbial populations are stimulated by low concentrations of phenolic acids where the phenolic acids are utilized as carbon sources (Kuiters 1990). In a study by Kuiters (1990), ferulic acid stimulated growth of fungi including *Dothichiza pityophila* and *Thysanophora penicilloides*. Syringic acid resulted in a reduction in richness, evenness and diversity indices of rhizosphere bacterial community (Zhou et al. 2014). This shows that salicylic acid plays a pivotal role in communication between plants and soil microorganisms. Syringic acid applied at 0.1 µmol/g resulted in an increase in abundance of bacterial phylum Proteobacteria and fungal classes *Leotiomycetes*, *Pezizomycetes*, *Tremellomycetes* and *Eurotiomycetes*. However, the syringic acid decreased the relative abundance of bacterial of phylum *Firinicutes* and fungal class *Sordariomycetes* (Wang et al. 2018). In another study, p-coumaric acid promoted *F. oxysporum* f. sp. Cucumerinum owen (FOC) (Zhou and Wu 2012). Ferulic acid inhibited the growth of *P. fluorescens* and *Glomus intraradices* (Medina et al. 2011, Lemos et al. 2014). Ferulic acid amendments stimulated soil dehydrogenase activity, lowered bacterial community while it caused an increase in fungal community sizes (Zhou and Wu 2012). In cucumber plants, p-HBA resulted in a decrease in Shannon-Wiener index for soil bacterial community and it caused an increase in rhizosphere fungal community (Zhou and Wu 2012). Addition of low concentrations of vanillic acid in soils stimulated soil microbial biomass while high concentrations resulted in marked reduction in the microbial biomass.

Phenolic acids also play an important role as they can be used as alternative carbon sources by the microsymbiont *Rhizobium* (Irisarri et al. 1996). Ferulic acid, p-coumaric acid, protocatechuic acid and vanillic acid have been effectively used as carbon sources by different rhizobial strains including *Rhizobium*, *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum* and *Azorhizobium caulinodans* (Irisarri et al. 1996, Seneviratne and Jayasinghearachchi 2003). Use of phenolic acids by the microsymbionts results in alterations in the microbes' biochemical and physiological properties and this in turn affects their persistence. Biological nitrogen fixation efficiency of the symbiotic legume plants is improved as the overall cost of nitrogen fixation is reduced (Seneviratne and Jayasinghearachchi 2003).

When subjected to abiotic stress, plants produce reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and these create oxidative stress (Hayat et al. 2010a, Marchiosi et al. 2020, Zafar-ul-Hye et al. 2020). These ROS may be detrimental to plants as they cause oxidative damage to biomolecules, nucleic acids and antioxidant systems in plants. To curb the challenge of oxidative damage, plants exude phenolic acids which enhance antioxidant enzymes activities and these help scavenge the reactive oxygen species

(Hayat et al. 2010b, Khan et al. 2010a, El-Awadi 2018, Riaz et al. 2019). Phenolic acids including caffeic acid, ferulic acid, gallic acid, p-coumaric acid and vanillic acid are known to exert strong antioxidant properties (Kiokias et al. 2020). Other phenolic acids including protocatechuic acid (Marchiosi et al. 2020), salicylic acid (Gunes et al. 2005) and *p*-hydroxybenzoic acid (Ahrabi et al. 2011) also have strong antioxidant properties. Given the different roles played by phenolic acids in promoting plant nutrient acquisition discussed above, it is important to understand the different phenolic acids produced by different *Vigna unguiculata* varieties grown in nutrient poor grassland and savanna ecosystems.

2.9 Significance of legume microbe symbiotic interaction in nutrient acquisition

2.9.1 Role of arbuscular mycorrhizal fungi in legume nutrient acquisition

Fungi develop mutualistic associations with plants whereby they penetrate plant roots and supply the plants with important nutrients including P and several other immobile nutrients, and in return the fungi get fixed C (Scheublin et al. 2004). About 80-90% of higher plants are infected by mycorrhizal fungi (Smith et al. 2010, Maseko and Dakora 2013). There are six types of mycorrhizal symbioses and these include arbutoid, ecto, monotropoid, ericoid, arbuscular and orchid mycorrhiza and of these the most are arbuscular and ectomycorrhizae (Smith and Read 2008).

Of particular interest are the arbuscular mycorrhizal fungi (AMF), which form mutual interaction with host plants and as a result the fungi proliferate their hyphae in order to provide an extended surface area for nutrient uptake (Smith and Smith 2012, Maseko and Dakora 2013). Nutrient acquisition as facilitated by AMF can be via two different pathways which are; direct pathway and mycorrhizal pathway (Smith et al. 2011). The direct pathway is found at the root-soil interface and here plants take up nutrients such as N and P directly from the rhizosphere (Smith et al., 2011). On the other hand, the mycorrhizal pathway promotes the scavenging of P from large quantities of soil and this P is then delivered to cortical cells which are located in the roots thus by-passing direct uptake (Smith et al. 2011). Mycorrhizal pathway is characterized by Pi absorption through extra radical mycelium (ERM) from the soil and this is facilitated by fungal phosphate importers (Hodge et al. 2010). Legumes may preferentially associate with specific AMF which are efficient in supplying P which is then used for nodulation and N_2 fixation (Scheublin et al. 2004). It has been confirmed that AMF contributes

large amounts of P, which can go up to 90% of the total plant P requirement (Van Der Heijden et al. 2006).

AMF have adopted several mechanisms which enable them to acquire large amounts of P and other mineral nutrients. Some AMF may exude organic acids and these enhance solubilization of insoluble mineral P (Muleta 2017). Arbuscular mycorrhizal fungi also contribute to enhancement of biological nitrogen fixation (Muleta 2017) in legumes as it enhances availability of P and other nutrients that could otherwise be immobile, such as zinc and copper and are important in N_2 fixation (Clark and Zeto 2000). Good supply of P ensures efficient photosynthesis and increased C flow to the nodules and this ensures efficient N_2 fixation (Muleta 2017). Arbuscular mycorrhizal fungi also help in altering microbial composition in the rhizosphere and they produce enzymes which help break down soil organic matter thereby releasing important organic compounds for uptake by plants.

It has been demonstrated that co-inoculation of AMF with rhizobia helps enhance legume growth, nodulation and biological N_2 fixation. Studies done by Kayode and Franco (2002) showed that co-inoculation of AMF *Gigaspora margarita* and *Scutellospora heterogama*, with rhizobial strains BR3609 and BR3617 enhanced nodulation and growth of *Acacia mangium.*

2.9.2 Significance of plant growth promoting *Bacillus* and *Paenibacillus*

Bacillus and *Paenibacillus* are important ubiquitous plant growth promoting bacteria that contribute significantly to enhanced nutrient availability for plant use either directly or indirectly (Govindasamy et al. 2010). Some plant growth promoting *Bacillus* and *Paenibacillus* exude enzymes directly into the soils for example chitinase, hydrolytic enzymes and these promote the breaking down of soil organic matter and this causes improved cycling of essential mineral nutrients such as C, N and P (Veres et al. 2015).

P deficiency is one of the most common stresses in the tropical and subtropical regions as the mineral complexes with Fe and Al compounds and this negatively affects crop production (Khan et al. 2010a, Marra et al. 2012). Phosphorus availability is promoted by *Bacillus* and *Paenibacillus* in plants as these microbes help mineralize organic P and they facilitate solubilization of precipitated phosphates (Chen et al. 2006, Govindasamy et al. 2010). The bacteria also excrete organic acids such as keto glutonic acids and gluconic acids. These work by dissolving phosphatic minerals or by chelating cationic partners of P ions, that is PO_4^{3-} and this results in the release of P for plant acquisition (Govindasamy et al. 2010). P solubilizing bacillus do include *Bacillus megatarium, B. circulans, B. polymyxa, B. subtilis* (Govindasamy et al. 2010) while examples of *Paenibacillus* include *Paenibacillus polymyxa, P. mucilaginous,*

P. *macerans* and *P*. *elgii* (Grady et al. 2016). *Bacillus* and *Paenibacillus* contribute to improved plant growth as they produce phytohormones which include auxins, gibberellic acid, cytokinins and ethylene (Govindasamy et al. 2010, Grady et al. 2016).

2.10 Significance of the tripartite symbiosis

The tripartite symbiosis which involves legumes, *Rhizobium* and arbuscular mycorrhizal fungi is regarded as one of the most important interactions in ecology (Abd-Alla et al. 2000, Abd-Alla et al. 2014, Muleta 2017). Arbuscular mycorrhizal symbiosis contributes to making immobile nutrients especially P available to host plants while bacterial symbiosis provides N through N_2 fixation (Abd-Alla et al. 2014, Magadlela et al. 2014). Arbuscular mycorrhizal fungi are also important because they promote growth and development of plants and help promote plant tolerance to both biotic and abiotic stresses (Abd-Alla et al. 2014). Dual inoculation of *Rhizobium* (*R. leguminosarum* bv. *viciae* STDF- Egypt 19) in combination with AMF (*Acaulospora laevis*, *Glomus geosporum*, *Glomus mosseae* and *Scutellospora armeniaca*) resulted in significant increase in the activity of nitrogenase enzyme, leghemoglobin content of nodule, number and mass of nodules, mycorrhizal colonization as well as dry mass of shoots and roots in faba bean (*Vicia faba* L.) in alkaline soils (Abd-Alla et al. 2014). In as much as the use of AMF in soils help enhance growth and yields of legume crops both in the fields and in greenhouses, there is need for compatibility between the AMF and the *Rhizobium* strains for effective N nutrition (Xavier and Germida 2002, Muleta 2017). Xavier and Germida (2002) assessed the significance of co-inoculating lentil (*Lens culinaris* cv. Laird) with different AMF and *Rhizobium* strains which had different efficacy levels. The authors used two AMF strains namely *Glomus clarum* NT4 and *G*. *mosseae* NT6 in combination with nine *Rhizobium* strains. Results indicated that growth and yield responses of lentil was influenced by the AMF-*Rhizobium* combinations; with some incompatible AMF species reducing lentil productivity. In concurrence with this assertion are the findings by Azcón et al. (1991) who evaluated efficacy of interactions between *Glomus mosseae*, *G. caledonium* and *G*. *fasciculatum* and six *Rhizobium meliloti* strains. The authors confirmed that different combinations AMF may modify legume host responses to different *Rhizobium* strains.

2.11 Nitrogen metabolism in legume nodules during phosphorus deficiency

The ammonia/ ammonium produced through biological nitrogen fixation is transported from the bacteroids and it moves through diffusion into the cytosol of the cells of host plants and

consequently it is assimilated (Liu et al. 2018). The legume nodules then synthesize amino acids and/or ureides which are transported to the shoots via two different routes depending on whether the roots are determinate or indeterminate (Foyer et al. 2011, Liu et al. 2018). Determinate nodules are found mainly in tropical legumes which include cowpea, soybean and common bean while indeterminate nodules are mainly found in legumes which originate in temperate regions such as lupins, clovers and peas (Valentine et al. 2017). The indeterminate nodules take up amides in the form of glutamine (Gln) and asparagine (Asn) while the determinate nodule legumes are mostly involved in transporting allantoate (ureides) and allantoin as fixed N_2 compounds (Sprent 2009, Valentine et al. 2017). The ureide exporting and amide exporting legumes have a similar initial ammonium assimilation step (Todd et al. 2006, Foyer et al. 2011) (Figure 2.4). In this initial step, there is fixation of N_2 in the bacteroids. Thereafter, it is transferred into the cell cytosol of the legume plants, then ammonia is converted to Gln and glutamate (Glu) and the processes are catalyzed by enzymes Gln synthetase (GS; EC 6.3.1.2) and Glu synthetase (NADH-GOGAT; EC 1.4.1.14) (Liu et al. 2018). For the indeterminate nodules, Gln and Glu are converted to aspartate (Asp) and Asn in the presence of Asp aminotransferase (AAT; EC 2.6.1.1) and Asn synthetase (As; EC 6.3.5.4). On the other hand, for the determinate nodules, Gln goes to purine synthesis pathway where it is changed into ureides.

Several researchers have shown that the ureide biosynthesis is a more economical pathway with regards to C expenditure (Magadlela et al. 2016, Valentine et al. 2017). This is because when N is transported in the form of ureide it has lower C:N ratio compared to when the N is transported as asparagine or glutamine (Smith and Atkins 2002). Some legumes have adopted mechanisms of exporting more ureides compared to amino acids during P deficiency and this is of significance since during P deficiency there is limited supply of C, so this photosynthetically derived C will be conserved (Valentine et al. 2017). It is estimated that the energy costs of producing ureides in terms of ATP costs per mole of N assimilated is around 50% compared to that of producing amino acids, asparagine and glutamine (Schubert 1986). Studies by Todd et al. (2006) showed that 1.4 gCg^{-1} fixed N_2 is required for ureide exporting cowpea while about 3.9 gCg^{-1} fixed N_2 is required in the amide exporting lupin. This is evidence that it is less costly to synthesize and export ureides as organic N relative to the amide pathway (Valentine et al. 2017). It is important to understand how legumes metabolize their acquired N compounds as this may contribute to energy saving strategies and their ability to thrive under nutrient stress conditions. Studies by Olivera et al. (2004) showed that *Phaseolus vulgaris* nodules resulted in the reduction of enzyme activities which are involved in synthesis

of amino acid during P stress conditions. This aligns with the findings by Magadlela et al. (2016) and Magadlela et al. (2017) which showed that at low P, *V. divaricata* exported more ureides than amino acids and ammonium.

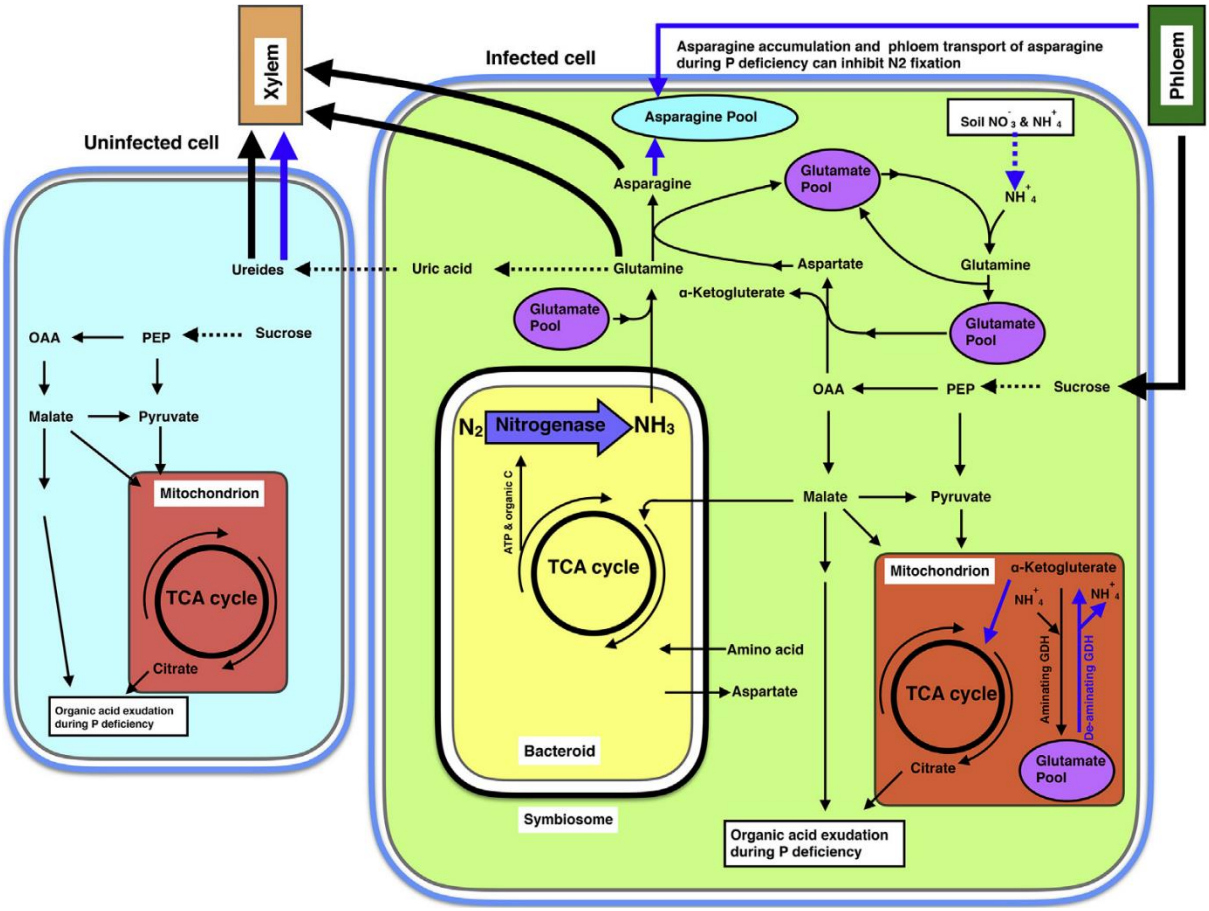

Figure 2.4 Nitrogen (N) metabolism in legume nodules showing the alterations in the pathway during phosphorus (P) deficiency. The blue arrows represent the enhanced engagement of pathways and routes of N nutrition during P stress in nodules (Valentine et al. 2017).

2.12 Overview on *Vigna unguiculata*

Cowpea (*Vigna unguiculata* [L]. Walp) is one of the most important legumes grown by smallholder farmers in the tropical regions of Sub-Saharan Africa (Ogunkanmi et al. 2006). Globally, more than 12.5 million tons of cowpea grains are produced, and the Sahel region and Africa accounts for 64% of total cowpea production. The legume has high nutritional value; about 25% protein, 64% carbohydrates and it contains vitamins and fiber (Fatokun 2002, Hall 2012). Cowpea can also be used as animal feed and as a cash crop (Hall 2012). Cowpea is a drought-tolerant legume which can fix N_2 in nutrient poor soils thus improving soil fertility (Hall 2004). *Vigna unguiculata* can also be included in cereal-legume cropping systems as the cereals can benefit from the residual N derived from the decayed legume leaf and root litter

and root nodules (Okereke et al. 2006). Cowpea production is relatively inexpensive because it fixes around 80% N from the atmosphere during growth (Asiwe 2009) reducing the N fertilizer demand. Despite all the positive benefits of cowpea, its production is still very low (Mohammed et al. 2018). Most small-scale farmers of Sub-Saharan Africa realize very low cowpea grain yields of less than 1 tone ha^{-1} which is significantly lower than the attainable potential (Mohammed et al. 2018). Low production of cowpea may be attributed to limited research in trying to improve cowpea varieties, drought, poor soil fertility (Timko et al. 2007) and inefficient symbiosis (Mohammed et al. 2018). In Africa, cowpeas are mostly grown on infertile soils under very low rainfall conditions (Timko et al. 2007). Adoption of the protein rich cowpeas, mainly by the resource poor small-scale farmers living will significantly ensure food security (Fatokun 2002). It is thus imperative to find ways of improving productivity of cowpea, through encouraging higher biological N_2 fixation of different locally grown cowpea varieties under nutrient stress conditions. This can be achieved through enhancement of root association of the cowpea plants with AMF and the symbiotic bacteria (Vance et al. 2003). Recent studies have confirmed that the tripartite association between legumes, AMF and N_2 fixing bacteria results in improved nutrient acquisition and water uptake (Porcel and Ruiz-Lozano 2004, Magadlela et al. 2016).

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Chapter 3: Soil nutrient dynamics in KwaZulu-Natal savanna and grassland ecosystems

Abstract

Savanna and grassland ecosystem arable soils are known to be acidic and nutrient poor, resulting in decreased crop productivity. Soil microbes and soil enzymes however play an important role in mineralizing soil nutrients and increasing their availability for plant uptake. In this experiment, the physicochemical properties, microbial composition and soil enzyme activities of soils from four geographically distinct regions of KwaZulu-Natal savanna and grassland ecosystems were analyzed. There were significant differences in all analyzed soil physicochemical parameters except soil N concentrations. Bergville soils were the most acidic and had the lowest carbon and total cation concentrations. Most soil bacterial strains identified belonged to the genera *Bacillus* and *Lysinibacillus* while most fungi strains belonged to the genera *Trichoderma* and *Fusarium*. There were significant differences in soil lignin degrading, N, P and C cycling enzyme activities. Hluhluwe soils had the highest β-D phosphatase, L-asparaginase, β-glucosaminidase, β-cellobioside, catalase and lacasse activities while Ashburton soils showed the least manganese peroxidase and catalase activities. Soil pH showed negative correlation with all enzyme activities except β-D phosphatase. β-D phosphatase showed a negative correlation with soil P. Therefore, the presence of diverse soil microbes and variation in enzyme activities in savanna and grassland ecosystem soils may regulate nutrient availability in these soils.

Key words: ecosystems, soil acidity, nutrient stress, soil microbes

3.1 Introduction

Rapid population increase, poor agricultural practices, uncontrolled burning and overgrazing have resulted in the drastic degradation of most savanna and grassland ecosystem soils (Conant et al. 2001, Augustine 2003, Sanderson et al. 2013). As a consequence, there has been a loss of as much as 90% of soil structure, increased soil acidity and depletion of soil nutrients in these ecosystems (Jastrow et al. 1998, Liebig et al. 2009, Parihar et al. 2019). Of all the mineral nutrients, P is regarded as the most limiting nutrient in most arid and semi-arid soils (Ahmad et al. 2018, Billah et al. 2019, Parihar et al. 2019, Thioub et al. 2019). The unavailability of P for plant uptake is mainly because P readily forms insoluble complexes with cations particularly iron (Fe^{3+}) and aluminium (Al^{3+}) in predominantly acidic soils (Vance et al. 2003, Abdalla et al. 2019). Despite the acidity and poor nutrition, these soils house microbes that play a significant role in recycling soil nutrition and these include bacteria of different genera such as *Rhizobium*, *Pseudomonas*, *Azotobacter*, *Bradyrhizobium*, *Bacillus*, *Paenibacillus* and fungi including *Trichoderma* (Govindasamy et al. 2010, Jaiswal and Dakora 2019, Parihar et al. 2019). Rhizospheric microbes are involved in key processes such as formation of soil structure, decomposition of organic matter and the cycling of important elements such as P, N, carbon (C), K and sulphur (S) through mobilization, mineralization, N_2 fixation and secretion of soil enzymes (Dardanelli et al. 2010, Billah et al. 2019, Parihar et al. 2019, Thioub et al. 2019).

Plants usually establish symbiotic interaction with arbuscular mycorrhizal fungi (AMF) whereby there is proliferation of mycelial hyphae in exchange for plant photosynthates (Smith and Read 2008, Rouphael et al. 2015, Parihar et al. 2019). Uptake of nutrients such as P, N, K, Fe, calcium (Ca), copper (Cu) is enhanced as a result of AMF-plant root interaction and this effectively promotes plant growth (Zhang et al. 2015). Most importantly, AMF such as *Trichoderma*, *Aspergillus*, *Penicillium* and *Glomus* species contribute significantly to enhancing P bioavailability for plant uptake and this consequently leads to high P use efficiency in plants (Sharma et al. 2013, Parihar et al. 2019, Thioub et al. 2019). Besides AMF, several bacterial strains also contribute to P cycling and these include *Bacillus*, *Paenibacillus* and *Pseudomonas* species (Govindasamy et al. 2010, Sharma et al. 2013, Belane et al. 2014, Parihar et al. 2019). These soil microbes solubilize P through a number of mechanisms such as releasing complex or mineral dissolving compounds such as siderophores, organic acid anions, production of extracellular enzymes and the release of P during their substrate degradation (Sharma et al. 2013).

In addition to solubilizing P in the rhizosphere, several soil *Bacillus* and *Paenibacillus* species exclusively fix atmospheric N_2 when they are in symbiotic association with plants and these include *B. cereus*, *B. megaterium*, *B. subtilis*, *B. pulilus* and *P. azotifigens* (Govindasamy et al. 2010, Veres et al. 2015, Martínez-Hidalgo and Hirsch 2017). The rhizospheric bacteria and fungi also contribute to soil fertility improvement through releasing enzymes extracellularly (Veres et al. 2015).

Soil enzymes help enhance soil fertility by breaking down organic matter into assimilable forms and they facilitate the processes of mineralization and cycling of essential nutrients including P, C and N (Veres et al. 2015, Martínez-Hidalgo and Hirsch 2017). Important enzymes involved in C cycling include dehydrogenases, β-D-Cellobiohydrolase, and β-D-Glucosidase (Henriksson et al. 1998). More so, cellulases are an important group of enzymes that catalyzes the degradation of cellulose and the polysaccharides buildup of β -1,4 linked glucose units (Deng and Tabatabai 1994). Phosphatases is another important group of soil enzymes which play a pivotal role in P cycling (Das and Varma 2010). Asparaginase and β-D-Glucosaminidase contribute to N mineralization and they facilitate the conversion of asparagine into aspartic acid and ammonia (NH_3) and hydrolyze chito-oligosaccharides (Mega et al. 1973).

It is important to note that different soils may contain different groups of microbes and enzymes and the activities of these components are dependent on the physical, chemical and biochemical properties of the soil (Das and Varma 2010). There is need to understand the soil health of ecosystems as this influences plant growth and ecosystem structure. Soil health can thus be viewed as a combination of the interaction between the physicochemical, microbial and soil enzyme components of the soil (Makoi and Ndakidemi 2008, Das and Varma 2010, Kotroczó et al. 2014). Therefore, the aim of this research was to study the different physicochemical properties, microbial composition and soil enzyme activities of soils from geographically distinct regions of KwaZulu-Natal (KZN) savannas and grasslands. We hypothesize that soil microbes and associated soil enzyme activities determine the nutrient status of KZN savanna and grassland ecosystem soils.

3.2 Materials and methods

3.2.1 Soil sampling and nutrient analysis

Soils were collected from four geographically distinct locations viz Hluhluwe location, Northern KZN (28 0′58″S 32 12′4″E, altitude 100 m); Harding/ Izingolweni location, Southern

KZN (30 43′32″S 30 6′10″E, altitude 450 m); Bergville location, Mountainous KZN (28 34′14″S 29 4′17″E, altitude 1040 m) and Ashburton/ Pietermaritzburg location, Midlands KZN (29 38′55″S 30 26′42″E, altitude 670 m).

From each of the four locations, five soil samples were randomly collected at a depth of 0-30 cm, 2 m apart and these were pooled together to form one composite sample per site. Thereafter, 50 mg of the homogenized soils were sieved to <2 mm and were sent for analysis (inorganic P (Olsen), total N, C (SOC), pH (water) and exchange acidity) at the KZN Department of Agriculture and Rural Development's Analytical Services Unit, Cedara, South Africa.

3.2.2 Arbuscular mycorrhizal fungi spore identification and enumeration

Fungi spore extraction

Fungi spores were isolated from the composite soil samples taken from the four sites: Hluhluwe, Izingolweni, Ashburton and Bergville. Spore isolation was done by slightly modifying the wet-sieving and decanting method as described by Gerdemann and Nicolson (1963) as follows; 10 g of each soil sample was added to 100 ml of sterile distilled water and thereafter the suspension was decanted through a three-layer sieve which had mesh of sizes (500 µm, 100 µm and 50 µm) respectively. The spore residues were then suspended in 100 ml of sterile distilled water in a 250 ml beaker for further purification.

Fungi spore purification and enumeration

Further purification of the fungi was done in accordance with a slight modification of the method by Daniel and Skipper (1982). A 25 ml sucrose solution (70% v/w) was added to 10 ml of spore suspension in a 50 ml Falcon tube. Spores were purified by centrifuging them at 13000 rpm for 5 min. Spores of arbuscular mycorrhizal fungi were then collected from the interface of sucrose solution, followed by washing them with tap water on a 32 µm sieve for 2 min. The purified spores were then transferred to Petri dishes. Enumeration of the purified spores was done under stereomicroscope at 40X magnification using a needle. Sporocarps and spore clusters were considered as a unit while other parameters such as shape, size, colour and wall ornamentation were measured as described by International culture collection of vesicular and arbuscular mycorrhizal fungi (INVAM) (https://invam.wvu.edu/methods/spores/enumeration-of-spores)

3.2.3 Molecular identification of fungi

Fungal DNA extraction procedure

The purified extracted spores were grown on PDA dishes and thereafter about 10 mm of fungal mycelia were transferred from a fresh dish to a 1.5 ml sterile Eppendorf tube, where its total genomic DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrep^{TM} kit (Zymo Research, South Africa) in accordance to the manufacturer's instructions.

PCR amplification

The extracted fungal DNA was used for PCR amplification of primers ITS4 (5′-T TCCTCCGCTTATTGATATG -3′) and ITS5 (5′- GGAAGTAAAAGTCGTAACAAG -3′). Fifty-microliter PCR reactions were prepared, and these comprised of 10 µl DNA, 5 µl of 10 X reaction buffer, 2 µl 25 mM $MgCl_2$, 2.5 µl of each primer ITS4 and ITS5, 0.25 µl of Taq DNA polymerase, 1 µl of 10 mM dNTP and 26.75 µl of RNAse free water. The target genes were amplified in a T100 Thermal Cycler (Biorad, USA) under the following conditions: Initial denaturation at 95 C for 2 min, 25 cycles of denaturation at 95 C for 30 s, annealing at 53 C for 45 s and elongation at 72 C for 8 min. A final elongation step was done at 72 C for 8 min. Electrophoresis of the amplified DNA was done in 1.0% (w/v) agarose gel (Seakem) and products were visualized after staining the gel with ethidium bromide (0.5 μgml^{-1}) using the Chemigenius Bioimaging System (Syngiene, England).

DNA sequencing and further analysis

Positive amplicons of 600-base pairs obtained were excised and sequenced at Inqaba Biotech Pty. Ltd., South Africa. The sequences were edited and then compared against the GenBank database using BLASTn program at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest homologues.

3.2.4 Isolation and enumeration of bacteria

The bacteria were isolated from 10 g of each soil sample through serial dilution method (three-fold). Enumeration of total bacterial colony forming units was done for each sample by spreading 100 µl of each dilution on pre-sterilized nutrient agar plates, which was then incubated for 18 h at 37 C and observed for the appearance of distinct bacteria colonies.

Molecular identification of bacteria

Template DNA were prepared from freshly grown cultures of the isolates on nutrient agar using the boiling method as previously described by Akinbowale et al. (2007), with slight modification. A total of 3 to 5 isolated colonies were suspended in 70 µl of sterile deionized

water, thereafter, boiled in a water bath at 100 C for 10 min and cooled on ice for a further 5 min. Thereafter, the suspension was centrifuged at 13000 rpm in a micro-centrifuge (Eppendorf) for 5 min. Thereafter, 50 µl of the supernatant was transferred to a sterile Eppendorf tube and used as a template in the PCR assay.

PCR amplification

The extracted bacterial DNA was used for PCR amplification of primers 63F (5'-CAGGCCTAACACATGCAAGTC -3′) and 1387R (5′- GGGCGGTGTGTACAAGGC -3′). A total of 50 µl PCR reactions were prepared consisting of 10 µl DNA, 5 µl of 10 X reaction buffer, 2 µl of 25 mM $MgCl_2$, 2.5 µl of each primer 63F and 1387R, 0.25 µl of Taq DNA polymerase, 1 µl of 10 mM dNTP and 26.75 µl of RNAse free water. Amplification of the target genes was performed in a T100 Thermal Cycler (Biorad, USA) under the following conditions: Initial denaturation at 94 C for 2 min, 30 cycles of denaturation at 92 C for 30 s, annealing at 56 C for 45 s and elongation at 75 C for 45 s with a final elongation temperature at 75 C for 10 min. Amplified DNA was electrophoresed in 1.0% (w/v) agarose gels (Seakem) and products were visualized after staining the gel with ethidium bromide (0.5 μgml^{-1}) using the Chemigenius Bioimaging System (Syngiene, England).

DNA sequencing and further analysis

Positive amplicons of 1324-base pairs obtained were excised and sequenced at Inqaba Biotech Pty. Ltd., South Africa. The sequence which was acquired was then compared against the GenBank database using the BLASTn program at the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest homologues.

3.2.5 Measurement of soil microbial biodiversity

Measurements of biodiversity were done based on Total CFU/ml, Total $sporeg^{-1}$, Percentage, relative density, relative abundance, isolation frequency, Simpson index of dominance (D), Shannon-Wiener index of diversity (H0), Evenness (E), $R_{margalef}$ and E_{pielou}.

3.2.6 Measurement of soil enzyme activities

Dehydrogenase assay

The activity of soil dehydrogenase was measured using the method described by Ezirim et al. (2017). In this method, 5 g of soil sample were mixed with 10 ml of 0.2% triphenyl tetrazolium chloride (TTC) and 10 ml of Tris buffer (0.1 N, pH=7.6) in a specimen bottle before it was then incubated at 37 C for 6 h. Thereafter the reduced triphenyl formazan (RTF) formed was

extracted by adding 10 ml of methanol and then centrifuging at 4000 *g* for 5 min. The decrease in absorbance was measured using spectrophotometer (Agilent Cary 60 UV-Vis) at 485 nm (ε=15433 cm^{-1} M^{-1}) of 0.1 nmol h^{-1} g^{-1} of the reaction mixture.

Laccase assay

Laccase activity (E.C. 1.10.3.2) was determined following the method used by Patel et al. (2009). This was done by measuring the oxidation of 2, 2- Azino-bis-3-ethyl-benzthiozoline-6-sulphonic acid (ABTS). The reaction mixture contained 100 µl of 50 mM ABTS and 800 µl of 20 mM Na-Acetate buffer (pH 4.5) and 100 µl of soil suspension. The reaction mixture was incubated at 30 C for 15 min and thereafter the reaction was stopped using 40 µl of 20% trichloroacetic acid. One unit of enzyme activity was expressed as an increase in spectrophotometric absorbance (Agilent Cary 60 UV-Vis) at 420 nm (ε=36,000 cm^{-1} M^{-1}) of 0.1 nmol h^{-1} g^{-1} of the culture filtrate.

Lignin peroxidase assay

Lignin peroxidase (EC 1.11.1.14) assay was done following a modified procedure described by Agrawal and Shahi (2017) which was based on the reduction of azure B. The reaction mixture was made up of 749.5 µl of 125 mM sodium tartrate buffer (pH 3.0), 83.5 µl of soil suspension and 0.16 mM of azure B. The reaction was catalyzed by 83.5 µl of 2 mM hydrogen peroxide (pH 3.0) and thereafter it was incubated at 30 C for 15 min. The colour change was measured using spectrophotometer (Agilent Cary 60 UV-Vis) at 651 nm (ε=85,000 cm^{-1} M^{-1}) of 0.1 nmol h^{-1} g^{-1} of the culture filtrate.

Manganese peroxidase assay

Manganese peroxidase activity (EC 1.11.1.13) was measured by observing the oxidation of 2, 6- dimethoxy phenol (DMP) at 469 nm as described by Patel et al. (2009). The reaction mixture contained 83.5 µl of soil suspension, 749.5 µl of sodium tartrate buffer (50 mM, pH 4.0), and 2 mM of 2,6-DMP. The enzymatic reaction was catalyzed with 83.5 µl of 0.4 mM hydrogen peroxide (pH 4.0) and thereafter the mixture was incubated at 30 C for 15 min. The reaction was catalyzed by the addition of 0.5 ml of 0.4 mM hydrogen peroxide. The enzyme activities were expressed as nmol h^{-1} g^{-1} with an extinction coefficient of 27,500 cm^{-1} M^{-1}.

β-Glucosaminide assay

This assay was carried out using fluorogenic substrate (4-MUB-N-acetyl-*β-D*-glucosaminide) according to the method described by Jackson et al. (2013).

L-Asparaginase assay

L-asparaginase (E.C. 3.5.1.1) activity was determined according to the method described by Shifrin and Parrott (1974) whereby the amount of NH_3 liberated from L-asparaginase hydrolysis was measured. The reaction mixture contained 100 µl of 50 mM Tris Buffer (pH 8.6), 800 µl of L-asparaginase (10 mM) and 50 µl of the soil suspension. The reaction mixture was then incubated at 37 C for 15 min, thereafter it was stopped using 50 µl of trichloroacetic acid (1.5 M). Thereafter, the reaction was clarified by centrifugation at 10000 *g* for 2 min before adding 20 µl of the supernatant to 4.3 ml of sterile double distilled water. The enzymatic reaction was catalyzed with 50 µl of Nessler's reagent before incubating at 30 C for 15 min. One unit of enzyme activity was expressed as an increase in spectrophotometric absorbance (Agilent Cary 60 UV-Vis) at 436 nm of 0.1-unit min^{-1} L^{-1} of the reaction mix using NH_3 as standard.

β-Phosphatase assay

This enzyme assay was carried out using fluorogenic substrates (4-MUB-Phosphate) according to the method described by Jackson et al. (2013).

3.2.7 Data analysis

IBM statistics 25 was used to test for differences in soil physicochemical, enzyme and microbial properties in four different soils from different locations of KZN using one-way analysis of variance (ANOVA). Where the ANOVA showed significant differences between treatments, Tukey's Post hoc test was used to separate the means (≤ 0.05).

3.3 Results

3.3.1 Soil physicochemical properties and arbuscular mycorrhizal fungi spore counts

Soils from the four sites were acidic. Bergville soils were the most acidic and had the highest P concentration which was almost three times higher than that of Hluhluwe soils. N concentrations showed no statistical differences among the soil sites (Table 3.1). Hluhluwe soils had the highest K concentration while Izingolweni soils had the lowest K concentration (Table 3.1). Ashburton soils had the highest C concentration while Bergville soils had the least C concentration. Bergville soils had the highest exchange acidity which was more than 90% greater than the other soil sites. Hluhluwe soils had the highest total cations while Bergville soils had the least total cations. Bergville soils had the highest number of AMF spores while Hluhluwe soils had the least (Table 3.1).

3.3.2 Soil enzyme activities

3.3.2.1 Organic matter and lignin degrading enzyme activities

Izingolweni soils had the highest dehydrogenase activity which was about six times higher than that of Bergville soils (Table 3.2). More so Izingolweni soils had the highest manganese peroxidase activity and lignin peroxidase activity. However, lignin peroxidase activity was not determined in Hluhluwe and Bergville soils. Hluhluwe soils recorded the highest laccase activity although this was not statistically different from the rest of the sites (Table 3.2).

3.3.2.2 Nitrogen and phosphorus cycling enzyme activities

β-D-glucosaminide activity was generally low across the four sites. However, Bergville soils had the highest β-D-glucosaminide activity while Ashburton soils had the lowest (Table 3.2). Hluhluwe soils had the highest β-D-phosphate activity and L-asparaginase activity. However, L-asparaginase activity was not detected in Bergville soils (Table 3.2).

3.3.2.3 Carbon cycling enzyme activities

Hluhluwe soils had the highest β-D-glucopyranoside activity, and this was significantly different from the other three sites (Table 3.2). Hluhluwe soils also had the highest β-D-cellobioside activity, and this was not statistically different from that of Izingolweni soils while Bergville and Ashburton soils had similar β-D-cellobioside activity (Table 3.2). There was no statistical difference on catalase activity among the four soil types.

3.3.3 Functional microbial diversity

The soils sampled from the four experimental sites showed variations in terms of functional microbial diversity. Ashburton soils had the highest total number of bacteria-colony forming units and it had the highest bacteria species richness (7 bacterial species) and a relatively low diversity index and species evenness. On the other hand, Hluhluwe soils had the highest diversity index and the highest species evenness, while Izingolweni soils had the lowest number of viable bacterial cells. Bergville soils had the lowest species richness, diversity index and species evenness (Table 3.3). Furthermore, there were differences in the AMF composition and distribution for different soils and Hluhluwe soils had the highest functional AMF diversity indices (Table 3.3).

3.3.4 Soil microbial composition

The different KZN soils housed a wide range of bacteria and fungi. Most of the fungi which were isolated and identified belonged to the family *Necriaceae*, *Mucoraceae* and *Hypocreaceae* while the identified bacteria largely belonged to *Bacillaceae* family (Table 3.4).

3.3.4.1 Relative abundance of arbuscular mycorrhizal strains

There was a diversity of AMF strains identified in the soils collected from the four sites and these occurred in different levels of abundance. Hluhluwe soils had seven different fungi strains and of these *Muco velutinous* had the highest relative abundance, closely followed by *Giberrella intermedia* and *Fusarium sinensis* (Figure 3.1). Izingolweni soils had two fungal strains which were *Amylomyces rouxii* and *Rhizopus stolonifer*. On the other hand, in Bergville soils three fungal strains were identified which were *Fusarium concntricum*, *Fusarium oxysporum* and *Amylomyces rouxii*. Ashburton soils were mainly dominated by *Rhizopus stolonifer* (Figure 3.1).

3.3.4.2 Relative abundance of bacteria strains

Hluhluwe soils had a total of six bacterial strains which were mainly *Bacillus* strains. Izingolweni soils comprised of six bacterial strains which were a mixture of *Bacillus* and *Lysinibacillus* strains. Bergville and Ashburton soils comprised mainly of *Lysinibacillus xylanilyticus* (Figure 3.2).

3.3.5 Correlation matrices between soil enzymes and soil physicochemical properties

Enzymes catalase, laccase and lignin peroxidase had no significant correlation with any of the soil properties. However, strong positive correlations were recorded between enzymes β-D-glucopyranoside, β-D-cellobioside, β-D-phosphatase and L-asparaginase with Ca, Mg and total cations. In contrast negative correlation was recorded between β-D-glucosaminide and Ca, Mg and total cations (Table 3.5). Enzyme β-D-phosphatase had a negative correlation with soil P and it was the only soil enzyme that had a positive correlation with soil pH. Enzyme laccase and lignin peroxidase had no significant correlation with any of the soil properties recorded. However, manganese peroxidase and dehydrogenase had positive correlation with soil K (Table 3.5).

3.4 Discussion

Soils collected from the four sites were acidic and had relatively low nutrient composition. Several studies have reported that tropical soils, especially in sub-Saharan Africa, are acidic, nutrient deficient, especially with regards to N and P and this results in reduced plant growth (Abd-Alla et al. 2014, Ferreira et al. 2016, Sulieman and Tran 2016). The differences in distribution and diversity of the various bacteria and fungi species observed in this study could be attributed to the variations in soil physicochemical and biological characteristics (Rengel

and Marschner 2005, Puozaa et al. 2019). It is suggested that nutrient deficiency in soils may prompt proliferation of specific soil microbes to enhance solubilization or remobilization of the required mineral nutrients (Duly and Nannipieri 1998, Rengel and Marschner 2005). Therefore, the occurrence of fungi and bacterial species known to promote N, P and C cycling could be due to the KZN soils nutrient deficiency.

In the present study, the differences in soil P observed could be attributed to differences in soil pH, soil microbial compositions and soil enzyme activities. In acidic soil conditions, P may form complexes with cations such as Al^{3+} and Fe^{3+} making it unavailable for plant assimilation (Valentine et al. 2018). Bergville soils were the most acidic and had high exchange acidity so it would be expected that these soils would contain insoluble soil P due to complexion with cations. However, Bergville soils housed several fungi and bacterial strains which contributed to P solubilization in soils. Microbe species such as *Fusarium*, *Bacillus* and *Lysinibacillus* which were identified in the Bergville soils play a pivotal role in P solubilization (Govindasamy et al. 2010, Jones and Oburger 2011, Sharma et al. 2013). Several fungi and bacteria species identified in the soils, such as *Bacillus, Lysinibacillus* and *Trichoderma* species could have enhanced P availability. These different microbes release mineral dissolving compounds such as organic acids, siderophores and extracellular enzymes such as β-D phosphatase (Yadav and Tarafdar 2007, Bünemann et al. 2010, Sharma et al. 2013, Billah et al. 2019). Studies by Saravanakumar et al. (2013) showed that 10 isolates of *Trichoderma* species derived from the rhizosphere of *Aricennia marina* had the ability to solubilize P *in vitro* as evidenced by increased concentrations of soluble phosphate in the culture filtrates. Furthermore, Hayat et al. (2013) reported that *L. xylanilyticus* strains have P solubilization properties. Hluhluwe soils had the lowest P content while it had the highest β-D phosphatase. The low soil P concentration could have prompted increased activity of β-D phosphatase enzyme for catalysis of substrates thus making more P available in the rhizosphere (Duly and Nannipieri 1998, Rudresh et al. 2005). Similarly, the negative correlation was observed in Ashburton soils where the soils had high P content and the lowest β-D phosphatase activity.

Soil microbes play a pivotal role in enhancing N availability in soils through mineralization and N fixation (Meena et al. 2016). N fixation has been shown to occur in several bacterial strains including *Azotobacter, Bacillus, Enterobacter* and *Paenibacillus* (Martínez-Hidalgo and Hirsch 2017) and some of the most important N fixing *Bacillus* include *B. cereus, B. megaterium, B. subtilis, B. aryabhattai, B. thuringiensis* and *B. pulilus* (Govindasamy et al. 2010, Martínez-Hidalgo and Hirsch 2017). This suggests that the presence of the different *Bacillus* species observed in the soils sourced from the savanna and grassland ecosystems of

KZN could have directly contributed to the soil N status. Furthermore, several *Lysinibacillus* species are known to have the capacity to fix N_2 (Mishra et al. 2015). For example, different strains of *Lysinibacillus sphaericus* species have been shown to possess genes for N fixing which include *nifA*, *ntrC*, *nifU* (Martínez and Dussán 2018, Aguirre-Monroy et al. 2019). It is therefore proposed that the *Lysinibacillus* species which were identified in the different soils played an important role in enhancing N cycling in the different sites.

Soil microbes contribute to the population of intracellular and extracellular enzymes found in the soils as most soil enzymes are derived from bacteria and fungi (Acosta-Martinez et al. 2007). Soil enzymes play a pivotal biochemical role in the process of organic matter decomposition into assimilable forms, they help in degrading litter in soils (Acosta-Martinez et al. 2007). Furthermore, soil enzymes act as catalysts in biochemical processes such as soil nutrient cycling and nutrient mineralization of such important nutrients like N, P and C (Acosta-Martinez et al. 2007, Makoi and Ndakidemi 2008, Veres et al. 2015, Ntoko et al. 2018). In this regard the presence of L-asparaginase could have contributed to N availability in the different soil types. Asparaginase enzyme is known to have *ansZ* gene which hydrolyses asparagine to aspartic acid and NH_3 (Ebrahiminezhad et al. 2011). Hluhluwe soils had the highest L-asparaginase activity and this could have contributed to the high soil N content observed in the Hluhluwe soils.

Soil enzyme activities are dependent on a number of factors including soil properties, soil microbe interactions and presence of activators or inhibitors (Nannipieri et al. 2011). In the present study, the positive correlation between organic acid and β-D phosphatase could be because the enzyme β-D phosphatase had caused increase in organic acid which helps chelate the cationic element that will have been associated with P and this results in the release of inorganic phosphate in the medium (Rudresh et al. 2005). A high negative correlation between β-D phosphatase and soil P concentration observed in this study is in confirmation to the suggestions made by other researchers that low soil P triggers the release of P cycling enzymes by soil microbes (Olander and Vitousek 2000, Allison et al. 2007). Phosphatase production and activity are linked to the biotic demand for P; therefore, the negative feedback mechanism could be a primary way in which plants and microbes regulate mineralization in response to nutrient supply (Olander and Vitousek 2000). However, contrary to our results, Bowles et al. (2014) observed that there was no significant association between P availability and the potential activity of P-cycling enzymes, alkaline phosphomonoesterase and phosphodiesterase. Of the factors which affect enzyme activity, soil pH has been identified as one of the most important factors (Acosta-Martinez and Tabatabai 2000, Dick et al. 2000, Rodríguez-Loinaz et

al. 2008). This is mainly because soil pH may influence the ionization and solubility of the enzymes, cofactors and substrates thus affecting rate of enzyme activity (Acosta-Martinez and Tabatabai 2000). Earlier studies by Dick et al. (1988) showed significant positive correlations between soil pH and enzyme activities such as alkaline phosphatase and urease but not with β-glucosidase activity. In parallel to this was a study by Rodríguez-Loinaz et al. (2008) who showed that soil pH was positively correlated with enzyme activities of amidase and arylsulphate.

The current findings were however contradicting to the above-mentioned findings as there was no positive correlation between soil pH and enzyme activities such as β-D-glucopyranoside, β-D-cellobioside, lignin peroxidase, dehydrogenase, L-asparaginase. The inconsistency could be attributed to other confounding intrinsic soil factors such as soil temperature (Rudresh et al. 2005, Acosta-Martinez et al. 2007). There was a positive correlation between soil N and carbon cycling enzymes (β-D-glucopyranoside, and β-D-cellobioside) and this is in parallel with findings by Bowles et al. (2014) where it was demonstrated that carbon cycling enzyme activities increased with increase in inorganic N availability.

3.5 Conclusion

From this study, it can be concluded that different savanna and grassland ecosystems of KZN have diverse soil microbial compositions and soil enzyme activities that could contribute to nutrient mineralization and sustain the growth of crops in these ecosystems. The interaction between soil physicochemical properties and soil enzyme activities affects in making essential nutrients available for plant growth. Good agronomic practices such as liming and adding supplementary soil organic amendments could help improve soil fertility and ensure sustainable crop production in the savanna and grassland ecosystems of KZN.

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Table and Figure legends

Table 3.1 Physicochemical properties of soils collected from Hluhluwe, Izingolweni, Bergville and Ashburton; means ± s.e. n=5. The significant differences are indicated by different letters (P≤0.05) for each parameter (Matiwane et al., 2019)

Parameter	Hluhluwe	Izingolweni	Bergville	Ashburton
Soil nutrient concentration ($\mu molg^{-1}$)				
Phosphorus	0.03 ± 0.00a	0.08 ± 0.02b	0.11 ± 0.01bc	0.07 ± 0.00a
Nitrogen	0.18 ± 0.01a	0.13 ± 0.01a	0.14 ± 0.01a	0.09 ± 0.00a
Potassium	10.07 ± 0.25d	1.20 ± 0.02a	4.63± 0.14b	6.49 ± 0.00c
Carbon	4.13 ± 0.02c	3.86± 0.006b	3.39± 0.006a	4.23±0.015d
Soil chemistry				
Exchange acidity ($cmolL^{-1}$)	0.07 ± 0.01a	0.06 ± 0.00a	1.50 ± 0.04b	0.06 ± 0.02a
Total cations($cmolL^{-1}$)	21.01 ± 0.18d	11.37 ± 0.10c	4.18 ± 0.08a	6.74 ± 0.07b
pH	4.96 ± 0.00c	4.63 ± 0.00b	4.06 ± 0.00a	5.07 ± 0.02d
Fungal spore count (no./100 g soil)	5.20±1.07a	11.60±2.68b	15.80±1.80b	13.40±1.40b

Table 3.2 Organic matter, lignin and soil nutrient (Nitrogen, phosphorus and carbon) enzyme activities ($nmolh^{-1}g^{-1}$) of soils collected from Hluhluwe, Izingolweni, Bergville and Ashburton; means ± s.e. n=20. The significant differences are indicated by different letters (P≤0.05)

	Hluhluwe	**Izingolweni**	**Bergville**	**Ashburton**
Organic matter and Lignin degrading enzyme activities ($nmolh^{-1}g^{-1}$)				
Dehydrogenase activity	36.7±16.8b	123.9±41.4c	19.9±3.17a	60.4±10.9b
Laccase	86.12±15.1b	28.5±29.5a	62.5±10.4b	50.7±21.9ab
Lignin peroxidase	ND	105.7±49.1b	ND	40.9±66.3a
Manganese peroxidase	220.9±57.2ab	338.8±121.7b	185.2±18.1ab	50.3±68.3a
Carbon cycling enzyme activities ($nmolh^{-1}g^{-1}$)				
β-D-Glucopyranoside	0.911±0.113a	0.546±0.095b	0.356±0.042b	0.335±0.012b
β-D-Cellobioside	0.190±0.023b	0.178±0.006b	0.072±0.003a	0.072±0.006a
Catalase	90.9±25.1a	64.3±11.9a	61.8±10.6a	56.1±3.01a
Nitrogen and Phosphorous cycling enzyme activities ($nmolh^{-1}g^{-1}$)				
β-D-Glucosaminide	0.637±0.007b	0.734±0.011c	0.981±0.016d	0.567±0.034a
L-Asparaginase	574.5±12.6c	66.2±4.51b	ND	15.5±0.201a
β-D-Phosphatase	1.149±0.05a	0.797±0.012b	0.686±0.020c	0.806±0.046b

ND: Not detected, Values in the same row with different letters as superscripts are significantly different at $p \leq 0.05$

Table 3.3 Functional microbial diversity index of the soil samples from four locations Hluhluwe, Izingolweni, Bergville and Ashburton

Microorganisms	**Site**	**Total CFUml^{-1}**	**Richness**	**Shannon diversity index (*H*)**	**Simpson Index (λ)**	**$R_{margalef}$**	**E_{pielou}**
Bacteria	Hluhluwe	$2.04X10^5$	6	1.180	0.622	0.409	0.659
	Izingolweni	$1.57X10^4$	6	0.959	0.563	0.518	0.535
	Bergville	$5.88X10^4$	5	0.224	0.096	0.364	0.139
	Ashburton	$3.31X10^5$	7	0.462	0.222	0.472	0.238
		Total spores/g					
AM Fungi	Hluhluwe	$2.65X10^3$	7	1.594	0.769	0.761	0.819
	Izingolweni	$6.89X10^2$	2	0.385	0.226	0.153	0.556
	Bergville	$2.24X10^3$	3	0.884	0.521	0.259	0.804
	Ashburton	$6.76X10^2$	2	0.471	0.295	0.153	0.679

Table 3.4 Endophytic fungi and bacterial strains isolated from soil samples collected from the four sites: Hluhluwe, Izingolweni, Bergville and Ashburton

	Strain Code	Family	Strain	Accession No.	Similarity (%)
Fungi	A	*Nectriaceae*	*Fusarium oxysporum* strain Strain LL-5	MK966308	100
	AB		*Fusarium sinensis* isolate RM63	MG652446	99.63
	D		*Gibberella intermedia* isolate F22	HQ379695	99.63
	E		*Fusarium concntricum* isolate F1	HQ379633	98.66
	B	*Mucoraceae*	*Mucor velutinosus* strain Beca_7a	KY203942	96.7
	C		*Amylomyces rouxii* CBS 416.77	Q118998	95
	F		*Rhizopus stolonifer* strain APBSDSF79	MG669211	99.12
	H	*Hypocreaceae*	*Trichoderma spirale* strain TU Graz 11TSM4	EU871034	99.49
	J		*Trichoderma koningiopsis* isolate CTCCSJ-ASC50272	KU896317	100
	K		*Trichoderma gamsii* isolate P2-18	KJ439112	99.31
Bacteria	A	*Bacillaceae*	*Bacillus thuringiensis* strain Eca8	KY952733	99.09
	B		*Lysinibacillus xylanilyticus* strain MAHUQ-40	MK680116	99.19
	C		*Bacillus cereus* strain RM	MG230318	99.28
	D		*Bacillus mycoides* strain NSII-15	JN993723	98.82
	E		*Bacillus aryabhattai* strain SO4H51	KP706808	98.41
	F		*Bacillus huizhouensis* strain CA98	MK618613	99.62
	H		*Bacillus methylotrophicus* strain HB5	KM659215	98.93
	I		*Lysinibacillus boronitolerans* strain MGB4045	MH261172	83.02
	K		*Bacillus simplex* strain P49_BA1H	MK883080	99.19
	L		*Bacillus arbutinivorans* strain 1404	JN645967	91.3

Table 3.5 Correlation matrices of the soil enzyme activities and the soil physicochemical properties

	1	2	3	4	5	6	7	8	9	10
11	-0.054 ns	-0.554*	-0.406 ns	0.675**	-0.688**	0.051 ns	0.147 ns	0 215 ns	0.042	-0.411 ns
12	0.406 ns	0.511*	0.524*	0.235 ns	0.544*	0.287 ns	-0.177 ns	0 343 ns	-0.133	0.349 ns
13	0.190 ns	0.512*	0.821**	-0.417 ns	0.479*	-0.095 ns	0.330 ns	0.449*	0.512*	0.390 ns
14	0.376 ns	0.821**	0.811**	-0.533*	0.886**	0.215 ns	-0.018 ns	0 189 ns	0.069 ns	0.639**
15	0.385 ns	0.828**	0.792**	-0.507*	0.900**	0.240 ns	-0.051 ns	0 173 ns	0.025 ns	0.641**
16	0.388 ns	0.835**	0.811**	-0.468*	0.890**	0.237 ns	-0.041 ns	0 202 ns	0.036 ns	0.634**
17	0.110 ns	0.312 ns	0.287 ns	-0.968**	0.575**	0.050 ns	0.080 ns	-0.174 ns	0.159 ns	0.442 ns
18	0.318 ns	0.601**	0.600**	0.289 ns	0.531*	0.253 ns	-0.149 ns	0 357 ns	-0.137 ns	0.329 ns

ns = Not significant, *= P≤0.05, ** = P≤0.01, 1= Catalase, 2= β-D-Glucopyranoside, 3= β-D-Cellobioside, 4= β-D-Glucosaminide, 5= β-D-Phosphatase, 6= Laccase, 7= Lignin peroxidase, 8= Manganese peroxidase, 9= Dehydrogenase, 10= L-Asparaginase, 11=Phosphorus, 12= Nitrogen, 13= Potassium, 14= Calcium, 15= Magnesium, 16= Total cations, 17= pH, 18= Organic acid

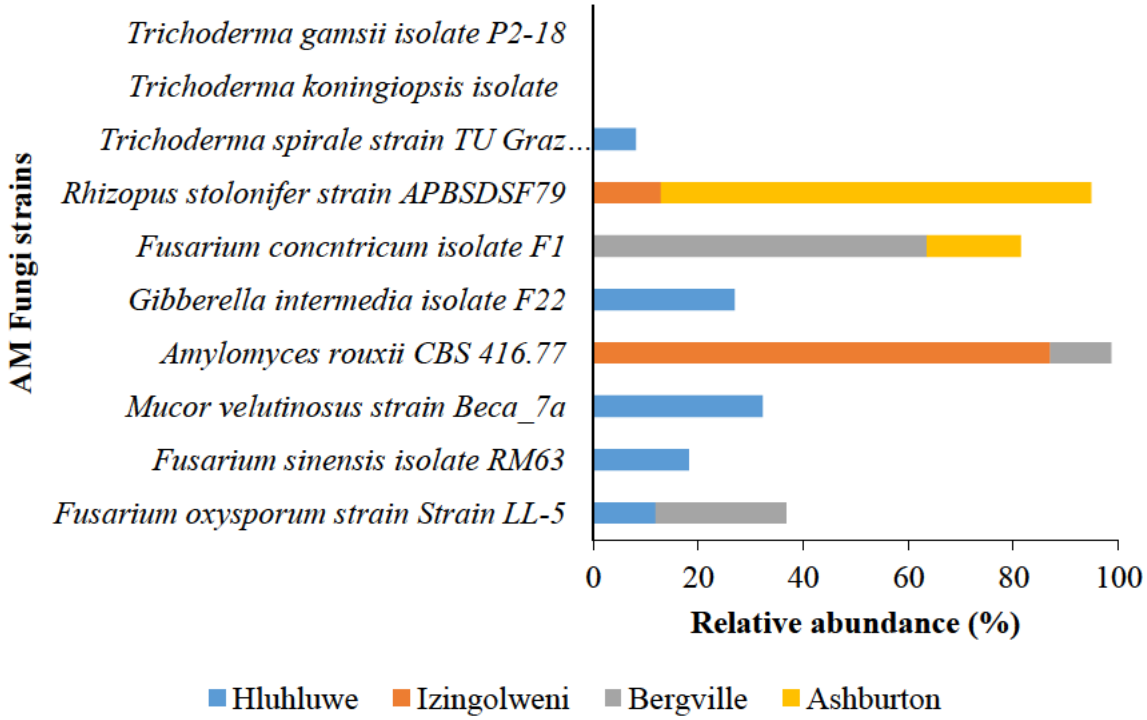

Figure 3.1 Relative abundance of the arbuscular mycorrhizal fungi strains isolated from the experimental soils of the four sites

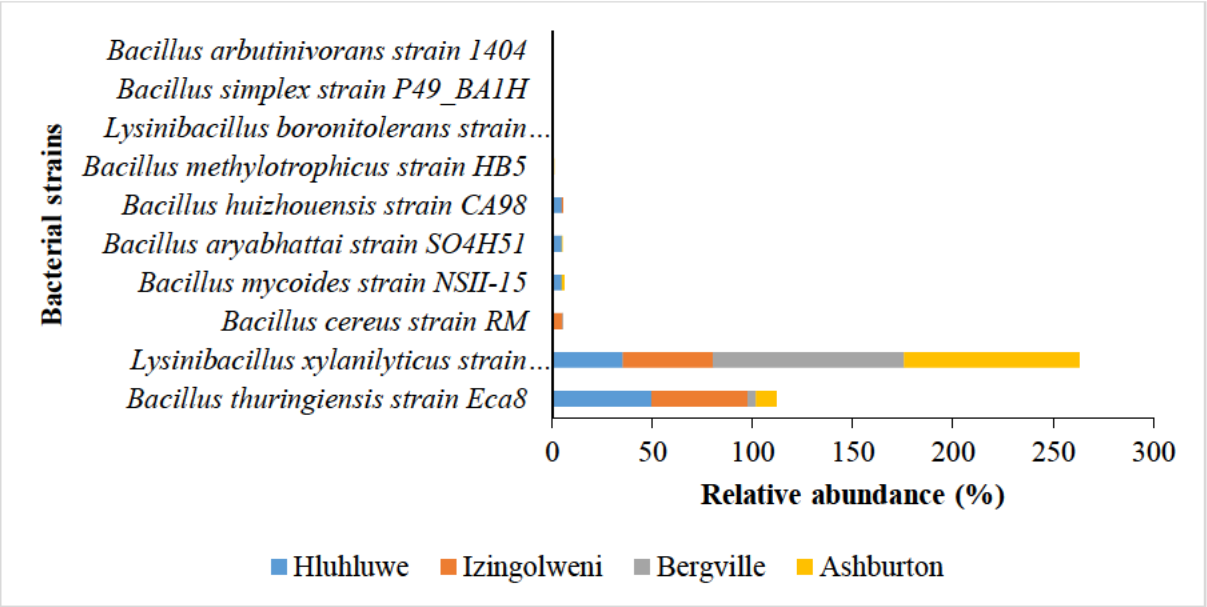

Figure 3.2 Relative abundance of bacterial strains isolated from the experimental soils of the four sites

Chapter 4: Soil nutritional status drives the co-occurrence of nodular bacterial species and arbuscular mycorrhizal fungi modulating plant nutrition and growth of *Vigna unguiculata* L. (Walp) in grassland and savanna ecosystems in KwaZulu-Natal, South Africa

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Soil nutritional status drives the co-occurrence of nodular bacterial species and arbuscular mycorrhizal fungi modulating plant nutrition and growth of *Vigna unguiculata* L. (Walp) in grassland and savanna ecosystems in KwaZulu-Natal, South Africa

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Abstract

Vigna unguiculata is a staple food with high nutritional value and commonly consumed among many rural communities in South Africa. Its success in low nutrient soil has been attributed to the establishment of efficient symbioses with soil bacteria and fungi. We investigated how legume-microbe symbiosis affect phosphorus (P) and nitrogen (N) nutrition, and growth of *V. unguiculata* grown in nutrient deficient soils of KwaZulu-Natal (KZN). Four *V. unguiculata* varieties were grown in nutrient-poor natural soils collected from four geographically different areas in KZN, under glasshouse conditions. From each site homogenized soils were analyzed for soil nutrient concentration, soil chemistry and arbuscular mycorrhizal (AM) fungal spore count. Seeds of four *V. unguiculata* L. (Walp) varieties (IT18, Batch white, Brown mix, Dr Saunders) commonly grown by local farmers in South Africa were used in the experiment. *Vigna unguiculata* was nodulated by several bacterial strains including *Bradyrhizobium*, *Rhizobium*, *Paenibacillus* and *Bacillus* strains. The legume effectively fixed more than 60% of its total N from the atmosphere in all soil treatments. Indicating the adaptation of the observed N fixing bacterial strains, which included the non-rhizobial strains, to the nutrient poor KZN soils. *Vigna unguiculata* varieties had symbiotic association with arbuscular mycorrhizal fungi (AMF); the AMF root colonization ranged from 58-100%, with Hluhluwe soil grown plants recording the highest for all *V. unguiculata* varieties. There were variations in growth kinetics, nutrient assimilation and utilization among the four *V. unguiculata* varieties grown in the soil-types. All the *V. unguiculata* varieties utilized both soil and atmospheric nitrogen (N) and they maintained their total plant N concentrations despite the differences in soil nutrient properties. *V. unguiculata* may adapt to nutrient poor ecosystems by establishing symbiotic interaction with naturally occurring soil bacteria and AMF and through its ability to switch N source preferences.

Key words: Nutrient poor ecosystems, cowpea, symbiosis, bacteria, fungi, biological nitrogen fixation

4.1 Introduction

Nutrient-poor soil, specifically low concentrations of nitrogen (N) and phosphorus (P) is amongst the major limiting factors in crop production in Sub-Saharan Africa (Jones and Oburger 2011, Liu et al. 2018). In order to overcome soil infertility and increase crop yields, most farmers apply synthetic fertilizers (Grady et al. 2016). However, these are costly and may cause pollution, resulting in terrestrial and water resource degradations (Tilman et al. 2001, Vance 2001, Jones and Oburger 2011). Approximately 30-50% and 10-45% of applied N and P fertilizer, respectively are assimilated by crops while the remaining is lost to the environment (Adesemoye and Kloepper 2009). However, symbiotic N fixation, an environmentally friendly N fixing process, could help alleviate the challenge of low soil N availability (Valentine et al. 2010, Martínez-Hidalgo and Hirsch 2017), especially for resource limited small-scale farmers who contribute significantly to global crop production (Khan et al. 2010a, Oruru and Njeru 2016, Chidebe et al. 2018). Symbiotic N fixation accounts for about 60% of fixed N on earth (Martínez-Hidalgo and Hirsch 2017) and occurs in root nodules of legumes mediated by rhizobia (Valentine et al. 2010). Several rhizobial and non-rhizobial bacteria such as *Rhizobium, Bradyrhizobium, Burkholderia, Paenibacillus* and *Bacillus* strains are known to nodulate legumes and fix atmospheric N (Grady et al. 2016, Martínez-Hidalgo and Hirsch 2017, Kawaka et al. 2018).

In terms of soil chemical properties, low P availability is one of the most limiting factors to symbiotic N fixation (Magadlela et al. 2014, Vardien et al. 2016, Sulieman et al. 2019). It plays an essential role in the metabolic processes that drive symbiotic N fixation into NH_3 and organic N by-products such as amino acid and ureides (Vance 2001, Valentine et al. 2017, Liu et al. 2018). As a result, legumes have a higher P demand compared to plants that are solely reliant on mineral soil N (Olivera et al. 2004, Magadlela et al. 2014). During limited P availability, legumes have developed adaptive mechanisms for efficient nutrient uptake, these include increased P partitioning to root tissue, increasing the carbon (C) acquisition and N fixing efficiency (Magadlela et al. 2014, Sulieman and Tran 2015). Some legumes alter N source preferences under P deficiency in order to save energy (Magadlela et al. 2016). Furthermore, some legumes have developed symbiotic association with soil microbes such as arbuscular mycorrhizal fungi (AMF) which maximize root surface area and solubilize soil bound P for plant uptake (Olivera et al. 2004, Parihar et al. 2019, Thioub et al. 2019). Also, the

association with P solubilizing non-rhizobial bacteria such as *Paenibacillus* and *Bacillus* enhance P assimilation by legumes thus promoting symbiotic N fixation (Govindasamy et al. 2010, Marra et al. 2012). However, limited studies have explored the symbiotic association of the rhizobia and phospho-bacteria with cowpea (*Vigna unguiculata* L. Walp) in nutrient-poor grassland and savanna ecosystems of KwaZulu-Natal (KZN), South Africa.

Vigna unguiculata is an important protein-rich crop legume which contributes immensely to human diets in rural South African communities and can be applied in cropping systems to enhance soil N via symbiotic N fixation (Oruru et al. 2018, Jaiswal and Dakora 2019). Studies have been conducted to explore the genetic diversity of *Rhizobium* strains which nodulate cowpea in Africa including South Africa (Steenkamp et al. 2008, Jaiswal and Dakora 2019, Puozaa et al. 2019). However, the association of *V. unguiculata* with phospho-bacteria and how this association affects atmospheric N fixing efficiency in nutrient stressed soil ecosystems remain speculative. Thus, the aim of this research was to investigate the effects of four distinct soil types with varying nutrient concentrations and pH on the plant-microbe symbiosis, biomass accumulation, N-source preference and plant nutrition of *V. unguiculata*. We hypothesize that *V. unguiculata* adapts to nutrient-poor soils by establishing symbiotic association with multiple microbes including phospho-bacteria to enhance plant nutrient acquisition.

4.2 Materials and methods

4.2.1 Soil collection sites

Soil sampling was done in four geographically distinct regions in KZN province, South Africa which covered grassland and savanna ecosystems. These sites were Hluhluwe, Northern KZN (28°0′58″S 32°12′4″, altitude 100 masl); Izingolweni, Southern KZN (30°43′32″S 30°6′10″E, altitude 450 masl); Bergville, Mountainous KZN (28°34′14″S 29°4′17″E, altitude 1040 masl) and Ashburton/Pietermaritzburg, Midlands KZN (29°38′55″S 30°26′42″E, altitude 670 masl). From each of the four sites, 20 soil samples were randomly collected at a depth of 0-30cm, 2 m apart and these were pooled together to form one composite sample per site.

4.2.2 Soil geochemistry and arbuscular mycorrhizal fungi analysis

We prepared five samples at 50 g of the homogenized soils sourced from the four sites by sieving to <2 mm and sent for analysis (inorganic P (Olsen), total N, C (SOC), pH (water) and exchange acidity) at the KwaZulu-Natal Department of Agriculture and Rural Development's Analytical Services Unit, Cedara, South Africa (Garrido and Matus 2012). Furthermore,

arbuscular mycorrhizal spore count analysis was conducted according to the protocol by Smith and Dickson (1991) at Mycoroot (Pty) Ltd, Rhodes University, South Africa.

4.2.3 Seed germination and seedling growth

Seeds of four *V. unguiculata* L. (Walp) varieties (IT18, Batch white, Brown mix, Dr Saunders) used in the experiment were sourced from a commercial supplier, AGT foods Africa, Marji Mizuri Farm, Ingomankulu, KZN, South Africa. These cowpea varieties are commonly grown by local farmers of South Africa. Seeds of *V. unguiculata* were surface sterilized in bleach solution (30% commercial bleach + 0.02% Triton X-100) for 15 min, washed 10 times with sterile water and air dried in a sterile laminar flow. Prior seed germination the surface sterilized seeds were soaked overnight in distilled water. The seeds were germinated and grown at a depth of 2 cm in 20 cm diameter pots filled with the soils from the four sites at a greenhouse at Pietermaritzburg campus of the University of KwaZulu-Natal, South Africa. The greenhouse conditions were as follows; the night and day temperature ranged from 12 to 14 °C and 30 to 35 °C, respectively, the humidity range was from 70 to 80% and the irradiance ~35% of full sunlight (i.e., 415.6 $mmolm^{-2}$ s^{-1}). Irrigation took place every day until the seeds germinated and thereafter plants were irrigated after every two days. The experiment was a completely randomized design with four treatments each *V. unguiculata* variety. Each soil treatment had 20 replicates.

4.2.4 Plant harvesting and nutrient analysis

Harvesting was done at 8 weeks after planting and 10 plants per treatment were rinsed with distilled water and separated into leaves, stem, roots and nodules. These separated plant parts were oven dried at 80 °C till constant weight and their dry weights were recorded. The dried plant material was ground to powder using a pre-chilled pestle and mortar with liquid N, after the liquid N had evaporated, the dry plant materials were ground to fine powder and analyzed for P, N and C concentrations using inductively coupled mass spectrometry (ICP-MS) at Central Analytical Facilities (CAF) at Stellenbosch University, South Africa. The isotope N analysis was done on the ground plant materials using a LECO-nitrogen analyzer at Archeometry Department, University of Cape Town, South Africa. Nodules were harvested from the remaining plants for bacterial extraction.

4.2.5 Extraction and identification of bacteria from root nodules

Root nodules were excised from five plants and the nodules were surface sterilized with 70% (v/v) ethanol for 30 seconds and further subjected to 3.5% (v/v) sodium hypochlorite solution

for 3 min. Root nodules were washed 10 times with sterilized distilled water (dH_2O). Root nodules from each soil treatment and variety were squashed separately in Eppendorf tubes and thereafter 100 µl of 15% glycerol was added. Suspensions were streaked onto yeast extract mannitol agar (YMA) plates containing 1% (m/v) mannitol (BDH GPR), 0.05% (m/v) K_2HPO_4 (Merck), 0.02% (m/v) $MgSO_4.7H_2O$ (Merck), 0.01% NaCl (BDH GPR), 0.1% (m/v) yeast extract and 2% (m/v) bacteriological agar. The plates were incubated at 28 °C and colony growth was monitored every day. Culture purity was verified by repeated streaking until pure cultures were obtained.

To identify the bacteria present in the pure colonies, a portion of the 16S rDNA gene was amplified for all the pure bacterial colonies through PCR reactions using the primers 27F (5'AGA GTT TGA TCC TGG CTC AG3') (Suau *et al*., 1999) and 485R (5'TAC CTT GTT ACG ACT TCA CCC CA3'). Each 25 µl PCR reaction contained 12.5 µl Emerald AMP master mix, 0.25 µl of the respective primers, 11 µl of sterile milliQ water, and 1 µl of diluted bacterial pure colony. DNA amplification was performed on BioRad Mini Opticon thermal cycler (BioRad, South Africa) using the following protocol: initial denaturation for 5 min at 94 °C, 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and elongation for 2 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. The acquired amplicons were viewed on a 1% agarose gel and were confirmed to be of the expected size (1500 base pairs). Thereafter, the amplicons of each treatment were sequenced at The Central Analytical Facilities, Sequencing Facilities (Stellenbosch University, South Africa). Bacterial identification was done on BLASTN (National Center for Biotechnology Information, NCBI (https://www.ncbi.nlm.nih.gov/genbank/).

4.2.6 Determination of root arbuscular mycorrhizal fungi colonization percentage

Five roots with intact nodules from each treatment were stored in glass vials containing 50% ethanol for AMF root colonization. The roots with nodules were firstly cleaned with 10% (g/v) potassium hydroxide (KOH) and stained with lactoglycerol trypan blue. Thereafter, microscopic evaluations of colonization percentages were performed using the modified gridline intersect method (Smith and Dickson 1991).

4.2.7 Nutrition and growth kinetics calculations

Growth rate and Relative growth rate

Growth rate and relative growth rate are used in plant physiology to quantify speed of plant growth. Growth rate is a function of amount of growing material present. Relative growth rate

is the total plant dry weight increase in a time interval in relation to the initial weight or dry matter. These were calculated according to Ågren and Franklin (2003) as follows;

$GR = [(W2 - W1) / (t2 - t1)]$

$RGR = [(\ln W2 - \ln W1) / (t2 - t1)]$

Where, W represents the total plant dry weight and t represents time taken for plant growth.

Specific Nitrogen absorption rate

Specific nitrogen absorption rate (SNAR) values were determined by calculating the total N absorbed by the plant through the roots (mg Ng^{-1} root dw day^{-1}). This was calculated using the formula modified from Hunt (1990);

$SNAR = \left[\frac{M2-M1}{t2-t1}\right] * [\frac{logeR2-logeR1}{R2-R1}]$ Where, M denotes the total nitrogen content in the plant, t is the time it took for the plant to grow and R, the root dry weight.

Specific Nitrogen utilization rate

Specific nitrogen utilization rate (SNUR) was determined by calculating the dry weight acquired by the plant during nitrogen uptake (g dw mg^{-1} N day^{-1});

$$SNUR = \left[\frac{W2 - W1}{t2 - t1}\right] * [\frac{logeM2 - logeM1}{M2 - M1})$$

Where, W is the plant's dry weight and M is the total nitrogen content in the plant (Hunt 1990).

Specific phosphorus assimilation rate

Specific phosphorus absorption rate (SPAR) values were determined by calculating the total P absorbed by the plant through the roots (mg Pg^{-1} root dw day^{-1}). This was arrived at using the formula;

$$SPAR = \left[\frac{M2 - M1}{t2 - t1}\right] * [\frac{logeR2 - logeR1}{R2 - R1}]$$

Where, M denotes the total phosphorus content in the plant, t is the time it took for the plant to grow and R, the root dry weight, as described in (Nielsen et al. 2001).

Specific phosphorus utilization rate

Specific phosphorus utilization rate (SPUR) value was obtained from calculation of the dry weight acquired by the plant during phosphorus uptake (g dw mg^{-1} Pday^{-1});

$$SPUR = \left[\frac{W2 - W1}{t2 - t1}\right] * [\frac{logeM2 - logeM1}{M2 - M1}]$$

Where, W is the plant's dry weight and M is the total phosphorus content in the plant (Nielsen et al. 2001).

4.2.8 Calculations of percentage nitrogen derived from the atmosphere (%NDFA)

The δ15N analyses was carried out on the ground, oven-dried whole plant material at the Archeometry Department (University of Cape Town, South Africa). The isotopic ratio of N was calculated as;

$$\delta = 1000\ (R\text{sample}/R\text{standard})$$

where *R* is the molar ratio of the heavier to the lighter isotope of the samples and standards. About 2.20 mg of each milled sample was weighed into a tin. The samples were combusted in a Fisons NA 1500 (Series 2) CHN analyzer (Fisons Instruments SpA, Milan, Italy). The nitrogen isotope values for the N gas released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which were connected to a CHN analyser by a Finnigan MAT Conflo control unit. Thereafter the %NDFA was calculated according to the formula by Shearer and Kohl (1986) as:

$$\%\text{NDFA} = 100\left(\frac{\delta 15\text{N reference plant} - \delta 15\text{N legume}}{\delta 15\text{N reference plant} - \beta}\right)$$

Where the reference plant was cowpea (*V. unguiculata*) grown under the same greenhouse conditions. β value is the δ15N natural abundance of the N derived from biological nitrogen fixation of the above-ground tissue of *V. unguiculata* grown in a nitrogen free culture and this was determined as −2.60‰.

4.2.9 Data analysis

Genstat 18.1 was used to test for the effects of variety and the soil geochemical and microbial factors on *V. unguiculata* biomass, plant mineral nutrition and growth kinetics using two-way analysis of variance (ANOVA). Where the ANOVA showed significant differences between treatments at ($P \leq 0.05$), Post hoc multiple mean comparison (Tukey's HSD test) was used for multiple comparisons. Relations between the measured values of soil physicochemical properties and the experimental variants of the four *V. unguiculata* varieties (plant biomass, plant nutrition and growth kinetics) were determined using Principal component analysis (PCA). PCA statistical procedures were performed using R (4.1.1) using statistical package gg plot, function *pr comp*.

4.3 Results

4.3.1 Soil geochemistry and arbuscular mycorrhizal fungi (AMF)

The geochemical analysis revealed variations in the soil nutrient concentrations, relative acidity and AMF properties in the different KZN soils (Matiwane et al. 2019). Soils from the four sites were generally acidic although Bergville soils were the most acidic. Bergville soils had the highest P concentration and exchange acidity but lowest total cations as shown in Chapter 3 (Table 3.1). Hluhluwe soils had the highest K concentration and total cations while it had the lowest P concentration. The highest C concentration was recorded in Ashburton soils. The N concentrations of the soil from the four sites were similar. Arbuscular mycorrhizal fungi spore count per 100 g of soil was relatively low in the four soils and ranged from 5.20 - 15.80 per 100 g of soil as shown in previous chapter (Table 3.1) (Matiwane et al. 2019).

4.3.2 Cowpea nodulating bacteria

Sequence comparison of the 16S ribosomal RNA partial gene sequence revealed the presence of multiple symbionts in nodules of the four *V. unguiculata* varieties grown in the four distinct KZN soils (Table 4.1). IT18 were nodulated by the largest variation of strains which were *Bacillus*, *Bradyrhizobium, Delftia*, and *Rhizobium*. Batch white were nodulated by *Bradyrhizobium* and *Paenibacillus* strains while Brown mix were nodulated by *Bacillus* and *Paenibacillus* strains. Dr Saunders were nodulated by *Bradyrhizobium* strains. *Bradyrhizobium* was the most common strain across all varieties except for Brown mix (Table 4.1). More so, *Bradyrhizobium* was the most common across all the four soil types. *Delftia* strain was only restricted to Bergville soil-grown plants (Table 4.1).

4.3.3 Plant biomass and growth kinetics

Hluhluwe soil-grown cowpea varieties had the highest plant biomass while Bergville soil grown plants had the least total biomass (Table 4.2). A similar trend was observed for plant growth rates (GR) across all soil types (Table 4.2). Within the cowpea varieties, plant biomass and growth rate (GR) was in this order; IT18>Batch white>Brown mix>Dr Saunders (Table 4.2). Relative growth rate (RGR) of three cowpea varieties was significantly higher in Hluhluwe soils except Batch white which had significantly higher RGR in Ashburton and Hluhluwe soils (Table 4.2). Overall, Brown mix had the least RGR across all soils while Batch white had the highest RGR (Table 4.2).

4.3.4 Plant nutrition and arbuscular mycorrhizal fungi root colonization (%)

Percentage nitrogen derived from the atmosphere (%NDFA) was maintained by the four *V. unguiculata* varieties across soil types (Table 4.3). Moreso, the NDFA showed no statistical differences across the different *V. unguiculata* varieties and the four soil types. Similarly, total plant N concentration was maintained across soil types while statistical differences were shown across varieties with IT18 recording the highest total plant N concentration (Table 4.3). Ashburton soil-grown Batch white, Dr Saunders and IT18 showed a significantly higher nitrogen derived from soil (NDFS), while Brown mix grown in Bergville soil showed a significantly higher concentration of nitrogen derived from soil (Figure 4.1A). Hluhluwe soil-grown plants had a significantly higher SNAR across all the four *V. unguiculata* varieties while Izingolweni soil-grown plants recorded the least, except for variety Batch white (Figure 4.1B). Similarly, Hluhluwe soil grown plants had a significantly higher SNUR across the four *V. unguiculata* varieties while Bergville soil grown plants had the least SNUR for all varieties except for variety Dr Saunders. Overall, Dr Saunders had distinctly lower SNUR relative to the other three cowpea varieties (Figure 4.1C).

Total plant P concentration of different *V. unguiculata* varieties showed statistical differences across the four soil types (Figure 4.1D). Overall IT18 had the highest total plant P concentration across all soil types while Dr Saunders recorded the least. Hluhluwe soil-grown plants of varieties Brown mix and IT18 had the highest total plant P concentration. Conversely for Batch white, Ashburton soil grown plants had the highest total plant P concentration. Bergville soil grown plants had the least total plant P concentration for varieties Batch white, Brown mix and IT18 (Figure 4.1D).

The AMF root colonization % showed significant differences for the four *V. unguiculata* varieties across the four soil types. Hluhluwe soil-grown cowpea varieties had 100% AMF root colonization, and this was closely followed by Izingolweni soil-grown plants while Bergville soil grown plants recorded the least across all *V. unguiculata* varieties (Figure 4.1E).

There were no marked differences of SPUR across the other three soil types for all the four *V. unguiculata* varieties (Figure 4.1F). Hluhluwe soil-grown Brown mix and IT18 plants had the highest SPAR while Ashburton and Bergville soil-grown plants had the highest SPAR for varieties Batch white and Dr Saunders respectively. Overall, Brown mix had the lowest SPAR (Figure 4.1G). Hluhluwe soil-grown plants had the highest SPUR across the four *V. unguiculata* varieties.

4.3.5 Correlations between the physicochemical properties (soil P, N, K, C, exchange acidity, total cations, pH) of four soil types (Bergville, Ashburton, Izingolweni, Hluhluwe) of KwaZulu-Natal

There were distinct variations in the soil physicochemical properties of Hluhluwe and Bergville soils. However, Izingolweni and Ashburton soils had similar properties as shown by the overlap in ellipses (Figure 4.2). The first two principal components of the soil physicochemical properties analysis explained 86.4% of the cumulative variability of the measured components with PCA 1 accounting for 65.1% and PCA 2 accounting for 21.3% of the total variation (Figure 4.2). Principal component (PCA 1) had strong positive loadings for P and exchange acidity, and strong negative loadings for Ca and Mg. The second axis (PCA 2) had strong positive loadings for N and exchange acidity and a strong negative loading for pH. Bergville and Hluhluwe soil properties were separated along PCA 1. Variations in Bergville soils were highly associated with soil P concentrations and exchange acidity values while variations in Hluhluwe soils were highly associated with total cations, and the concentrations of soil Mg, Ca and K.

4.3.6 Correlations between four *V. unguiculata* varieties grown under different soil types of KwaZulu-Natal

To investigate the correlations among the four *V. unguiculata* in terms of their growth and nutrition response to different soil properties, the data were subjected to principal component analysis (PCA). The positions of the different varieties grown under the four different soils and the relations among the soil properties and the measured plant parameters are as shown in the PCA biplots (Figure 4.3). There was separation of overall soil type clusters and this clearly shows that the *V. unguiculata* varieties responded considerably differently to different soil types. The first PCA explained 43.8% of the total variation while PCA2 explained 18.3% of the total variation. The cluster of Hluhluwe soil grown plants was separated from the cluster of Bergville soil grown plants by PCA1 while there was an interaction in terms of response between Ashburton soil grown plants and Izingolweni soil grown *V. unguiculata* plants as shown by the overlap of the ellipses. Variation within Hluhluwe soil grown plants was mainly associated with soil Mg, Ca and soil pH and to a lesser extent by soil K and soil N. Variation in Bergville soil grown plants was mainly explained by exchange acidity and soil P. Ashburton soil grown IT18 and Batch white were separated from Ashburton soil grown Dr Saunders along PCA1. Variation in Izingolweni soil grown IT18, Ashburton soil grown IT18 and Ashburton soil grown Batch white was explained by NDFS, NDFA, SPAR, total plant N and total plant P. The PCA plot also showed the relationships of the measured response parameters of *V.*

unguiculata varieties to different soil properties. The groups closer together showed high correlations.

4.4 Discussion

The 16S rRNA gene sequence analysis demonstrated co-occurrence of different bacterial species in the nodules of the four *V. unguiculata* varieties grown in the sampled soils. The presence of *Bradyrhizobium, Rhizobium, Delftia, Bacillus* and *Paenibacillus* is an indication that cowpea may be a promiscuous legume nodulated by a wide range of N_2 fixing bacterial species (Pule-Meulenberg et al. 2010, Puozaa et al. 2019). Furthermore, the sequence analysis revealed that all the *V. unguiculata* varieties apart from Brown mix were predominantly nodulated by *Bradyrhizobium* strains particularly *B. japonicum* and *B. elkanii, B. yuanmingense*. Similar *Bradyrhizobium* strains were found to nodulate *V. unguiculata* grown in South Africa ((Mohammed et al. 2018, Puozaa et al. 2019). Symbiotic association of *V. unguiculata* with the various *Bradyrhizobium* and *Rhizobium* strains could have enhanced biological nitrogen fixation of the four varieties (Hayat et al. 2010b) as evidenced by the high %NDFA of around 60% in this study. On the other hand, Jaiswal et al. (2017) and Pule-Meulenberg and Dakora (2009) alluded that cowpea can effectively fix atmospheric nitrogen and thereby derive up to 66% or more of its N nutrition from the symbiotic fixation. It is interesting to note that there was high % NDFA for all the cowpea varieties grown in the different soil types despite the absence of *Rhizobium* and *Bradyrhizobium* strains in the nodules of some cowpea varieties.

Variety IT18 grown in Bergville soils was nodulated by *Delftia* strains; IT18 plants grown in Ashburton soils was nodulated by *Bacillus* strains, Batch white plants grown in Izingolweni soils were nodulated by *Paenibacillus* strains while Brown mix was nodulated by *Bacillus* and *Paenibacillus* strains. This suggests that these non-rhizobial bacteria have the capacity to fix nitrogen. Our findings are in agreement with the reports by several authors who alluded that some non-rhizobial bacteria can effectively fix nitrogen making it available for plant uptake (Elkoca et al. 2010, Govindasamy et al. 2016, Martínez-Hidalgo and Hirsch 2017). *Delftia* strains have the capacity to fix nitrogen as they possess the *nifH* gene (Han et al. 2005, Morel et al. 2011, Agafonova et al. 2017). In addition, several *Bacillus* and *Paenibacillus* strains have the capacity to fix nitrogen (Govindasamy et al. 2010, Martínez-Hidalgo and Hirsch 2017, Siddiqi et al. 2017, Kawaka et al. 2018, Ngeno et al. 2018). The association of *V. unguiculata* with phospho-bacteria including *Bacillus* and *Paenibacillus* strains could have enhanced P

bioavailability, and this could have resulted in enhanced nodulation and increased biological nitrogen fixation (Agafonova et al. 2017).

The tripartite symbiosis of *V. unguiculata*, nodulating bacteria and AMF possibly played an important role in enhancing biological nitrogen fixation of the legume. The AMF likely enhanced P availability for *V. unguiculata* uptake thereby promoting nodulation. An enhanced nitrogenase activity and increased leghemoglobin content of nodules improved biological nitrogen fixation efficiency of legumes (Javaid 2010, Abd-Alla et al. 2014, Hack et al. 2019, Musyoka et al. 2020). Symbiotic interaction between legumes and AMF causes physiological changes in legumes which influence the formation and behavior of rhizobial nodules. The increased P mineralization and solubilization by AMF could have enhanced P levels in the legume tissues which has the potential to alter the quality and quantity of root exudates. Changes in rhizospheric microbial populations promote some nodulating rhizobia (Linderman 1992, Javaid 2010). Furthermore, biological nitrogen fixation could have been promoted by AMF as the fungi increase photosynthetic efficiency of the legumes due to improved P uptake by the roots. Evidence indicate that P plays an important role as it supplies energy in form of NADPH and ATP and it is also involved in regeneration of carbon dioxide acceptor RUBP (De Groot et al. 2003, Javaid 2010). Consequently, the legume photosynthetic efficiency is improved and higher proportions of the photosynthates are channeled to nodule development which in turn results in enhanced biological nitrogen fixation (Linderman 1992). Our results are in agreement with those of other researchers which suggest that legumes may have mutual symbiotic association with AMF and nodulating bacteria; through enhanced nodulation and improved biological nitrogen fixation (Abd-Alla et al. 2014, Hack et al. 2019, Musyoka et al. 2020). Synergistic effects of AMF and rhizobia was demonstrated in *Phaseolus vulgaris* (Ibijbijen et al. 1996). In the study, inoculation with AMF increased the dry matter production, P concentration and nitrogen derived from atmosphere.

All the four *V. unguiculata* varieties were able to switch N sources between the soil and atmospheric N across all soil types as evidenced by results on nitrogen derived from atmosphere (NDFA) and nitrogen derived from soil (NDFS). Biological N_2 fixation is an energy requiring process (Magadlela *et al*., 2016; Valentine *et al*., 2010) and the associated carbon costs range between 3.3 and 6.6 g Cg^{-1} N depending on the legume-*Rhizobium* combination while the cost of NO_3^- reduction does not exceed 2.5 g Cg^{-1} N (Minchin and Witty, 2005). The ability of *V. unguiculata* to switch N sources is an important adaptation mechanism as it ensures survival of the legume even under nutrient stress conditions as more energy is made available for photosynthesis and biomass accumulation by the legume (Magadlela et al.

2016, Magadlela et al. 2017). The ability of *V. unguiculata* to utilize soil N could have contributed to reduced carbon costs by the legume as less energy is required to acquire soil N than atmospheric N (Valentine et al. 2010). As a result of the reduced carbon costs, more energy was likely channeled to photosynthesis and plant biomass accumulation hence the high total plant biomass observed. Despite differences in soil pH, N and P, the four *V. unguiculata* varieties were able to maintain total plant N across the different soil types. This could be attributed to the ability of the legume to utilize both soil N and atmospheric N.

In the current study, high below ground biomass observed in the different *V. unguiculata* varieties could be an important survival strategy. Modification of root architecture enhances nutrient acquisition for legumes under nutrient stress conditions (Vance et al. 2003, Vardien et al. 2016). High below ground biomass provides a large surface area for nutrient absorption. The large root surface area resulted in high SNAR and SPAR that resulted in high N and P acquisition by *V. unguiculata*. This could have contributed to high plant N and P respectively observed in the current study. High below ground surface area could have increased contact area between *V. unguiculata* and the soil microbes for nutrient uptake. The low plant biomass attained in Bergville soil grown plants could be as a result of the high acidity in the Bergville soils which could have resulted in nutrient immobilization (Sulieman and Tran 2015) and reduced nutrient uptake and reduced growth rate of *V. unguiculata.*

Symbiotic association of *V. unguiculata* with AMF could have resulted in P solubilization and increased root surface area for enhanced soil P uptake (Parihar et al. 2019). As established by Elbon and Whalen (2015), AMF has the potential to release organic acids that break P bound complexions and scavenge P and resulting in an increased nutrient absorption rates by the plants. Also, AMF could have released siderophores and extracellular enzymes which contribute to P solubilization (Sharma et al. 2013, Hafeez et al. 2019). The highest AMF root colonization (%) for all the *V. unguiculata* varieties were in plants grown in Hluhluwe soils, and these soils had the lowest soil P concentrations. This could be an important strategy to increase soil P for plant uptake. In the current study, IT18 and Brown mix plants grown in Hluhluwe soils had the highest plant P compared to the respective varieties grown in the other three soils. This suggests that the symbiotic association of AMF with the legume in P deficient soils enhanced P availability and uptake by the *V. unguiculata*. Furthermore, the symbiotic association of *V. unguiculata* with phospho-bacteria such as *Delftia, Bacillus* and *Paenibacillus* strains in this study could have contributed to enhanced P availability and assimilation by *V. unguiculata*. The phospho-bacteria could have solubilized some precipitated phosphates by excreting organic acids such as gluconic acids and keto-glutonic acids which help dissolve

phosphatic minerals (Vance et al. 2003, Govindasamy et al. 2010, Marra et al. 2012). The phospho-bacteria could have directly dissolved mineral P by chelating metal ions, causing anion exchange or by lowering soil pH resulting in the release of P from mineral complexes (Sharma et al. 2013, Grady et al. 2016). Phosphorus solubilization by *Delftia* strains has been reported by Agafonova et al. (2017) and Prasannakumar et al. (2015) while other researchers confirmed P solubilizing capacity of *Bacillus* and *Paenibacillus* strains (Govindasamy et al. 2010, Grady et al. 2016). Rhizospheric P solubilizing bacteria identified in this study could have excreted extracellular enzymes such as phosphatases and phytases into the soils thereby contributing to P cycling (Sharma et al. 2013, Kotroczó et al. 2014, Veres et al. 2015, Martínez-Hidalgo and Hirsch 2017). For each variety, high SPAR also corresponded with high plant P concentrations. The P solubilizing microbes could have enhanced P assimilation rates and may have contributed to increased total plant P and increased plant biomass in the four *V. unguiculata* varieties. In this study, plants with high plant P concentrations also had high total plant biomass across the four *V. unguiculata* varieties. This may be due the importance of P macronutrient which plays a pivotal role in as an energy source in important processes such as photosynthesis and biological nitrogen fixation (Billah et al. 2019).

The high growth rate for all the different varieties translated to high biomass and this was generally highest in Hluhluwe soil grown plants. This may imply that there was high photosynthetic efficiency and more of the photosynthates were channeled to plant biomass accumulation. High concentration of soil K in Hluhluwe soil could have promoted cowpea growth as K plays a critical role in plant growth and metabolism. Particularly, it is known to contribute immensely to the survival of plants under stress conditions including nutrient stress (Wang et al. 2013). Similar results were obtained by Haro et al. (2018) who demonstrated that co-inoculation of *V. unguiculata* with native rhizobial and mycorrhizal strains resulted in increased plant biomass, mineral nutrition and yield of the legume. Izingolweni soils had the least K and it would be expected that the *V. unguiculata* grown in these soils would have the lowest biomass as K plays a pivotal role in plant growth, photosynthesis and enzymatic activities (Meena et al. 2016, Etesami et al. 2017, Verma et al. 2017). However, the symbiotic association of the legume with soil microbes could have enhanced K solubilization thereby making the macronutrient available for plant uptake and improving plant growth and biomass accumulation. Rhizospheric bacteria including those of genera *Bacillus*, *Paenibacillus*, *Delftia*, *Rhizobium* have been identified to promote K solubilization (Meena et al. 2016, Verma et al. 2017). Furthermore, AMF help increase solubility of mineral K thus enhancing its bioavailability for crop use (Meena et al. 2016).

4.5 Conclusion

In the current study, *V. unguiculata* established multiple root symbiosis with AMF and several strains of bacteria which enhanced the survival of the legume. This was attributed to improved nutrient uptake and biological nitrogen fixation and enhanced growth rate in nutrient poor grassland and savanna ecosystems. In addition, *V. unguiculata* has the capacity to switch N source preference by relying on both soil and atmospheric N. We identified a rich pool of microbes that nodulate *V. unguiculata* which can be explored as biofertilizers in nutrient-poor soils thus contributing to sustainable agriculture. For future studies, the identified native microbes may be isolated and multiplied then inoculated to different legumes and other crops of agricultural interest in field experiments to test their potential in the presence of interactions with other edaphic microorganisms and other soil components. Furthermore, it will be pertinent to establish the specific plant growth promoting processes that are promoted by the identified *V. unguiculata* nodulating bacterial strains.

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Declarations

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Data Availability The data can be made available upon request from the corresponding author.

Code availability Not applicable

Authors' contributions

Experimental work and draft manuscript were done by BTM and AM. AM and AOA assisted with experimental design. AM and AOA reviewed and edited the manuscript. All the authors have read the manuscript and agreed to submit this version for publication.

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

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Table and Figure legends

Table 4.1 Tentative identification of nitrogen fixing bacterial species from the root nodules harvested from plants of four *Vigna unguiculata* varieties (IT18, Dr Saunders, Brown mix, Batch white) grown in nutrient-poor KwaZulu-Natal soils

VARIETY	SITE	GENBANK BLAST	ACCESSION NUMBER	IDENTITY %
IT18	**Bergville**	*Delftia* sp. strain QW16-4	MH769486.1	99.86
		Delftia acidovorans JCM 2784	LC420057.1	99.86
		Delftia acidovorans JCM 5833	LC462156.1	99.86
		Delftia lacustris strain FSB_FG2	MG322199.1	99.86
		Bacterium KR 1979/21_108	GQ288844.1	99.86
		Bacterium KR 1996/1_85	GQ288833.1	99.86
		Delftia lacustris strain MB38	MH675503.1	99.71
	Ashburton	*Bacterial strains not determined*		
	Izingolweni	*Bacterial strains not determined*		
	Hluhluwe	*Bradyrhizobium zhanjiangense* strain CCBAU 51778 chrom	CP022221.1	100.00
		Bradyrhizobium zhanjiangense strain CCBAU 51778	CP022221.1	99.84
		Bradyrhizobium kavangense strain IC3195	MG016482.1	100.00
		Bradyrhizobium iriomotense	LM994098.1	100.00
		Bradyrhizobium canariense strain BRUESC629	KT880606.1	99.86
		Bradyrhizobium ingae strain BR 10250	NR_133985.1	99.86
		Bradyrhizobium japonicum strain SEMIA 6439	FJ025098.1	99.86
		Bradyrhizobium japonicum strain BVVRRN67	MK559521.1	99.84
		Bradyrhizobium yuanmingense strain IND 18	MN197778.1	99.84
		Bradyrhizobium subterraneum strain IND 17	MN197777.1	99.84
BATCH WHITE	**Bergville**	*Bradyrhizobium guangxiense* strain CCBAU 53363	CP022219.1	99.71
		Bradyrhizobium centrosematis strain CB46g	MH603873.1	99.71
		Bradyrhizobium japonicum NR-40	LC386880.1	99.71
		Bradyrhizobium stylosanthis strain BR 510	KU724143.1	99.71
		Bradyrhizobium ingae strain BR 10250	NR_133985.1	99.71
		Bradyrhizobium arachidis strain CzR2	KJ125399.1	99.71
		Bradyrhizobium liaoningense strain Y36	KF787800.1	99.71
		Bradyrhizobium japonicum strain BD05	KY284085.1	99.57
		Bradyrhizobium stylosanthis strain BR 510	KU724143.1	98.00
		Bradyrhizobium stylosanthis strain BR 446	NR_151930.1	98.00
		Bradyrhizobium arachidis strain CzR2	KJ125399.1	98.00
	Ashburton	*Bradyrhizobium jicamae* strain G177	MK817575.1	99.29
		Bradyrhizobium sp. strain BRUESC1011	MH636081.1	99.29
		Bradyrhizobium embrapense strain UFLA03-689	MF495737.1	99.29
		Bradyrhizobium embrapense TrV2B	LC460915.1	100.00
		Bradyrhizobium sp. strain BR13648	MH094213.1	99.29
		Bradyrhizobium namibiense strain 5-10	KX661401.2	99.29
		Bradyrhizobium elkanii strain CUR_S25#2	MK228880.1	100.00
		Bradyrhizobium sp. strain BR13665	MH094227.1	100.00
		Bradyrhizobium sp. strain TUTRAH8A	MF140379.1	100.00
	Izingolweni	*Paenibacillus chondroitinus* strain SESRK1	KU605697.1	99.43
		Paenibacillus aceris strain 128	MH910232.1	99.29
		Paenibacillus nebraskensis strain JJ-59	NR_159223.1	99.29
		Paenibacillus nebraskensis strain BT240 16S	MN204046.1	99.43
		Paenibacillus pectinilyticus partial	LN881694.1	98.57
		Paenibacillus pectinilyticus strain BJC15-C22	JX464213.1	98.00

		Paenibacillus marchantiophytorum strain R55	NR_148618.1	98.29
		Paenibacillus alginolyticus strain GYB9	JQ342918.1	98.15
		Paenibacillus sp. NBRC 13632	AB680469.1	97.86
		Paenibacillus pocheonensis strain Gsoil 1138	NR_112565.1	97.29
		Paenibacillus alginolyticus strain GYB9	JQ342918.1	99.29
	Hluhluwe	*Bradyrhizobium zhanjiangense* strain CCBAU 51778	CP022221.1	99.86
		Bradyrhizobium zhanjiangense strain CCBAU 51778	CP022221.1	99.14
		Bradyrhizobium sp. strain 391-11	MH201294.1	99.86
		Bradyrhizobium kavangense strain IC3195	MG016482.1	99.86
		Bradyrhizobium kavangense strain 14-3	NR_145925.1	99.71
		Bradyrhizobium iriomotense	LM994098.1	99.86
		Bradyrhizobium iriomotense strain BRUESC667	KT880634.1	99.71
		Bradyrhizobium canariense strain CCBAU 51257	GU433455.1	99.86
		Bradyrhizobium japonicum NR-40	LC386880.1	99.71
		Bradyrhizobium japonicum strain SEMIA 6192	AY904772.1	99.71
		Bradyrhizobium ingae strain BR 10250	NR_133985.1	99.71
		Bradyrhizobium yuanmingense strain IND 18	MN197778.1	99.14
		Bradyrhizobium subterraneum strain IND 17	MN197777.1	99.14
		Bradyrhizobium yuanmingense	LT986177.1	99.14
	Bergville	*Paenibacillus chondroitinus* strain SESRK1	KU605697.1	99.43
		Paenibacillus chondroitinus strain CJ62	EU290158.1	97.15
		Paenibacillus sp. KC016-AaL	KJ192339.1	99.43
		Paenibacillus sp. MH67	EU182895.1	99.57
		Paenibacillus aceris strain 128	MH910232.1	99.29
		Paenibacillus aceris strain 18JY4-6	MH497637.1	97.15
		Paenibacillus nebraskensis strain JJ-59	NR_159223.1	99.29
		Paenibacillus pectinilyticus strain RCB-08	NR_044487.1	97.86
		Paenibacillus pectinilyticus	LN867249.1	98.57
		Paenibacillus alginolyticus strain GYB9	JQ342918.1	98.15
		Paenibacillus sp. NBRC 13632	AB680469.1	97.86
		Paenibacillus pocheonensis strain Gsoil 1138	NR_112565.1	97.29
	Ashburton	*Bacillus proteolyticus* strain A56	MN252089.1	99.57
		Bacillus cereus strain A25	MN252078.1	99.57
		Bacillus tropicus strain mar1-12	MN249501.1	99.57
		Bacillus anthracis strain Sihong_667_3	MN240434.1	99.57
		Bacillus paramycoides strain ANK10	MN044865.1	99.57
		Bacillus thuringiensis strain 384	MN108016.1	99.57
BROWN MIX		*Bacillus paramycoides* strain UACC 1	MN093301.1	99.57
	Izingolweni	*Bacillus thuringiensis* strain BACT-MZ02	MN252353.1	96.58
		Bacillus proteolyticus strain A56	MN252089.1	96.58
		Bacillus cereus strain A25	MN252078.1	96.58
		Bacillus toyonensis strain G31	MN134491.1	96.58
		Bacillus licheniformis strain DLSB-13	MK795391.1	96.58
	Hluhluwe	*Paenibacillus alvei*	LS992241.1	99.86
		Paenibacillus alvei strain FJAT-45851	KY038666.1	99.71
		Paenibacillus alvei	LS992241.1	99.86
		Paenibacillus taiwanensis strain ADG20	KP326367.1	99.86
		Paenibacillus sp. MER_181	KT719765.1	99.86
		Paenibacillus sp. MER_184	KT719768.1	99.86
		Paenibacillus sp. strain S3	KU697352.1	99.86
		Bacillus flexus strain ASR-9	KP866883.1	99.14
	Bergville	*Bacterial strains not determined*		
DR SAUNDERS	**Ashburton**	*Bradyrhizobium elkanii* strain Cte-495	MH938226.1	97.43
		Bradyrhizobium sp. strain TUTMGSA133	MK183856.1	97.43
	Izingolweni	*Bradyrhizobium* sp. strain WR23	MK259090.1	98.43
		Bradyrhizobium embrapense TrV2B	LC460915.1	98.43

	Bradyrhizobium embrapense LaR3	LC460901.1	98.43
Hluhluwe	*Bradyrhizobium elkanii* strain Cte-504	MH938235.1	98.43
	Bradyrhizobium elkanii strain Cte-503	MH938234.1	98.43
	Bradyrhizobium elkanii strain Cte-502	MH938233.1	97.15
	Bradyrhizobium embrapense TrV2B	LC460915.1	97.15
	Bradyrhizobium sp. strain TUTMGSA140	MK183857.1	97.15

Table 4.2 Plant biomass and growth kinetics of four *Vigna unguiculata* varieties (IT18, Dr Saunders, Brown mix and Batch white) grown in soils from four sites; Bergville, Ashburton, Izingolweni and Hluhluwe

	Sites			
	Bergville	**Ashburton**	**Izingolweni**	**Hluhluwe**
Varieties	**Total Plant Biomass (g)**			
IT18	7.45±0 99 abcde	10.92±1.11 def	9.03±1.13 cdef	13.69±1.56 f
Dr. Saunders	4.82±0.62 abc	3.10±0.47 a	3.31±0.55 a	6 20±0.64 abcd
Brown mix	4.00±0 96 ab	6.68±0.79 abcd	6.72±0.54 abcd	10.33±0.78 def
Batch white	4.73±1 24 abc	8.23±1.27 bcde	7.04±0.67 abcde	11.87±1.05ef
	Aboveground Biomass (g)			
IT18	6.54±0.86 abcde	9.47±1.11 def	7.69±0.92 bcdef	11.88±1.43 f
Dr. Saunders	4.24±0 57 ab	2.67±0.41 a	2.84±0.53 a	5.56±0.50 abcd
Brown mix	3.12±0.85 a	5.59±0.69 abcd	5.04±0.48 abc	9.12±0.56 cdef
Batch white	3.73±1 14 ab	6.67±1.00 abcde	5.51±0.47 abcd	10.57±0.92 ef
	Belowground biomass (g)			
IT18	0.918±0 13 abcde	1.45±0.11 cde	1.34±0.36 bcde	1.81±0.16 e
Dr. Saunders	0.59±0.05 abc	0.43±0.07 ab	0.38±0.04 a	0.64±0.15 abcd
Brown mix	0.88±0 12 abcde	1.09±0.12 abcde	1.69±0.17 e	1.21±0.26 abcde
Batch white	1.00±0 15 abcde	1.56±0.28 de	1.52±0.32 cde	1.30±0.15 abcde
	Growth rate (g day^{-1})			
IT18	0.12±0.02 abcde	0.18±0.02 def	0.15±0.02 cdef	0.23±0.03 f
Dr. Saunders	0.08±0.01 abc	0.05±0.01 a	0.05±0.01 a	0.10±0.01 abcd
Brown mix	0.07±0.02 ab	0.11±0.01 abcd	0.11±0.01 abcd	0.17±0.01 def
Batch white	0.08±0.02 abc	0.14±0.02 bcde	0.12±0.01 abcde	0.20±0.02 ef
	Relative Growth rate (gg^{-1}day^{-1})			
IT18	0.08±0.01 abc	0.08±0.00 abcd	0.08±0.00 abcd	0.09±0.00 bcde
Dr. Saunders	0.09±0.00 cde	0.08±0.01 abc	0.08±0.01 abc	0.10±0.00 e
Brown mix	0.06±0.01 a	0.06±0.00 a	0.07±0.00 ab	0.07±0.00 ab
Batch white	0.07±0.07 abc	0.10±0.10 e	0.08±0.08 abcde	0.10±0.10 de

Results are presented as averages ± SE, n=10. The different letters indicate significant differences among the treatments at $P<0.05$ based on Tukey's HSD test.

Table 4.3 Plant nutrition of four *Vigna unguiculata* varieties (IT18, Dr Saunders, Brown mix and Batch white) grown in soils from four sites; Bergville, Ashburton, Izingolweni and Hluhluwe

			Sites	
	Bergville	**Ashburton**	**Izingolweni**	**Hluhluwe**
Varieties	**Total Plant N concentration (mmol N g^{-1})**			
IT18	2.26± 0.25	2.61±0.18	2.26±0.29	2.50±0.20
Dr. Saunders	1.98±0.11	1.87±0.22	1.83±0.02	2.11±0.10
Brown mix	2.03±0.11	1.95±0.24	1.70±0.28	2.33±0.32
Batch white	2.07±0.16	2.27±0.35	2.08±0.21	2.02±0.28
P-values	V *			
	S ns			
	V X S ns			
	Standard corrected $^{15}N/^{14}N$			
IT18	-0.18±0.30	0.24±0.23	-0.12±0.30	0.19±0.62
Dr. Saunders	0.17±0.40	0.51±0.52	-0.39±0.31	-0.56±0.22
Brown mix	0.33±0.68	-0.47±0.11	0.62±0.69	-0.20±0.09
Batch white	0.21±0.50	0.21±0.39	0.21±0.37	-0.33±0.40
P-values	V ns			
	S ns			
	V X S ns			
	%Nitrogen Derived From Atmosphere			
IT18	65.18±4.35	59.14±3.34	64.23±4.37	59.84±9.01
Dr. Saunders	60.13±5.82	55.14±7.60	68.18±4.43	70.69±3.24
Brown mix	57.71±9.84	69.41±1.56	53.58±10.06	65.41±1.32
Batch white	59.50±7.22	59.54±5.67	59.53±5.33	67.34±5.74
P-values	V ns			
	S ns			
	V X S ns			
	Nitrogen Derived From Atmosphere (mmol Ng^{-1})			
IT18	1.47±0.17	1.54±0.11	1.45±0.18	1.50±0.12
Dr. Saunders	1.19±0.07	1.03±0.12	1.25±0.02	1.49±0.070
Brown mix	1.17±0.07	1.35±0.17	0.91±0.15	1.53±0.21
Batch white	1.23±0.10	1.35±0.21	1.24±0.13	1.36±0.19
P-values	V *			
	S ns			
	V X S ns			

Results are presented as averages ± SE, n=10, (*, **, ***) denote that means are significantly different at P<0.05; <0.01 and <0.001 respectively, ns means not significant, V=variety, S=soil type

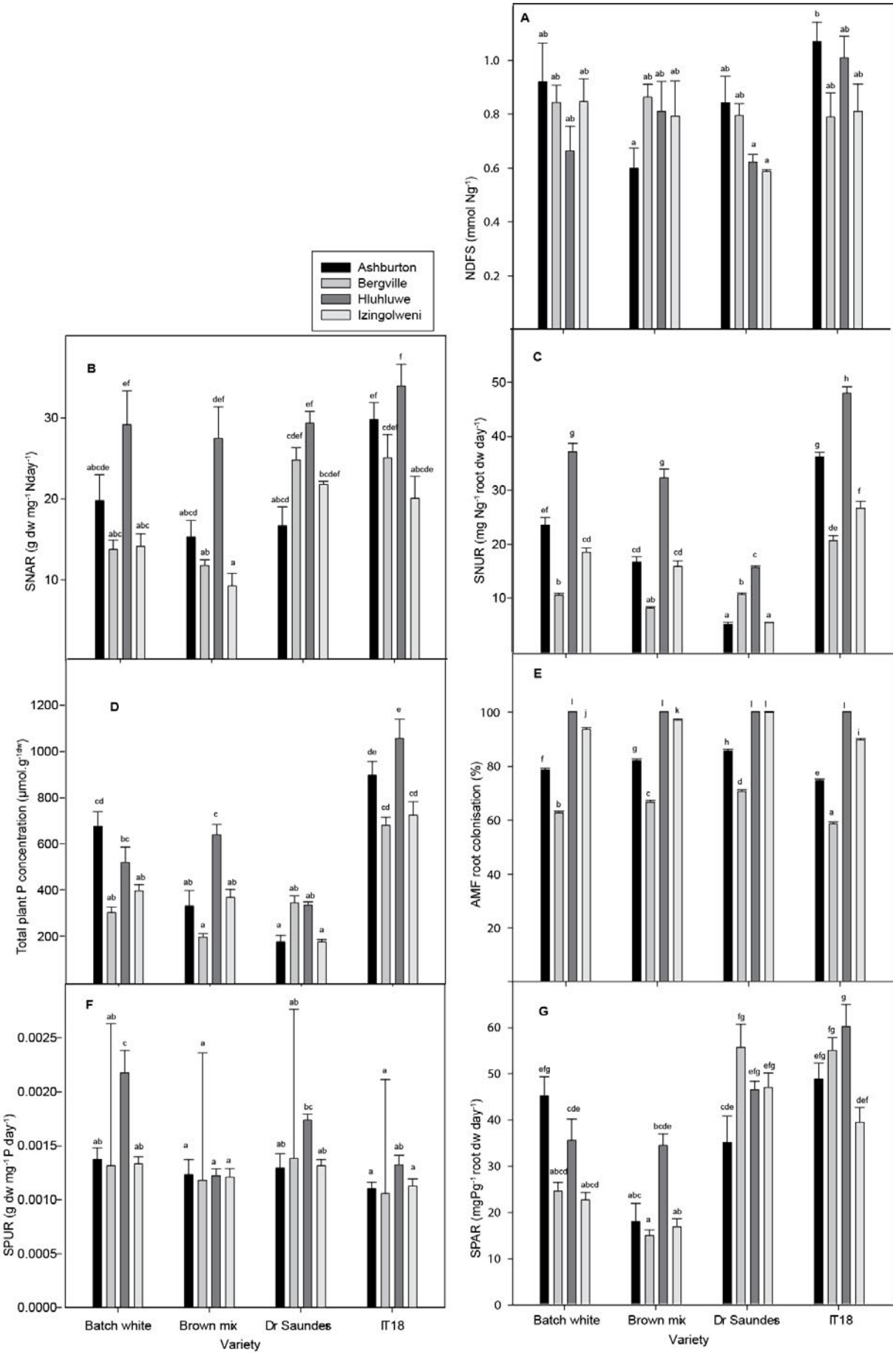
Ashburton
Bergville
Hluhluwe
Izingolweni
A
NDFS (mmol Ng⁻¹)
B
SNAR (g dw mg⁻¹ Nday⁻¹)
C
SNUR (mg Ng⁻¹ root dw day⁻¹)
D
Total plant P concentration (µmol.g⁻¹dw)
E
AMF root colonisation (%)
F
SPUR (g dw mg⁻¹ P day⁻¹)
G
SPAR (mgPg⁻¹ root dw day⁻¹)
Batch white
Brown mix
Dr Saunders
IT18
Variety

Figure 4.1 Effects of variety and soil type on: a) Nitrogen derived from soil (NDFS) b) Specific nitrogen absorption rate (SNAR) c) Specific nitrogen utilization rate (SNUR) d) Total plant P concentration e) AMF root colonization (%) f) Specific phosphorus utilization rate (SPUR) of *Vigna unguiculata* g) Specific phosphorus absorption rate (SPAR). Results are presented as averages ± SE, n=10. The different letters indicate significant differences among treatments at $P<0.05$ based on Tukey's HSD test

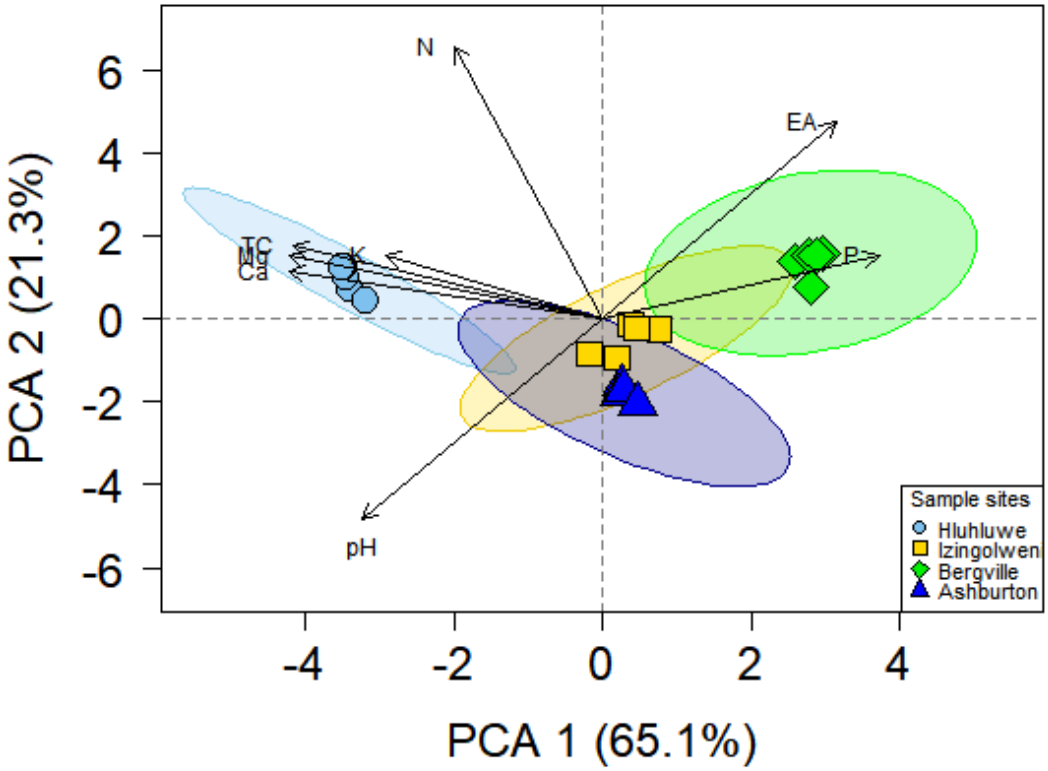

Figure 4.2 Correlations among the physicochemical properties (phosphorus (P), nitrogen (N), potassium (K), carbon (C), exchange acidity (EA), total cations (TC) and pH) of four soil types (Bergville, Ashburton, Izingolweni, Hluhluwe) of KwaZulu-Natal

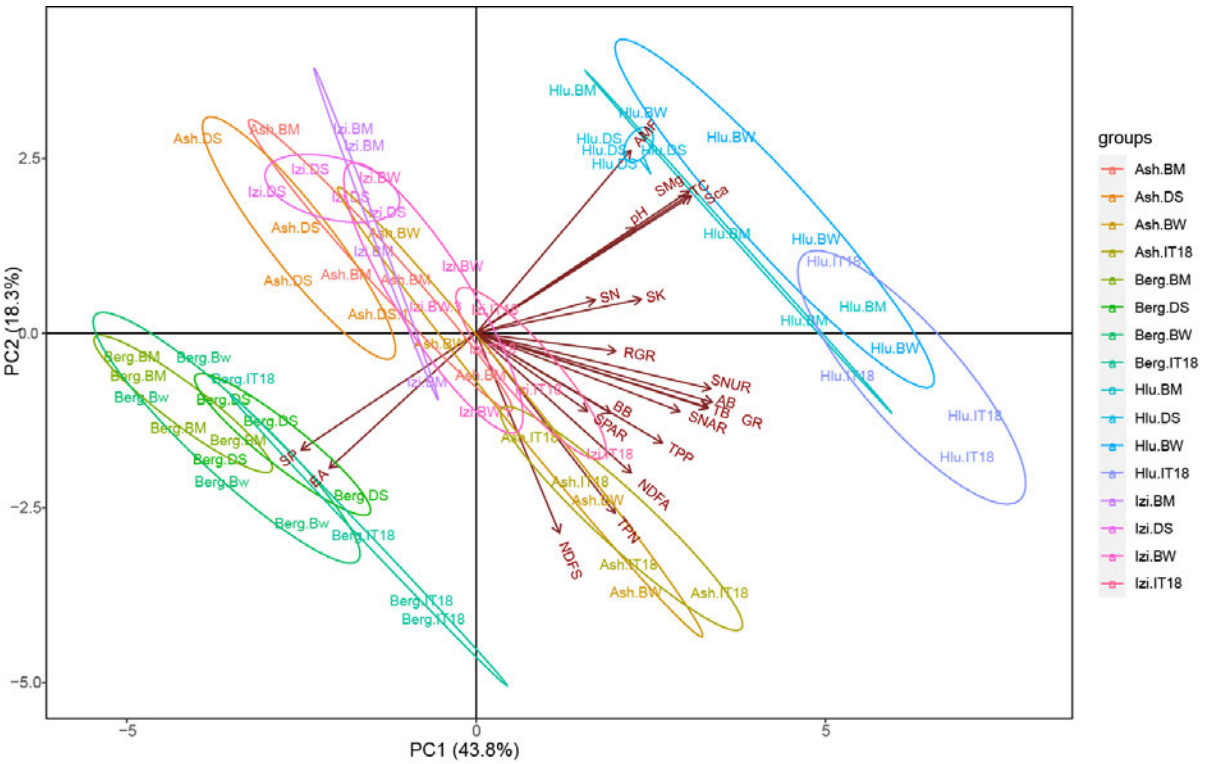

Figure 4.3 Correlations among four *Vigna unguiculata* varieties grown under different soil types of KwaZulu- Natal. Soil types are represented as; Ash- Ashburton, Berg- Bergville, Hlu- Hluhluwe, Izi-Izingolweni. Varieties are represented as BM- Brown mix, DS- Dr Saunders, BW- Batch white, IT18- IT18. The measured soil parameters are represented as; pH- soil pH, SMg- soil magnesium, SCa- soil calcium, SN- soil nitrogen, SK- soil potassium, TC- total cations, AMF- AMF root colonization (%). The plant parameters are represented as; RGR- relative growth rate, GR- growth rate, AB- above ground biomass, BB- below ground biomass, TB- total plant biomass, TPN- total plant N concentration, SNAR- specific nitrogen assimilation rate, SNUR- Specific nitrogen utilization rate, TPP- Total plant P concentration, SPAR- Specific phosphorus absorption rate, SPUR- Specific phosphorus utilization rate, NDFS- Nitrogen derived from soil, NDFA- Nitrogen derived from atmosphere.

Chapter 5: Phenolic acids and antioxidant regulation enhances growth, nutrition and plant-microbe interaction of *Vigna unguiculata* L. (Walp) grown in acidic and nutrient deficient grassland and savanna soil

Phenolic acids and antioxidant regulation enhances growth, nutrition and plant-microbe interaction of *Vigna unguiculata* L. (Walp) grown in acidic and nutrient deficient grassland and savanna soils

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Abstract

Soil acidity and nutrient stress in grassland and savanna ecosystems are the major abiotic factors affecting crop production thus contributing to food insecurity. Some plants including crop legumes may thrive in acidic and nutrient stressed soil environments by regulating their secondary metabolites. In this study, we investigated how four *Vigna unguiculata* varieties regulated their phenolic acid concentrations and antioxidant capacities to better adapt to acidic and nutrient deficient grassland and savanna ecosystems. The *V. unguiculata* varieties included IT18, Batch white, Brown mix and Dr Saunders and these were grown in soils collected from four geographically distinct areas in KwaZulu-Natal (Bergville, Ashburton, Izingolweni, Hluhluwe) covering grassland and savanna ecosystems. All four *V. unguiculata* varieties had varying concentrations of phenolic acids across all soil types with vanillic acid and protocatechuic acid being the most abundant and these constituted 22.59% and 17.22% respectively of the total phenolic acids identified and quantified. Principal component analysis (PCA) showed that the four *V. unguiculata* varieties responded differently to the different soil types with respect to phenolic acid production. There were variations in correlations between the phenolic acids and plant biomass, plant nutrition, soil nutrition and arbuscular mycorrhizal fungi infection. Varieties IT18 and Batch white had relatively higher oxygen radical absorbance capacity (ORAC) across the four soil types. Overall, the current findings demonstrated the influence of phenolic acids and antioxidant activities on the adaptability of *V. unguiculata* in acidic and nutrient stressed grassland and savanna soils.

Key words: Legumes, soil nutrients, antioxidant activities, secondary metabolites, correlation

5.1 Introduction

Soil acidity and nutrient deficiency negatively affect plant growth and productivity reducing food production especially in developing countries (Bagheri et al. 2017, Jaiswal and Dakora 2019, Sharma et al. 2019). In southern Africa, these challenges contribute to food insecurity in smallholder farming systems that are mostly practiced in acidic and nutrient-deficient grassland and savanna ecosystems (Bello et al. 2018, Jaiswal and Dakora 2019). Regulation of secondary metabolites (e.g., phenolic acids) to enhance the growth and nutrient uptake is one of the strategies plants use to acclimatize to soils which are nutrient-stressed (Singh et al. 2014, Per et al. 2017, Hafez et al. 2019, Sharma et al. 2019). Phenolic acids including syringic, caffeic, protocatechuic and salicylic acids contribute to mineral nutrient acquisition by plants through the modification of the rhizosphere to access the unavailable soil nutrients such as P and N (Per et al. 2017, El-Awadi 2018, Hafez et al. 2019, Sharma et al. 2019). Furthermore, these phenolic acids that are often categorized as hydroxybenzoic and hydroxycinnamic acid derivatives enhance nutrient uptake by chelating metallic ions, improving active absorption sites and enhancing mobilization of mineral elements such as Mg, Ca, K, Cu, Mo and Fe (Makoi and Ndakidemi 2007, Youssef et al. 2017, Pontigo et al. 2018, Sharma et al. 2019). Some phenolic acids contribute to enhanced soil nutrient availability by promoting plant-soil microbe interactions (Juszczuk et al. 2004, Mandal et al. 2010). Phenolic acids facilitate symbiotic associations of arbuscular mycorrhizal fungi, and rhizobia with legumes thus enhancing biological nitrogen fixation (Harrison 1999, Werner 2000, Juszczuk et al. 2004, Mandal et al. 2010).

However, phenolic acids may have negative effects on plant mineral nutrient availability in soils and on plant growth depending on their concentrations and plant species (Makoi and Ndakidemi 2007, El-Awadi 2018). Some phenolic acids may have antifungal and antibacterial characteristics and as a result they modulate soil fungal and bacterial communities, respectively thus affecting bioavailability of essential mineral nutrients (Zhou and Wu 2012, Badri et al. 2013, Zhou et al. 2014, Chen et al. 2018). The resultant enhanced bioavailability of mineral nutrients for plant uptake is of paramount importance as this ensures improved nutrient acquisition and assimilation especially for N and P by plants. Therefore, there is need to understand how secondary metabolites, specifically phenolic acids, affect legume N and P nutrition. Moreso, it is pertinent to understand how the production of phenolic acids by crop legumes contribute to their acclimatization during cultivation in acidic and nutrient-deficient grassland and savanna ecosystems (Dakora and Phillips 2002, Pontigo et al. 2018).

Under abiotic stress conditions, plants exude large amounts of reactive oxygen species (ROS) which may result in oxidative damage on lipids, proteins and other cellular components (Rodrigues et al. 2013, Kasote et al. 2015, Akram et al. 2017, Santos et al. 2018). The imbalance in ROS metabolism may result in oxidative damage in legume root nodules and reduce N_2 fixing efficiency (Silveira et al. 2011). However, legumes have established strategies to counter oxidative damage by activating antioxidant defense systems, which may be enzymatic or non-enzymatic (Rodrigues et al. 2013). These antioxidants help scavenge the ROS and thus promote plant tolerance to the abiotic stresses including nutrient stress (Silveira et al. 2011, Santos et al. 2018). Phenolic acids are among the non-enzymatic antioxidants which have radical scavenging properties against the ROS (Zia-Ul-Haq et al. 2013, Kasote et al. 2015).). The antioxidant activities of phenolic acids help protect legume root nodules against oxidative damage thus promoting efficient biological nitrogen fixation and enhanced N acquisition by plants (Silveira et al. 2011). Given this background it is important to understand how antioxidant activities contribute to legume N nutrition under acidic and nutrient stressed conditions.

Vigna unguiculata L. (Walp) (Cowpea) is a legume crop of high importance as it has high protein content and contributes towards food security especially in Africa (Bello et al. 2018, Jaiswal and Dakora 2019). It is a crop widely grown by resource constrained smallholder farmers whose arable lands are often nutrient deficient (Timko et al. 2007, Mohammed et al. 2018). In the present study, we examined the effects of soil acidity and limited nutrition on phenolic acid concentrations in four *V. unguiculata* varieties grown in grassland and savanna ecosystem soils. Furthermore, the study determined the antioxidant capacity of the four *V. unguiculata* varieties after their cultivation. We hypothesized that different *V. unguiculata* varieties will show varying phenolic acids concentrations and antioxidant capacity to maintain plant nutrition and growth in acidic and nutrient-deficient grassland and savanna ecosystem soils.

5.2 Materials and methods

5.2.1 Seed collection and soil analysis

Seeds of four *V. unguiculata* varieties commonly grown by local farmers of South Africa (Brown mix, Dr Saunders, IT18 and Batch white) were obtained from AGT foods Africa, in KwaZulu-Natal, South Africa. Soils were collected from four geographically discrete areas in KwaZulu-Natal (KZN) province, South Africa. These sites included Izingolweni, Southern KZN (30°43′32″S 30°6′10″E, altitude 450 masl); Hluhluwe, Northern KZN (28°0′58″S

32°12′4″E, altitude 100 masl); Ashburton/Pietermaritzburg, Midlands KZN (29°38′55″S 30°26′42″E, altitude 670 masl) and Bergville, Mountainous KZN (28°34′14″S 29°4′17″E, altitude 1040 masl). Twenty soil samples were collected at random from each site. These were 0-30 cm deep and 2 m away from each other. Thereafter, the soil samples were pooled together into composite samples for each site. The composite soil samples were air-dried at room temperature before they were crushed in a soil crusher. Concentration of macronutrients (N, P, K) and pH were measured in the soil samples. The Automated Dumas dry combustion method was used to determine total nitrogen using an LECO CNS 2000 (Leco Corporation, Michigan, USA). Atomic absorption method was used to determine the amount of P and K in the soil according to reported methods by Manson and Roberts (2000).

The procedure consisted of extracting 2.5 ml of soil solution with 25 mL of ambic-2 solution at a pH of 8. The liquid was then agitated at 400 rpm for 10 min with a multiple stirrer before being filtered through Whatman No.1 paper. A KCL solution was used to determine the pH of the soil. The geochemical assessment revealed differences in soil nutrient contents and relative acidity in different KZN soils as shown by (Matiwane et al. 2019). The soils were acidic with low levels of macronutrients as described in Chapter 3 (Table 3.1).

5.2.2 Experimental design and seedling growth

Vigna unguiculata seeds were surface sterilized for 15 min in a bleach solution (30% commercial bleach + 0.02% Triton X-100), rinsed 10 times in sterile water, and air dried in a sterile laminar flow. Prior to germination, the seeds of the four *V. unguiculata* varieties were soaked overnight in distilled water. Seeds were then germinated and cultivated in 20 cm diameter pots filled with soils from the four sites at a greenhouse on the University of KwaZulu-Natal, Pietermaritzburg campus in South Africa.

Each cowpea variety had 30 seedlings for every soil treatment and the experiment was arranged in a randomized complete block design. The greenhouse conditions were as follows; humidity (70 to 80%), the irradiance was approximately 35% of full sunlight, night temperatures reached 12 to 14 °C while day temperatures spanned from 30 to 35 °C. Before seedling emergence, irrigation was done every day with 200 ml of distilled water applied to each pot and thereafter the seedlings were irrigated after every two days. The experiment was carried out from September to December 2018.

5.2.3 Plant harvesting and nutrient analysis

Ten plants per treatment were harvested 12 weeks after seedling emergence and these were separated into stems, leaves, nodules and roots after rinsing with distilled water. Thereafter the

separated plant parts were dried in an oven at 80 °C until all moisture was removed. Recording of dry weights of the samples was then done. A pre-chilled pestle and mortar was used to pulverize the plant material to fine powder. The ground plant material was then sent for plant isotope N and P concentration analysis at commercial laboratories using the LECO-nitrogen analyzer and inductively coupled mass spectrometry (ICP-MS) at the Archaeometry Department, University of Cape Town, and Central Analytical Facilities (CAF), Stellenbosch University, South Africa, respectively.

There were differences in plant biomass and nutrition across all varieties in the four soil types. In terms of the biomass accumulation of the different varieties, the order was as follows; IT18, Batch white, Brown mix and Dr Saunders; from highest to lowest across the four soil treatments. Total plant N concentrations differed statistically among varieties with IT18 recording the highest total plant N concentration. Variety IT18 also had the highest total plant P across the four soil types (Table 4.3).

5.2.4 Determination of arbuscular mycorrhizal fungi colonization percentage and identification of *V. unguiculata* root nodulating bacteria

To determine the AMF root colonization percentage, six roots which had nodules were taken per treatment and these were put in glass vials which contained 50% ethanol. The procedure which involved cleaning and dyeing of the roots and thereafter, microscopic evaluations of colonization structures were done following experimental methods described by Smith and Dickson (1991). There were significant differences in AMF colonization percentages across the four varieties in the different soil treatments and this ranged from 58.5% to 100% (Figure 4.1).

To identify the *V. unguiculata* root nodulating bacteria, five plants were harvested, and nodules were excised from them. Thereafter, surface sterilization of the nodules was done using ethanol and sodium hypochlorite solution. The root nodules were then thoroughly washed using sterilized distilled water (dH_2O) before they were squashed. This was followed by the addition of 100 µl of 15% glycerol to the suspensions. Thereafter, streaking of the suspensions was done on yeast extract mannitol agar plates and incubated at 28 °C which was checked daily for growth of colonies. Repeated streaking was done on yeast extract mannitol agar plates to get pure cultures. Identification of bacteria present in the pure cultures was done by amplifying part of the 16S rDNA gene using PCR reactions. DNA amplification was done, and the acquired amplicons were viewed on 1% agarose gel and they met the expected size (1500 base pairs). Sequencing of the amplicons was done per treatment at the Central Analytical Facilities,

Sequencing Facilities (Stellenbosch University, South Africa). Bacterial strains were identified using BLASTN (National Centre for Biotechnology Information, NCBI (https://www.ncbi.nlm.nih.gov/genbank/). Several bacterial strains were identified in the *V. unguiculata* nodules and these included *Bacillus*, *Paenibacillus*, *Delftia*, *Nitrobacter*, *Rhizobium* and *Bradyrhizobium* (Table 4.1).

5.2.5 Quantification of phenolic acids using Ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS)

For each of the four *V. unguiculata* varieties in each soil treatment, 9 plants were harvested and divided into above-ground (stems and leaves) and below ground (roots and nodules) parts. The above-ground and below ground parts were each pooled into 3 biological samples, and these were immediately freeze-dried and lyophilized to prevent further physiological changes. Phenolic acids extraction was done using 30 mg of the freeze-dried material. Firstly, there was preparation of standard solutions in methanol at a concentration of 10-3 molL-1, thereafter, these were serially diluted to working concentrations. Internal standards of deuterium labelled 4- hydroxybenzoic acid (2,3,5,6 -D4) and salicylic acid (3,4,5,6 -D4) were then added at a final concentration of 10-5 molL-1. UPLC–MS/MS analyses were done using an ACQUITY Ultra Performance LCTM system (Waters, Milford, MA, USA) linked simultaneously to a PDA 2996 photo diode array detector (Waters, Milford, MA, USA) and a Micromass Quattro microTM API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionisation (ESI) source operating in negative mode. To control the instruments and to acquire and process data, MassLynxTM software (version 4.0, Waters, Milford, MA, USA) was used. Thereafter, identification and quantification of the phenolic acids was done using UHPLC-MS/MS according to the method described by Gruz et al. (2008).

5.2.6 Determination of Oxygen Radical Absorbance Capacity of the different *Vigna unguiculata* varieties grown in different soil types of KZN

Determination of oxygen radical absorbance capacity (ORAC) was done using an existing protocol (Ou et al. 2001). Firstly, the above-ground (leaves and stem) and below ground (roots and nodules) cowpea extracts (25 µl) and fluorescein (150 µl, 250 nM) were added in triplicates to the 96-well microplate before shaking. Thereafter, 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was added and shaken for 30s before it was subjected to pre-incubation at a temperature of 37 °C. The fluorescence was incubated at 40 °C, thereafter it

was read every 2 min and this was done for 30 cycles in a microplate reader. Antioxidant capacity (µmol TE/g) was then determined by using the net area under the curve.

5.2.7 Data analysis

Data on plant biomass, plant and soil nutrition, concentrations of phenolic acids and ORAC were analyzed using a two-way analysis of variance (ANOVA) using IBM SPSS Statistics 25 software. For statistical significance ($p \leq 0.05$), the mean values were separated using Tuckey's post hoc test. Phenolic acids data was processed using MassLynxTM software (version 4.0, Waters, Milford, MA, USA).

Relations among the measured values of phenolic acids, the experimental variants of the four cowpea varieties (plant biomass, plant nutrition) and soil nutrition were examined by Principal Component Analysis (PCA). For the PCA statistical procedures PAST version 4.02 was used. Correlations matrix of the variables were determined by Pearson coefficients (p <0.05 and p <0.01).

5.3 Results

5.3.1 Phenolic acids in four *Vigna unguiculata* varieties grown in four soil types

The below ground parts of *V. unguiculata* had higher concentrations of phenolic acids than the above-ground parts. Among the below ground parts, the variety Batch white had the highest phenolic acids while variety IT18 had the least concentration. On the other hand, variety Brown mix above-ground parts had the highest (311.82 $\mu g g^{-1}$) phenolic acid while variety Batch white had the least concentration (96.98 $\mu g g^{-1}$) (Table 5.1).

The quantified phenolic acids were grouped into two classes which were hydroxybenzoic acids (protocatechuic acid (PA); gallic acid (GA), vanillic acid (VA); 4-hydroxybenzoic acid (4HBA); syringic acid (SyA); salicylic acid (SA)) and hydroxycinnamic acids (caffeic acid (CaA); 4-coumaric acid (4-CoA); ferulic acid (FA)). *V. unguiculata* below ground parts had higher concentrations (271.01 $\mu g g^{-1}$) of hydroxybenzoic acids than above-ground parts (139.25 $\mu g g^{-1}$). Bergville soil-grown plants had the highest hydroxybenzoic acids among the below ground parts (Table 5.1). The above-ground parts of the four *V. unguiculata* varieties had higher concentrations of hydroxycinnamic acid than the below ground plant parts (Table 5.1).

In total, 9 phenolic acids were identified in both above-ground and below ground parts of the four *Vigna unguiculata* varieties evaluated. Overall, VA had the highest concentrations, the second one being PA and these constituted 22.59% and 17.22%, respectively of the phenolic

acids identified. Phenolic acids such as 4HBA, 4CoA, FA were moderately abundant, each constituting slightly more than 12% of the phenolic acids while SA and GA were relatively lower constituting 7.73% and 6.99% of the phenolic acids, respectively. SyA and CaA were the least abundant phenolic acids constituting 4.88% and 3.67%, respectively (Figure 5.1A-I). Generally, higher proportions of PA, GA, 4CoA and FA were observed in the above-ground parts while 4CaA, 4HBA, VA, SyA and SA were more concentrated in the below ground parts. The variety IT18 had the lowest concentrations of most of the identified phenolic acids in both below ground and above-ground parts. However, varieties Batch white and Dr Saunders recorded the highest phenolic acid concentrations in below ground parts while varieties Brown mix and Dr Saunders had the highest phenolic acid concentrations in above-ground parts.

5.3.2 Oxygen Radical Absorbance Capacity (ORAC) of the four *V. unguiculata* varieties grown in four different soil types of KZN

Among the four varieties, IT18 and Batch white had higher ORAC compared to varieties Brown mix and Dr Saunders. Izingolweni soil-grown Dr Saunders variety had the highest ORAC in the below ground parts relative to other treatments. Variety IT18 had the highest ORAC in the above-ground parts (Figure 5.3A). As shown in Figure 5.3B and 5.3C the above-ground parts exhibited higher ORAC (69.52 µmol TEg^{-1}) than the below ground parts (44.2 µmol TEg^{-1}).

5.3.3 Correlations between phenolic acids, cowpea varieties and soil properties

The first two principal components of Ashburton soil-grown plants explained 71% of the cumulative variability of measured traits with principal component (PC)1 accounting for 42% and PC2 accounting for 29% of the total variation. Batch white and Dr Saunders were separated along PC1 while IT18 was separated from Brown mix along PC1. However, IT18 was separated from Batch white along PC2. Brown mix was separated from Dr Saunders along PC2. The main factor that separated Batch white from Dr Saunders was phenolic acids RFA, LPA, LSA, L4-CoA, RSA, RGA. Variation in response of IT18 relative to Brown mix was because of soil P, LCaA and total plant N (TPN) (Figure 5.4A).

The first two principal components of Bergville soil-grown plants accounted for 64% of total variation with PC1 contributing 42% and PC2 contributing 22%. IT18 was separated from Dr Saunders along PC1 and the variation in IT18 was as a result of LPA, LSyA, total plant P (TPP) and soil N (Soil N). Variation in Dr Saunders was mainly as a result of soil K, L4HBA, RFA, LVA and LSA. Batch white and Brown mix were separated along PC1 with phenolic acids RSA, LCaA and LSyA being more associated with Batch white while L4CoA, R4HBA, LFA,

RGA, RCaA and LGA were associated with Brown mix. Varieties IT18 and Batch white were closer to each other showing that their response to treatments were not very different (Figure 5.4B).

PC1 and PC2 of Izingolweni soil-grown plants accounted for 65% of the total variation of measured traits. PC1 contributed 49% of the total variation while PC2 contributed 16%. Batch white was separated from IT18 along PC2 with variation in Batch white being as a result of phenolic acids RFA, R4CoA, RCaA, RSA while variation in IT18 was mainly as a result of soil P. Dr Saunders was separated from IT18 along PC1 and the variation in Dr Saunders was as a result of phenolic acids LSA, LVA, L4HBA, LCaA, LGA, L4CoA while the variation in IT18 was mainly associated with soil P (Figure 5.4C).

Cumulatively PC1 and PC2 for Hluhluwe soil-grown plants contributed 69% of the total variation with PC1 contributing 40% and PC2 contributing 29%. Batch white was separated from Brown mix along PCA1 with variations in Batch white being as a result of phenolic acids RSA, RVA and RCaA. However, variations in Brown mix were because of LFA, RGA and LGA (Figure 5.4D).

The correlation matrix shows the relationship between both the above-ground and below ground phenolic acids, soil nutrient compositions, plant biomass and nutrition (Table 5.2). Total plant biomass was negatively correlated to above-ground SyA and below ground PA while it was positively correlated with below ground 4HBA. Total plant N was negatively correlated to above-ground PA and SyA while it was positively correlated to below ground 4HBA. Similarly, total plant P was negatively correlated to above-ground PA, SyA and to below ground PA. There was no significant correlation between the phenolic acids and soil N and soil K. However negative correlations were recorded between soil P and above-ground CaA and FA (Table 5.2). There was a negative correlation between most phenolic acids and plant absorption and utilization rates, growth rates and plant biomass (Table 5.3). However, CaA was positively correlated with specific phosphorus utilization rate.

5.4 Discussion

Phenolic acids perform an essential role in plant metabolic and physiological processes especially in response to abiotic stress (Per et al. 2017, Hafez et al. 2019, Sharma et al. 2019, Perchuk et al. 2020). The high levels of phenolic acids observed in the four cowpea varieties may have been synthesized to solubilize mineral nutrients especially N, P and K which were deficient or may have been in insoluble complex forms in these experimental soils (Hafez et

al. 2019, Sharma et al. 2019). The enhanced bioavailability of these soil nutrients thus contributed to the growth of *V. unguiculata* under these acidic and nutrient poor conditions. Plants grown in highly acidic and infertile soils are reported to synthesize large quantities of phenolics as a survival mechanism to enhance nutrient uptake and tolerance to the low nutrient environment (Northup et al. 1998, Rengel 1999, Juszczuk et al. 2004). Also, soil nitrogen and phosphorus deficiency have been shown to enhance the biosynthesis of phenolic acids by different plant tissues (Dakora and Phillips 2002, Rengel 2002).

In the present study, the phenolic acids which were identified in the four *V. unguiculata* varieties are similar to those observed by other researchers in different *V. unguiculata* studies, some under optimal, saline and drought stress conditions (Gao et al. 2017, Moreira-Araújo et al. 2017, Alidu et al. 2020, Perchuk et al. 2020). However, variations were observed in phenolic acid concentrations in the present study relative to previous studies and this can be attributed to differences in cowpea genotypes and environmental growth conditions (Gao et al. 2017, Alidu et al. 2020). The high concentrations of total hydroxybenzoic acids in the below ground parts of Bergville soil-grown plants could be as a result of the elevated acidity of the Bergville soils and their low nutrient status. Such response by the *V. unguiculata* likely enhances bioavailability of soil nutrients for plant uptake. The current study also demonstrated that VA and PA are the major phenolic acids in the four *V. unguiculata* varieties making up 39.81% of the phenolic acid pool.

In the current study, the presence of SyA, CaA and PA in the different cowpea varieties could have enhanced solubilization and accumulation of inorganic P thereby enhancing it availability for plant acquisition, as hypothesized by Makoi and Ndakidemi (2007). Furthermore, SA plays a significant role as it regulates several physiological processes in *V. unguiculata* including acquisition of mineral nutrients, photosynthesis and stress tolerance (Khan et al. 2003, Per et al. 2017, El-Awadi 2018, Hafez et al. 2019). Particularly, SA enhanced the acquisition of Mg, N, P and K in *Zea mays* under boron and drought (Gunes et al. 2007) as well as enhancing the nutrient uptake in *Brassica* species (Muhal et al. 2014). (Hafez et al. 2019) reported that SA promoted N, P and K uptake in wheat resulting in increased biomass accumulation. In the present study, there was a negative correlation between some phenolic acids (PA, 4HBA and 4CoA) and nitrogen utilization rate and between SA and nitrogen assimilation rates which is an indication that these phenolic acids promote plant nitrogen utilization at low concentrations of soil N.

The phenolic acids (CaA, FA, 4CoA and 4HBA) identified in the present study could have enhanced plant nutrient uptake especially P and N through promoting plant-microbe

interactions (Kumar and Goel 2019). These phenolic acids act as signaling molecules at the start of arbuscular mycorrhizal and legume-*Rhizobium* symbioses (Mandal et al. 2010, Kumar and Goel 2019). It is also known that CaA promotes plant-arbuscular mycorrhizal symbiotic interactions which promotes P acquisition by plants in the nutrient stressed environments (Zafar-ul-Hye et al. 2020). In our study, the positive correlation between phosphorus utilization rate and CaA implies that high concentrations of CaA promoted P use by the *V. unguiculata*. The phenolic acids such as FA (Kuiters 1990), 4CoA and 4HBA (Mandal et al. 2010) could stimulate spore germination, hyphal growth, branching and enhanced arbuscular mycorrhizal establishment (Mandal et al. 2009, Mandal et al. 2010, Klein et al. 2015, Kumar and Goel 2019). This could have contributed to the high arbuscular mycorrhizal fungi (AMF) colonization percentages which were observed in the *V. unguiculata* roots (Figure 4.1). There was, however, no significant correlation between AMF infection and phenolic acids except for VA which had a negative correlation. The negative correlation could imply that high concentrations of VA inhibit AMF establishment and its infection in the roots of *V. unguiculata*.

The phenolic acids such as PA, VA, CaA, 4CoA and 4HBA identified in this study could have contributed to enhanced biological nitrogen fixation by promoting rhizobial growth and nodule development. Several bacterial strains were identified in the *V. unguiculata* nodules and these included *Bacillus, Paenibacillus, Rhizobium, Bradyrhizobium and Delftia* strains. The phenolic acids could have been used as alternative carbon sources by the *Rhizobium*. This is evidenced by the study by Seneviratne and Jayasinghearachchi (2003) who confirmed that PA, p-CoA, FA and VA were effectively used as carbon sources by different rhizobial strains (*Bradyrhizobium elkanii*, *B. japonicum* and *Azorhizobium caulinodans*). The use of phenolic acids by *Rhizobium* as carbon sources has been reported by Irisarri et al. (1996). When *Rhizobium* utilize phenolic acids, this leads to biochemical and physiological alterations of the *Rhizobium* which in turn affects their persistence in the soil and their biological nitrogen fixation efficiency (Seneviratne and Jayasinghearachchi 2003). The high rates of biological nitrogen fixation in the four *V. unguiculata* varieties across the four acidic and nutrient poor soils in our study may be partially attributed to these phenolic acids.

The phenolic acids PA, VA, CaA, 4CoA and 4HBA exuded by *V. unguiculata* could have helped in nod gene expression regulation of the *Rhizobium* and they could have modified the legume-*Rhizobium* symbiosis (Schultze and Kondorosi 1998, Seneviratne and Jayasinghearachchi 2003). In earlier studies, PA and p-HBA acids were shown to effectively induce nod gene in root nodules of *Mimosa pudica* (Mandal et al. 2016). It is evidenced that

CaA enhanced the enzymatic levels in biological nitrogen fixation and it increased the levels of carbohydrates and proteins in legume nodules (Dhir et al. 1992). Furthermore, CaA enhanced the function of leghemoglobin in root nodules (Zafar-ul-Hye et al. 2020). There was however no significant correlation between nitrogen derived from atmosphere and the phenolic acids in this present study and this could be attributed to the concentrations or the environment (El-Awadi 2018). Some phenolic acids which are hydroxybenzoic derivatives such as 3,4HBA and p-HBA function as chemo attractants which guide rhizobial cells to legume root hairs (Aguilar et al. 1988, Bhattacharya et al. 2010, Mandal et al. 2010). The phenolic acids PA and 4HBA which were relatively in high concentrations in the *V. unguiculata* varieties in the current study could have possibly regulated nod gene expression of the *Rhizobium* and thus enhancing legume-*Rhizobium* symbiosis and nodule development (Dakora and Phillips 1996, Mandal et al. 2010, Sharma et al. 2019). This in turn could have contributed to the high biological nitrogen fixation rates of >60% which were observed in this study across all soil types. The positive correlation between below ground 4HBA and total plant N shown in the present study could thus be attributed to the enhanced *Rhizobium* functioning and increased biological nitrogen fixation efficiency (Bhattacharya et al. 2010). There was a negative correlation between total plant N and phenolic acids such as 4CoA and SA. This is in agreement with findings of several researchers who suggested that high phenolic acids concentrations may inhibit growth of plants, uptake of nutrients and the plants' oxidative capacity (Seneviratne and Jayasinghearachchi 2003, Khan et al. 2010b, Afshari et al. 2013).

In the present study, FA, SyA, 4HBA and VA could have regulated the abundance and distribution of different species of both bacteria and fungi in the rhizosphere and thus affecting plant nutrient uptake especially N and P. In a study by Kuiters (1990), FA stimulated growth of fungi including *Dothichiza pityophila* and *Thysanophora penicilloides*. It is also known that SyA resulted in a reduction in richness, evenness and diversity indices of rhizosphere bacterial community (Zhou et al. 2014). SyA applied at 0.1 $\mu molg^{-1}$ resulted in an increase in abundance of bacterial phylum Proteobacteria and fungal classes *Leotiomycetes*, *Pezizomycetes*, *Tremellomycetes* and *Eurotiomycetes*. In addition, SyA decreased the relative abundance of bacteria of phylum *Firinicutes* and fungal class *Sordariomycetes* (Wang et al. 2018). Ferulic acid inhibited growth of *Pseudomonas fluorescens* and *Glomus intraradices* (Medina et al. 2011, Lemos et al. 2014). Furthermore, p-HBA resulted in a decrease in soil bacterial community and increased rhizosphere fungal community in cucumber plants (Zhou et al. 2012). In the present study, there was no correlation between *V. unguiculata* N and P assimilation rates and all phenolic acids, except SA which showed a negative correlation with

N assimilation rate (Table 5.4). This suggests that low concentrations of SA promote N acquisition by *V. unguiculata* while high concentrations have an inhibitory effect. This is in line with the sentiments suggested by several researchers that some phenolic acids may exert inhibitory effect on nutrient availability, plant-microbe interaction and plant growth depending on their concentrations (Kuiters 1990, Hättenschwiler and Vitousek 2000, El-Awadi 2018).

In the current study, the high concentrations of FA, 4CoA and VA in cowpea variety Dr Saunders possibly discouraged growth of the legume resulting in reduced biomass and lower plant P content (Table S5.2). A negative correlation was observed between phenolic acids PA, SyA and plant nutrients N and P (Table 5.3) implying that high concentrations of PA and SyA could have lowered N and P uptake by *V. unguiculata*. Phenolic acids are known to have either stimulatory or inhibitory effects on plant nutrient uptake depending on their concentrations, and this may ultimately affect plant growth (El-Awadi 2018). High phenolic acid concentrations may inhibit uptake of nutrients such as N, P, Ca, Mo and Fe by plants (El-Awadi 2018). Our findings are in line with previous findings by Glass (1973) who demonstrated that hydroxybenzoic acid derivatives including benzoic, 2-hydroxybenzoic, 4-hydroxybenzoic, 3.4 dihydroxybenzoic and cinnamic acids inhibited P uptake in barley (*Hordeum vulgare* L.) by altering membrane properties of the crop. Para-hydroxybenzoic acid inhibited growth of cucumber roots through reduction of meristematic activity and by reducing reactive oxygen species levels (Huang et al. 2020). In addition, VA, FA and p-CoA inhibited P uptake in cucumber (Lyu et al. 1990, Lehman and Blum 1999). Application of FA resulted in reduced uptake of net P and K by 75 and 29%, respectively in seedlings of cucumber plants (Lyu et al. 1990). In the current study, cowpea varieties IT18 and Batch white had relatively lower concentrations of the phenolic acids but these had the highest plant biomass (Table 4.2), thus suggesting that the lower phenolic acid concentrations had a stimulatory effect on nutrient uptake and plant growth. The principal component analysis showed distinct separation among the four *V. unguiculata* varieties in the four soil treatments, and this confirms variation in the response of the cowpea varieties to nutrient stress with respect to phenolic acid exudation. The grouping of the specific phenolic acids closer to specific *V. unguiculata* varieties suggests a positive role of the respective phenolic acids in enhancing adaptability of the legume to nutrient stress.

Plants produce reactive oxygen species (ROS) and under stress conditions, the ROS are produced in higher quantities which may hinder growth and development of plants (Kasote et al. 2015, Santos et al. 2018). To counteract this, plants naturally produce antioxidants, such as phenolic acids, which reduce ROS production by scavenging the free radicals (Kumar and Goel

2019). Thus, the concentrations of phenolic acids quantified in the current study could have contributed to the strong antioxidant capacities of the four *V. unguiculata* varieties. In our study, the ORAC values were from 102.38 to 127.74 µmol TEg^{-1}. These values were relatively higher than those observed in four *V. unguiculata* varieties grown in Pakistan which ranged from 83.8 to 96.2 µmol TEg^{-1} (Zia-Ul-Haq et al. 2013). In a related study in Japan, two *V. unguiculata* varieties which were evaluated had higher ORAC values of 367.5 and 540.6 µmol TEg^{-1} for red and black cowpea types, respectively (Orita et al. 2019). The differences in ORAC values could be attributed to differences in genotypes, growing conditions and differences in the cowpea parts evaluated. The high antioxidant activity exerted by the different *V. unguiculata* varieties grown in the nutrient-stressed soils is a positive attribute as this helps the legume reduce ROS levels thus enabling the plants to adapt to nutrient stress conditions and to grow optimally (Santos et al. 2018). Furthermore, *V. unguiculata* may effectively fix nitrogen through their nodules as they are protected from oxidative damage that may otherwise be caused by excessive ROS (Silveira et al. 2011). Varieties IT18 and Batch white had relatively higher ORAC values than varieties Dr Saunders and Brown mix. Such high antioxidant capacity of IT18 and Batch white could have contributed to better nutrient uptake and higher biomass accumulation (Table 4.2).

5.5 Conclusion

From this study, it was evident that *V. unguiculata* has the potential of adapting to acidic and nutrient stress conditions through production of phenolic acids and enhanced antioxidant capacity. The four *V. unguiculata* varieties produced different phenolic acids at different concentrations with vanillic acid and protocatechuic acid being the most abundant while caffeic acid and syringic acid were the least abundant. The phenolic acids may have contributed to *V. unguiculata* survival through enhanced nutrient solubilization and utilization, altered bacterial and fungal communities, enhanced plant microbe interaction and enhanced biological nitrogen fixation.

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Authors' contributions BTM, AM and AOA conceived research idea. BTM conducted the growth experiments and prepared the draft manuscript. BTM, AM and AOA and JG performed analysis of phenolic acids, analyzed data and edited the manuscript. All authors read and agreed to the final version of the manuscript.

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Consent for publication All authors agree for publication

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Table and Figure legends

Table 5.1 Concentrations (µgg-1) of phenolic acids for the below ground and above-ground parts of four *Vigna unguiculata* varieties (IT18, Dr Saunders, Brown mix and Batch white) grown in four soil types (Bergville, Ashburton, Izingolweni, Hluhluwe) of KwaZulu-Natal

Variety	**Soil type**	**Hydroxybenzoic acid concentration (µgg^{-1})**		**Hydroxycinnamic acid concentration (µgg^{-1})**		**Total**
		Belowground	Above-ground	Belowground	Above-ground	
IT 18	Bergville	336.01±107.48	91.90±8.44	50.64±7.36	56.43±4.22	534.98
	Ashburton	139.10±51.78	60.37±8.47	31.88±3.22	64.26±6.64	295.61
	Izingolweni	199.49±26.88	93.61±17.68	55.38±5.45	57.95±2.18	406.43
	Hluhluwe	143.01±34.67	83.76±8.28	42.36±11.10	74.50±3.66	343.63
	Total	817.61	329.64	180.26	253.14	1580.65
Dr	Bergville	301.02±50.97	148.55±17.39	124.71±26.01	62.67±2.38	636.95
Saunders	Ashburton	249.50±5.77	115.45±12.73	142.08±4.79	66.58±7.96	573.61
	Izingolweni	142.01±36.60	238.68±48.58	31.60±11.48	226.45±70.11	638.74
	Hluhluwe	211.97±47.03	188.71±17.97	143.61±23.63	77.42±17.36	621.71
	Total	904.50	691.39	422.00	433.12	2451.01
Brown	Bergville	426.91±42.20	127.69±12.53	96.56±19.19	94.34±17.46	745.5
mix	Ashburton	224.75±67.45	187.65±32.24	39.50±2.48	103.95±21.59	555.85
	Izingolweni	215.56±6.577	239.06±56.30	54.8±4.39	105.57±8.35	614.99
	Hluhluwe	165.79±43.72	265.50±80.46	58.84±19.48	123.53±8.91	613.66
	Total	1033.01	819.90	249.70	427.39	2530
Batch	Bergville	395.67±85.52	106.49±8.59	32.22±4.98	79.04±14.08	613.42
white	Ashburton	531.58±94.50	70.46±11.38	55.12±13.62	81.01±3.53	738.17
	Izingolweni	421.11±39.05	125.73±16.42	62.45±4.78	99.02±7.19	708.31
	Hluhluwe	232.69±6.94	85.24±2.62	88.92±4.96	82.49±3.62	489.34
	Total	1581.05	387.92	238.71	341.56	2549.24
***p* values**	V	<0.001*	<0.001*	<0.001*	<0.05*	
	S	<0.01*	<0.05*	<0.01*	<0.01*	
	V x S	ns	Ns	<0.001*	<0.01*	

Data are presented as mean ± SE, n=3. *Significantly different and ns= not significant at $p \leq 0.05$. Hydroxybenzoic acids consisted of protocatechuic acid, gallic acid, vanillic acid, 4-hydroxybenzic acid, syringic acid and salicylic acid. Hydroxycinnamic acids consisted of caffeic acid, 4-coumaric acid and ferulic acid. V = variety, S = soil type, V x S = variety x soil type.

Table 5.2 Correlations between the above-ground and below ground phenolic acids and plant biomass, plant nutrient properties of four *Vigna unguiculata* varieties and soil nutrient compositions.

	TPB	**TPN**	**TPP**	**Soil P**	**Soil N**	**Soil K**
LPA	-.483	-.515*	-.573*	.799	.274	-.355
LGA	-.070	-.129	-.219	.071	.199	-.605
L4HBA	-.336	-.283	-.265	-.654	.201	.021
LCaA	-.219	-.157	-.120	-.989*	.185	.729
LVA	-.296	-.287	-.279	-.413	.755	.060
LSyA	-.647**	-.646**	-.612*	.767	.106	-.820
L4CoA	-.393	-.388	-.411	-.159	.948	.494
LFA	.016	-.046	-.227	-.970*	.108	.751
LSA	-.415	-.434	-.440	-.393	.835	.124
RPA	-.593*	-.570*	-.530*	.853	.215	-.552
RGA	-.254	-.294	-.266	.442	.044	-.834
R4HBA	-.604*	-.595*	-.456	.900	.109	-.534
RCaA	.338	.213	.020	.064	.555	-.408
RVA	-.163	-.158	-.079	.740	.065	-.122
RSyA	-.281	-.303	-.270	.851	.182	-.413
RCoA	-.478	-.468	-.452	.892	.103	-.690
RFA	-.368	-.356	-.388	-.306	.392	-.263
RSA	-.117	-.189	-.117	.599	.263	-.744

Descriptions of the phenolic acids are as shown in Figure 5.1. TPB- Total plant biomass, TPN- Total plant nitrogen, TPP- Total plant phosphorus, Soil P- soil phosphorus, Soil N- soil nitrogen, Soil K- soil potassium. *Correlation is significant at 0.05 level, **Correlation is significant at 0.01 level.

Table 5.3 Correlation analysis of phenolic acids with plant biomass, plant nutrition, growth rate and arbuscular mycorrhizal fungi colonization

	Plant N	NDFA	NDFS	SNAR	SNUR	Plant P	SPAR	SPUR	AMF %	Plant Biomass	Growth rate
PA	-.493	-.235	-.563*	-.097	-.617*	-.654**	-.033	.208	.146	-.605*	-.605*
GA	-.332	-.200	-.322	-.300	-.113	-.226	-.485	-.262	.322	-.081	-.081
4HBA	-.338	-.338	-.148	-.408	-.648**	-.490	-.040	-.069	-.428	-.645**	-.645**
CaA	-.144	-.034	-.212	.196	.291	.001	-.083	.699**	.487	.322	.322
VA	.032	-.186	.308	-.400	-.191	-.092	-.231	.000	-.572*	-.178	-.178
SyA	-.149	-.224	.036	-.495	-.320	-.281	-.380	.023	-.347	-.293	-.293
4CoA	-.534*	-.323	-.518*	-.141	-.601*	-.624**	.018	.039	.174	-.620*	-.620*
FA	-.455	-.287	-.426	-.038	-.392	-.570*	-.180	.381	.458	-.391	-.391
SA	-.535*	-.478	-.309	-.547*	-.401	-.365	-.321	-.046	.107	-.351	-.351

Descriptions of the phenolic acids are as shown in Figure 5.1. Plant N- total plant nitrogen, NDFA- percentage of nitrogen derived from atmosphere, NDFS- percentage of nitrogen derived from soil, SNAR- specific nitrogen absorption rate, SNUR- specific nitrogen utilization rate, Plant P- total plant phosphorus, SPAR- specific phosphorus absorption rate, SPUR- specific phosphorus utilization rate, AMF%- arbuscular mycorrhizal fungi colonization rate. **Correlation is significant at the 0.01 level. *Correlation is significant at the 0.05 level.

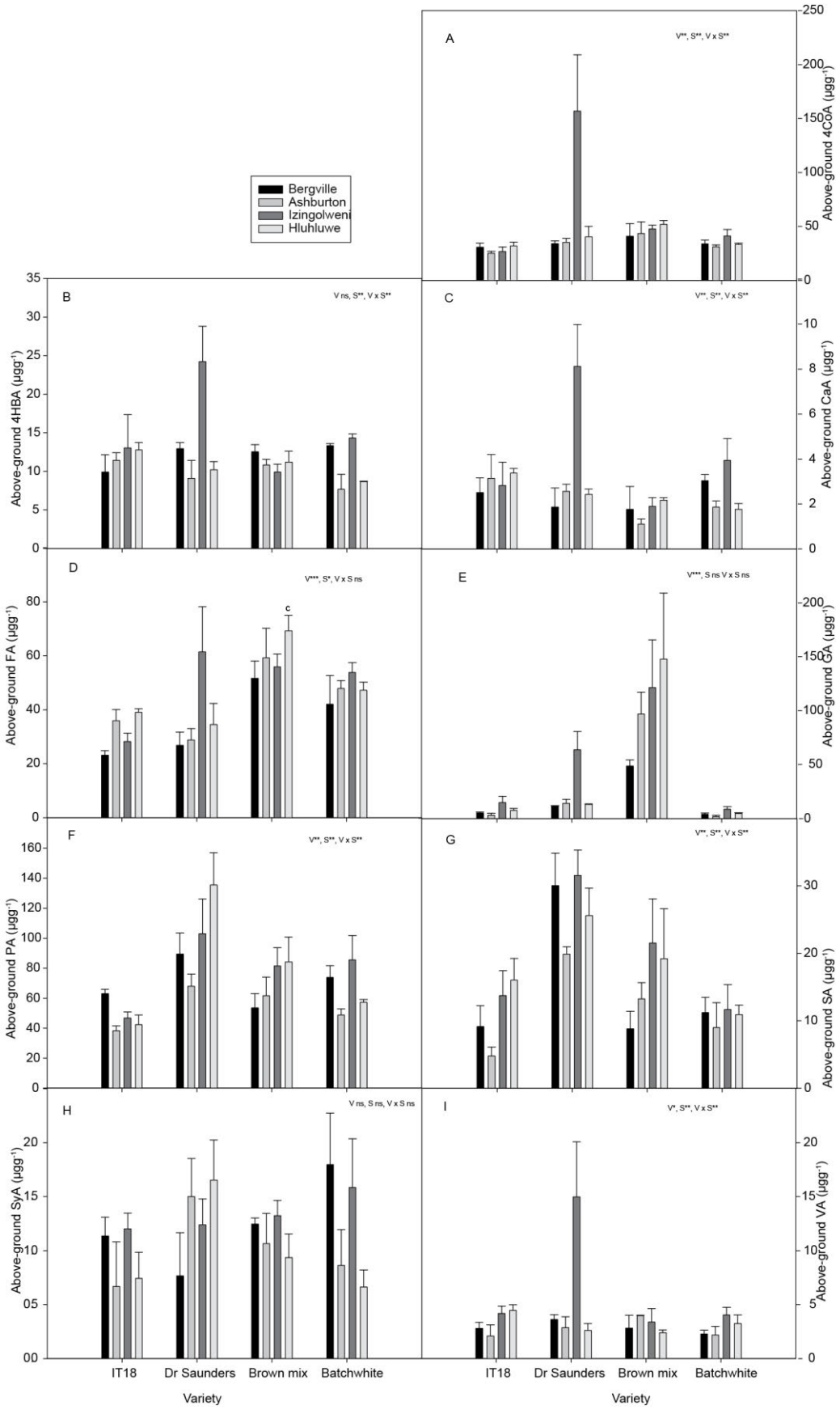
Bergville
Ashburton
Izingolweni
Hluhluwe
A
V**, S**, V x S**
Above-ground 4CoA (µgg-1)
B
V ns, S**, V x S**
Above-ground 4HBA (µgg-1)
C
V**, S**, V x S**
Above-ground CaA (µgg-1)
D
V***, S*, V x S ns
Above-ground FA (µgg-1)
E
V***, S ns V x S ns
Above-ground CA (µgg-1)
F
V**, S**, V x S**
Above-ground PA (µgg-1)
G
V**, S**, V x S**
Above-ground SA (µgg-1)
H
V ns, S ns, V x S ns
Above-ground SyA (µgg-1)
I
V*, S**, V x S**
Above-ground VA (µgg-1)
IT18
Dr Saunders
Brown mix
Batchwhite
Variety

Figure 5.1 Concentrations of specific phenolics (Protocatechuic acid (PA); gallic acid (GA); 4-hydroxybenzoic acid (4HBA); caffeic acid (CaA); vanillic acid (VA); syringic acid (SyA); 4-coumaric acid (4CoA); ferulic acid (FA); salicylic acid (SA)) in the above-ground parts of four *Vigna unguiculata* varieties (IT18, Dr Saunders, Brown mix and Batch white) grown in soils sourced from four sites of KwaZulu-Natal (Bergville, Ashburton, Izingolweni, Hluhluwe). Results are presented as averages ± SE, n=3, (*, **, ***) denote that means are significantly different at p <0.05; <0.01 and <0.001 respectively, ns=means not significant, V=variety, S=soil type, V x S= variety x soil type.

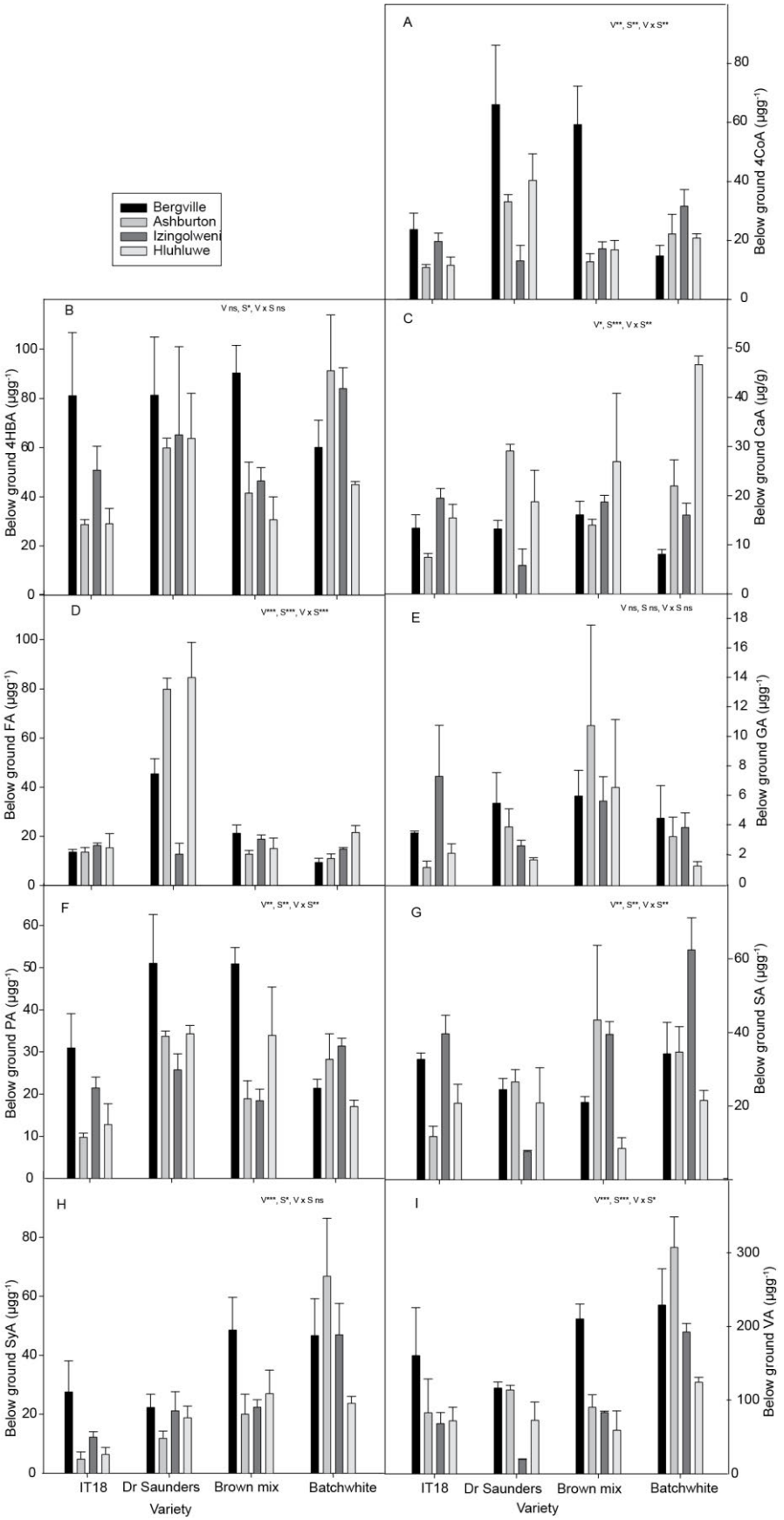

Figure 5.2 Concentrations of specific phenolics (Protocatechuic acid (PA); gallic acid (GA); 4-hydroxybenzoic acid (4HBA); caffeic acid (CaA); vanillic acid (VA); syringic acid (SyA); 4-coumaric acid (4CoA); ferulic acid (FA); salicylic acid

(SA)) in the below ground parts of four *Vigna unguiculata* varieties (IT18, Dr Saunders, Brown mix and Batch white) grown in soils sourced from four sites of KwaZulu-Natal (Bergville, Ashburton, Izingolweni, Hluhluwe). Results are presented as averages ± SE, n=3, (*, **, ***) denote that means are significantly different at p <0.05; <0.01 and <0.001 respectively, ns=means not significant, V=variety, S=soil type, V x S= variety x soil type.

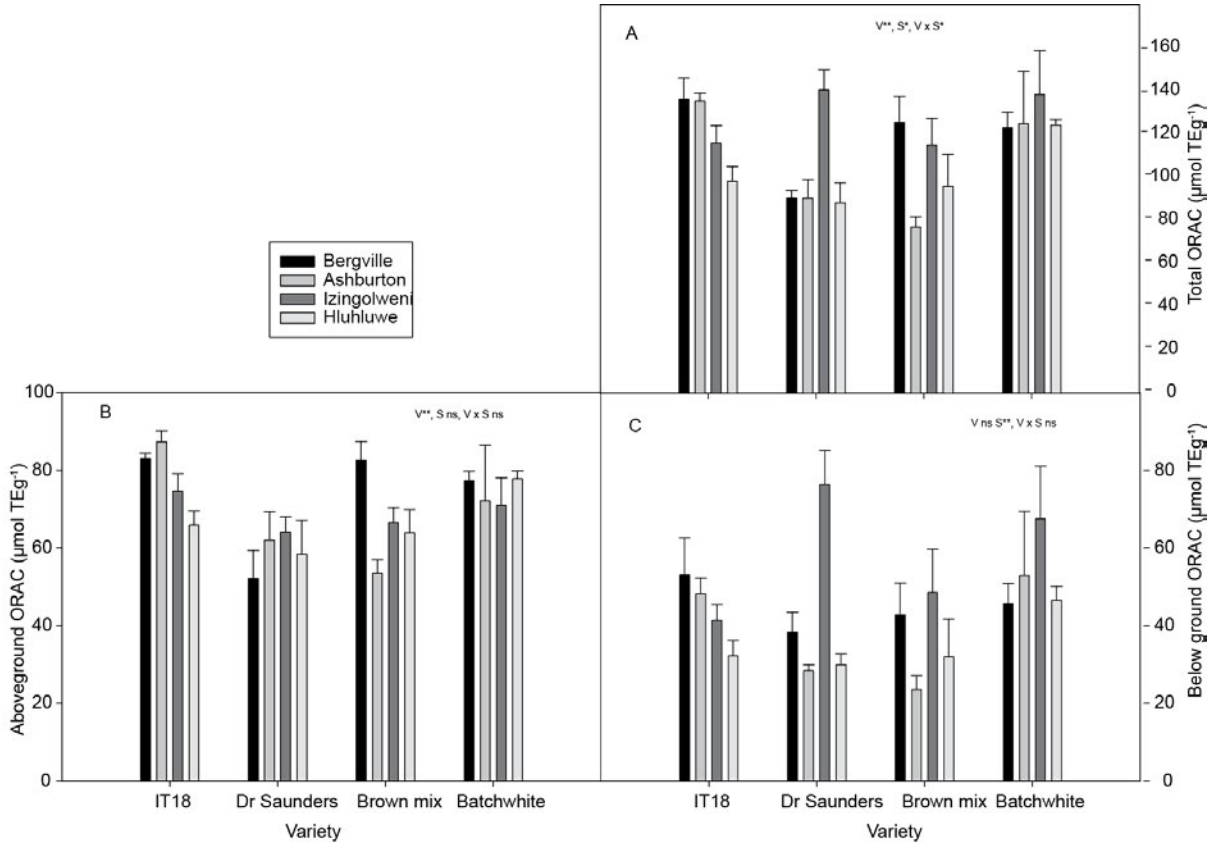

Figure 5.3 (A) Total (B) Above-ground and (C) Below ground oxygen radical absorbance capacity (ORAC) of four *Vigna unguiculata* varieties (IT18. Dr Saunders. Brown mix and Batch white) grown in four soil types (Bergville, Ashburton, Izingolweni, Hluhluwe) sourced from KwaZulu-Natal. Results are presented as averages ± SE. n=3. (*, **, ***) denote that means are significantly different at p <0.05; <0.01 and <0.001 respectively. ns=means not significant. V=variety. S=soil type. V x S= variety x soil type.

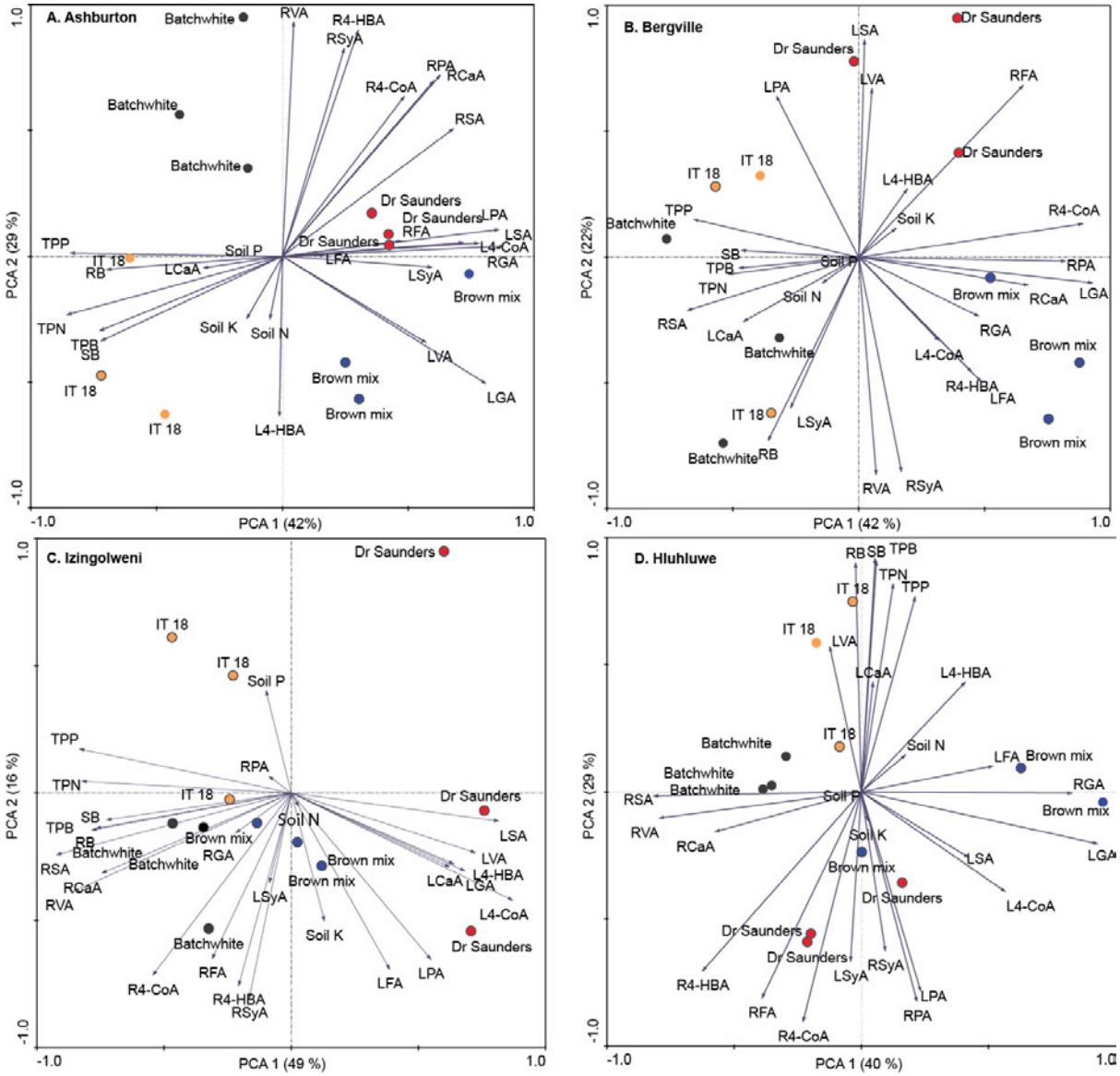

Figure 5.4 Correlations between above-ground and below ground phenolic acids of four *Vigna unguiculata* varieties (IT18, Dr Saunders, Brown mix, Batch white) grown in different soils from four regions (**A**- Ashburton; **B**- Bergville; **C**- Izingolweni; **D**- Hluhluwe) of KwaZulu-Natal. Description of the phenolic acids is as shown in Figure 5.1. The below ground phenolic acids are prefixed with 'R' while the above-ground phenolic acids are prefixed with 'L' The soil properties were soil potassium (K), phosphorus (P) and nitrogen (N).

Chapter 6: General Conclusion and Recommendations

Most savanna and grassland ecosystems are characterized by acidic and nutrient poor soils which do not support crop productivity (Abd-Alla et al. 2014, Liu et al. 2018, Jaiswal and Dakora 2019, Parihar et al. 2019). These conditions are exacerbated by rapid population increase, poor agricultural practices and overgrazing thereby contributing to global food insecurity (Conant et al. 2001, Sanderson et al. 2013). Nitrogen and phosphorus are among the most limiting nutrients in the savanna and grassland ecosystems (Liu et al. 2018, Billah et al. 2019) and their limited availability poses as a serious threat especially to resource constrained small-scale farmers who cannot afford to buy synthetic fertilizers (Khan et al. 2010a, Chidebe et al. 2018, Oruru et al. 2018). More so, the continued use of synthetic fertilizers causes pollution to the environment (Abd-Alla et al. 2014, Valentine et al. 2018). To mitigate the challenge of poor soil fertility, legumes can be utilized as they can fix atmospheric N_2 to NH_4^+ which can be used by plants (Oruru et al. 2018, Valentine et al. 2018). Biological nitrogen fixation is an economic, environmentally friendly process which contributes nearly 65% of N used in agriculture globally (Dupont et al. 2012). *Vigna unguiculata* L. (Walp) is an important protein rich legume which can be used in cropping systems as it effectively fixes N_2 through developing symbiotic association with both rhizobial (Oruru and Njeru 2016, Jaiswal and Dakora 2019) and non-rhizobial species (Wahyudi et al. 2011, Grady et al. 2016). For effective biological nitrogen fixation, there is need for a good supply of soil P as this promotes nodulation and provides energy for biological nitrogen fixation process (Magadlela et al. 2016, Vardien et al. 2016). However, most of the agricultural soils are P deficient as the mineral complexes with Al^{3+} and Fe^{3+} under acidic conditions. To improve P acquisition under nutrient stress conditions, legumes including *V. unguiculata* have developed several adaptation mechanisms including tripartite symbiosis with rhizospheric bacteria and AMF. There is limited information on how *V. unguiculata* interacts with symbiotic microbes to optimize nutrient uptake under nutrient stress conditions. The present study focused on increasing knowledge and understanding on the microbes associated with *V. unguiculata* grown in savanna and grassland ecosystems, the mechanisms of nutrient acquisition by the legumes, energy saving strategies adopted by the legume and the phenolic acids produced by *V. unguiculata*.

Chapter 3 explored the different physicochemical properties, microbial composition, and soil enzymes activities of soils from geographically distinct regions of KZN savanna and grassland ecosystems. All the soils were found to be very low in the essential nutrients including N, P

and K. More so, the soils were acidic with Bergville soils being the most acidic. These findings concur with many researchers who reported that most of the tropical soils in sub-Saharan Africa are acidic and nutrient-deficient (Khan et al. 2009, Magadlela et al. 2016, Vardien et al. 2016). The soils housed a wide range of soil enzymes which are important in nutrient cycling. The identified soil enzymes included organic matter and lignin degrading enzymes (dehydrogenase, laccase, manganese peroxidase, lignin peroxidase), nitrogen cycling enzymes (β-D-glucosaminide, L-asparaginase), phosphorus cycling enzymes (β-D-phosphatase) and carbon cycling enzymes (β-D-glucopyranoside, β-D-cellobioside). Similar soil enzymes were identified in soils collected from Nashua, Iowa, in a study by Acosta-Martinez and Tabatabai (2000). The identified soil enzymes included α and β-galactosidases, amidase, urease, L-glutaminase, L-asparaginase, L-aspartate and alkaline phosphatase (Acosta-Martinez and Tabatabai, 2000). The presence of the soil enzymes in the present study could be a good opportunity for improvement of soil nutrition as these could catalyze rhizospheric biochemical processes thus enhancing nutrient cycling and mineralization especially of N, P, C in soils as shown by Veres et al. (2015), Ntoko et al. (2018) and Acosta-Martinez et al. (2007). Furthermore, the variations in soil enzyme activities, diversity and distribution of bacteria and fungi in the soils could be attributed to the differences in the physicochemical properties of the soils (Puozaa et al. 2019). Differences in soil pH was shown to affect soil enzyme activities, with all the identified soil enzymes being negatively correlated with soil pH except for acid phosphatase (Acosta-Martinez and Tabatabai 2000). Nutrient deficiency is known to trigger proliferation of specific microbes which enhance solubilization or remobilization of the required nutrients (Rengel and Marschner, 2005).

Despite its acidic nature, the KZN soils had several rhizospheric microbes including strains of *Fusarium*, *Trichoderma*, *Bacillus*, *Lysinibacillus*. These soil microbes could potentially enhance soil nutrition through P solubilization and N mineralization and fixation (Govindasamy et al. 2010, Sharma et al. 2013, Martínez-Hidalgo and Hirsch 2017, Aguirre-Monroy et al. 2019). Tropical soils of sub-Saharan Africa are known to harbor a great diversity of symbiotic bacteria despite the pressure in agricultural resources, acidity and poor nutrient status of the soils (Kawaka et al., 2018). In their study Kadyan et al. (2013) identified several bacterial strains belonging to different genera including *Bacillus, Brevibacillus, Lysinibacillus* and *Paenibacillus*. This confirms that agricultural soils harbor diverse bacterial species which could play an important role as biofertilizers, contributing to enhanced plant growth Sharma et al. 2013, Kaur and Reddy 2014, Kawaka et al. 2018). The significance of a diversity of *Bacillus* species found in the rhizosphere was extensively discussed in a review by Meena et al. (2016)

and these included *B. mojavensis*, *B. subtilis*, *B. megaterium*. *Bacillus* species are an important integral component of soil microbial community and they play important in processes such as biological nitrogen fixation, P, K, Zn and Fe cycling in soils and ultimately contribute to enhanced crop growth and yield (Meena et al. 2016).

This study demonstrated that the KZN savanna and grassland ecosystems have diverse soil microbial compositions and soil enzymes which could potentially enhance nutrient mineralization thus improving their bioavailability and, in turn, promote crop productivity. The natural abundance and diversity of soil microbes available in grassland and savanna ecosystems is an important benefit as resource constrained small-scale farmers can use these microbes for crop improvement.

In chapter 4; the research was aimed at understanding the effects of four distinct soil types with varying nutrient concentration and pH on plant-microbe symbiosis, N nutrition and biomass accumulation of *V. unguiculata*. In the current study, *V. unguiculata* was nodulated by several bacterial species including different *Bacillus*, *Paenibacillus*, *Bradyrhizobium* and *Delftia* strains. In the present study, *V. unguiculata* clearly thrives under acidic and nutrient stressed conditions through developing symbiotic associations with a wide range of N_2 fixing and P solubilizing bacteria, as earlier reported by other researchers (Pule-Meulenberg et al. 2010, Mohammed et al. 2018, Puozaa et al. 2019). In separate studies carried out in different regions in South Africa, phylogenetic analysis revealed that *V. unguiculata* was nodulated by a wide range of *Bradyrhizobium* species closely related to *Bradyrhizobium elkanii*, *B. japonicum*, *B. yuanmingense* and *B. subterraneum* (Pule-Meulenberg, 2010, Mohammed et al. 2018, Puozaa et al. 2019). This is in tandem with the observations made in the present study. Similarly, Jaiswal and Dakora (2019), in their review, extensively reported on a wide range of microsymbionts which nodulate *V. unguiculata* in different geographic regions of Africa and among them *Bradyrhizobium* species are the most popular.

Besides the symbiotic association between *V. unguiculata* and different bacterial species, *V. unguiculata* also developed symbiotic association with arbuscular mycorrhizal fungi (AMF) as evidenced by the %AMF colonization results. This interaction could have contributed to increased P acquisition by the legume which in turn could have improved biological nitrogen fixation efficiency of *V. unguiculata* (Andrade et al. 2013, Valentine et al. 2018). The association between the AMF and *V. unguiculata* could have helped improve plant nutrient availability especially N and P thus promoting the growth of the legume (Ngakou et al. 2007, Yaseen et al. 2011, Andrade et al. 2013, Omirou et al. 2016, Billah et al. 2019). Several studies have shown that dual inoculation of *V. unguiculata* with nitrogen fixing bacterial strains such

as *Bradyrhizobium* and *Rhizobium* species and arbuscular mycorrhizal fungi result in improved mineral nutrition (including N, Fe, P, Ca), plant nutrient content, improved crop growth and increased yields (Ngakou et al. 2007, Andrade et al. 2013, Omirou et al. 2016, Haro et al. 2018, Oruru et al. 2018). Dual inoculation of *Bradyrhizobium* isolate and commercial mycorrhizal inoculum in cowpea was shown to enhance the shoot biomass and plant N and P (Omirou et al. 2016). In an earlier study dual inoculation of rhizobia and arbuscular mycorrhizal fungi in cowpea resulted in enhanced nutrient uptake by cowpea shoots and roots and this accounted for up to 17% for total N, 52% for available P, 19% for Ca, 55% of Mg and 46% of K (Ngakou et al. 2007).

The current findings substantiate the potential of using bacteria and AMF as biofertilizers, an alternative option which is less costly and environmentally friendly. This can be adopted even by the resource constrained small-scale farmers and hence promote food security. In further research, in-depth focus on housekeeping genes analysis could be done to confirm the *V. unguiculata* nodulating bacteria which were identified in the current study. Furthermore, there is need for in-depth molecular based studies to better understand the mechanisms adopted by the identified symbiotic bacteria and fungi to ascertain the exact roles they play in enhancing nutrient acquisition and growth of *V. unguiculata*.

Results of the present study also demonstrated that *V. unguiculata* has a capacity to switch N sources between atmospheric N_2 as evidenced by results on percentage of nitrogen derived from the atmosphere, and soil N as depicted by the %N derived from soil. These findings are a further substantiation on the adaptation mechanisms of *V. unguiculata* as the legume can optimize on N acquisition. The ability to switch N source preferences is an important adaptation mechanism which allows the legume to conserve energy as less energy is utilized during soil N uptake whereas biological nitrogen fixation is an expensive process, which requires a lot of energy from the host plant (Minchin and Witty 2005, Valentine et al. 2018). The potentially lower cost of utilizing soil N could have contributed to saving of energy which could have otherwise been consequently channeled to biomass accumulation (Magadlela et al. 2016, Magadlela et al. 2017). The ability of *Virgilia divaricata* to switch N source preference between soil N and atmospheric N_2 was an important energy saving adaptation mechanism that enabled the legume to survive in nutrient poor Mediterranean ecosystems (Magadlela et al., 2017). The current findings further showed that the *V. unguiculata* were able to fix N_2 with biological nitrogen fixation efficiency of >55% even in instances where the legume was nodulated by non-rhizobial bacteria such as *Delftia*, *Bacillus* and *Paenibacillus*. These findings are in agreement with the assertions that non-rhizobial bacteria can effectively fix N_2 (Elkoca et al.

2010, Govindasamy et al. 2010, Govindasamy et al. 2016, Agafonova et al. 2017, Martínez-Hidalgo and Hirsch 2017). In a study by Kawaka et al. (2018) it was shown that common bean was nodulated by both rhizobial and non-rhizobial strains including among others, *Bacillus* species. Govindasamy et al. (2016) and Martinez-Hidalgo and Hirsch (2017) reported extensively on a number of non-rhizobial bacteria species which are important in nitrogen fixation and these include *B. megaterium*, *B. cereus*, *B. pumilus*, *B. subtilis*, *B. brevis*, and several *Paenibacillus* species such as *P. polymyxa*, *P. graminis*, *P. azotofixans.*

The availability of diverse nodulating bacterial strains housed by savanna and grassland ecosystems demonstrated in this research presents as an opportunity for enhanced adaptation of *V. unguiculata* and other legumes to nutrient stressed conditions which are a common characteristic in most agricultural systems, most importantly in Southern Africa. There is need for further research to establish other energy saving strategies that are adopted by *V. unguiculata* which grows in association with soil microbes. This could be explored by investigating P, C and N metabolism pathways used by native *V. unguiculata* varieties grown under acidic and nutrient-stressed conditions. Such information is vital as it helps researchers to have a comprehensive appreciation of the overall survival mechanisms of *V. unguiculata* under acidic and nutrient stress.

The third experiment was conducted to have an understanding on the phenolic acids produced by *V. unguiculata* grown under acidic and nutrient stressed conditions and to explore how these could contribute to the legume's adaptability to nutrient stress (Chapter 5). Furthermore, the experiment also aimed at determining the antioxidant capacity of the four *V. unguiculata* varieties grown in the nutrient stressed soils. It is known that some plants regulate their secondary metabolites such as phenolic acids to improve their adaptability to nutrient stress conditions. The phenolic acids enhance bioavailability of mineral nutrients through mobilizing the mineral nutrients such as Mg, Ca, K (Youssef et al. 2017, Sharma et al. 2019), some may solubilize nutrients such as P (Makoi and Ndakidemi 2007) while some may promote plant microbe interaction (Juszczuk et al. 2004, Mandal et al. 2010) hence promoting P solubilization and biological nitrogen fixation. Under stress conditions including nutrient stress, plants tend to produce reactive oxygen species which may be detrimental to crop growth as they cause oxidative damage (Akram et al. 2017, Santos et al. 2018). Phenolic acids play an important role in plants as they act as non-enzymatic antioxidants; they have radical scavenging properties against reactive oxygen species (Kasote et al. 2015).

Results of the experiment revealed variations in the phenolic acid concentrations of the four *V. unguiculata* varieties across all soil types with the below ground parts having the higher

concentrations when compared to the above-ground parts. The phenolic acids identified included hydroxycinnamic acids (caffeic acid, 4-coumaric acid, ferulic acid) and hydroxybenzoic acids (gallic acid, protocatechuic, 4-hydroxybenzoic acid, salicylic acid, syringic acid and vanillic acid). The current research further confirms findings by other researchers who identified similar phenolic acids at varying concentrations in other varieties of *V. unguiculata* (Alidu et al. 2020, Perchuk et al. 2020). The availability of phenolic acids such as syringic acid, caffeic acid, protocatechuic and salicylic acids is an important survival strategy as these help in enhancing nutrient solubilization especially N and P for plant uptake. Phenolic acids are known to mobilize mineral elements, improve active absorption sites and to promote plant-soil microbe interaction. The exudation of phenolic acids by *V. unguiculata* could have influenced the dynamics of rhizospheric microbe diversity thus promoting survival of specific legume nodulating bacteria. In the current study, symbiotic association of *V. unguiculata* with the microsymbionts could have been thus, enhanced by the identified phenolic acids and this could have promoted P acquisition and biological nitrogen fixation. However, there is need for further molecular research to have a better understanding on the exact roles of the identified phenolic acids in the growth and development of *V. unguiculata*. Generally, a negative correlation existed between the phenolic acids concentration and plant biomass accumulation in the different *V. unguiculata* varieties. This observation is not surprising as it has been shown in several studies that high phenolic acid concentrations may have inhibitory effects on plant growth (Khan et al. 2010b, Mandal et al. 2010, Zhou and Wu 2012). Under abiotic stress conditions plants tend to produce reactive oxygen species which may have detrimental effects on plant organizational components and root nodules thus reducing nitrogen fixation efficiency of legumes. Some phenolic acids have the capacity to serve as antioxidants thereby scavenging the reactive oxygen species. In the current research, the different *V. unguiculata* varieties grown under acidic and nutrient stressed conditions had high anti-oxidative capacities which varied slightly among varieties and soil types. Results of the current research are a confirmation that *V. unguiculata* is highly adaptable to acidic nutrient-stressed conditions. The legume can be adopted in cropping systems especially by resource constrained small-scale farmers as it can effectively fix N and enhance acquisition of other essential nutrients including P. Our findings are in line with those reported by Zia-UlHaq et al. (2013) who observed that there were variations in the total phenolic contents and antioxidant activities of four *V. unguiculata* varieties. It was suggested that the antioxidant potential of cowpea seeds could be an important opportunity to advocate them as nutraceuticals

for human health. The legume could contribute to management of degenerative diseases associated with free radical damage (Zia-Ul-Haq et al. 2013).

There is need for further efforts through molecular biological research on plant-microbe symbiosis to establish how phenolic acids alter the AMF-plant symbiosis and *Rhizobium*-legume symbiosis and to understand the effects of phenolic acids on expression of *nod* genes. Further research is essential to understand how other phytochemicals and phytohormones produced by *V. unguiculata* contribute to enhanced nutrient solubilization and acquisition.

In conclusion, findings of the current research are useful as they contribute to development of sustainable agricultural practices to improve food security in farming systems in KZN and beyond South Africa. Promotion of legume-microbe association in agricultural systems will contribute to nutrient amendments in the nutrient stressed and acidic savanna and grassland ecosystems beyond South Africa. Furthermore, the research substantially contributes to the United Nations sustainable development goal number 2 which aims to achieve food security, enhance nutrition and encourage sustainable agriculture.

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