Varying soil nutrition in grassland and savanna ecosystems affect plant-microbe symbiosis, nitrogen nutrition and growth of *Pisum sativum* L.

By

Ntuthuko S. Zungu

A research dissertation presented in fulfilment of the requirements for the degree of Master of Science in the discipline Biological Sciences

School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Pietermaritzburg

December 2019

Supervisor: Dr A. Magadlela

Co- supervisors: Drs T. Khoza and P. Mathabe

SUMMARY

Sustainable agriculture is associated with soil fertility which promotes quality vegetation and higher yield potential for sustenance as either food or feed. In many developing countries with low gross domestic product (GDP) per capita, households mostly rely on agricultural produce and livestock to meet their dietary requirements. However, most of the regions are characterized by low fertility soils which negatively affect agricultural yield and overall quality of produce. The grassland and savanna ecosystems in which the majority of the population in these countries reside, are nutrient deficient and acidic. This imposes a burden on both small and large-scale farmers since they have to rely on commercial fertilizers to mitigate the effects. Furthermore, the use of these fertilizers can negatively affect the immediate environment through leaching and runoff.

Therefore, there is a need for eco-friendly, low-input high-yield farming systems that are also cost effective. This has increased research aimed at the use of biofertilizers such as leguminous plants to remediate soil nutrition in grassland and savanna ecosystems. Since biofertilizers are naturally occurring, their use has minimal threat to the environment and ecosystems. Legumes and their symbionts, whether endophytic and/or associative, might be a suitable sustainable farming system to mitigate soil quality degradation. More so, legumes are a rich source of proteins and minerals that improves the quality of feed in pastures, hence improving the quality of livestock. This project was aimed at investigating the functional adaptations of a winter legume, *Pisum sativum* L., in KwaZulu-Natal (KZN) grassland and savanna nutrient deficient ecosystem soils. The four soil collection sites were Hluhluwe, Izingolweni, Bergville, and Ashburton.

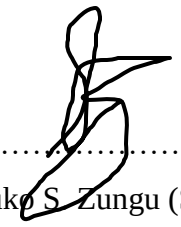
The first experiment examined soil nutrients, microbial composition and diversity and enzyme activities of KZN grassland and savanna soils. This was achieved by analyzing the soil geochemistry (nutrient concentration, pH, total cation concentrations and exchange acidity), microbial composition, arbuscular mycorrhizal (AM) fungi spore count and extracellular enzyme activities. The soils were found to vary in all aspects, ranging from nutrient concentration to the Biological Index for soil Fertility (BIF). Endophytic/associative microbes identified in the soils belonged to the families *Bacillaceae* (*Bacillus* and *Lysinibacillus*), *Nectriaceae*, *Mucoraceae*, and *Hypocreaceae*. Bergville soil had the lowest pH, dehydrogenase activity, microbial diversity, BIF, and total cations. The combined limitations led to us to postulate that this soil type was the least suitable for the growth of *P. sativum* L.

In the second experimental chapter we investigated the effects of soil nutrient availability and microbial associations on plant nutrition, growth kinetics, N-source preference and metabolite synthesis in *P. sativum* L. Soils from the four KZN regions were used as inoculum and growth medium, making up four treatments. Nodulation was only observed in Izingolweni grown plants and the root nodule inhabiting microbes were from the genera *Cupriavidus*, *Paenibacillus*, *Bacillus*, *Ralstonia*, and *Saccharibacillus*. However, plants from all four treatments relied on combined N for growth but at different extents. Bergville soil grown plants decreased growth, nutrient concentration, growth-promoting metabolites (kinetin, salicylic acid, and active cytokinins), but had high antioxidant and storage cytokinin concentrations. The current findings suggest that *P. sativum* L. is adaptable to nutrient deficient soils by associating with plant growth-promoting microbes, resource re-allocation, and using combined N.

PREFACE

The data presented in this dissertation was collected at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, Republic of South Africa from 2018 to 2019. Experimental work was carried out while registered at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Dr. Anathi Magadlela.

This dissertation submitted for the degree of Master of Science in the College of Agriculture, Engineering and Science, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, represents original work by the author and has not been submitted in any form for a degree or diploma at any other university.



…….

Ntuthuko S. Zungu (Student)

December 2019

I certify that the above statement is correct and as the candidate's supervisor I have approved this dissertation for submission.

Anathi Magadlela

……

Dr Anathi Magadlela (Supervisor)

December 2019

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE DECLARATIONS: PLAGIARISM

I, Ntuthuko S. Zungu (student no. 211517931) declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.
4. This dissertation does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a. Their words have been re-written, but the general information attributed to them has been referenced

b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced.

5. This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: …….

Ntuthuko S. Zungu

December 2019

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE DECLARATION: PUBLICATIONS

Applied soil ecology APSOIL_2019_1308 (Under review)

Soil nutrition, microbial composition and associated soil enzyme activities in KwaZulu-Natal grasslands and savannah ecosystems soils

Ntuthuko S. Zungu[a], Samson O. Egbewale[b], Ademola O. Olarinan[ab], María Pérez-Fernández[c]*, Anathi Magadlela[a]

[a]*School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, KwaZulu-Natal, South Africa*

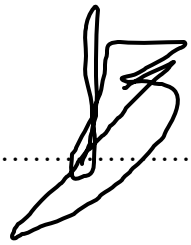
[b]*School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa*

[c]*Ecology Area, University Pablo de Olavide, Carretera de Utrera Km 1, 41013 Sevilla, Spain*

***Correspondence:** María Pérez-Fernández **email:** maperfer@upo.es

Author contributions:

Ntuthuko S. Zungu conducted the study, analyzed results and wrote the manuscript. Samson O. Egbewale and Ademola Olarinan analyzed the soil endophytic microorganisms and extracellular enzyme activities. María Pérez-Fernández and Thandeka Khoza were involved in editing the manuscript. Anathi Magadlela was the study leader, planned and funded the experiments and all analysis. He was involved in all data collection, analysis and interpretation as well as the manuscript write-up.

Student: 

Supervisor: 

Formatted for Journal of plant physiology

Pisum sativum L. can acclimatize to nutrient deficient soils from KwaZulu- Natal grasslands and savannah ecosystems, South Africa.

Ntuthuko S. Zungu[a], Thandeka Khoza[a], Adeyemi O. Aremu[a,b], Jiri Gruz[c], María Pérez-Fernández[d], Anathi Magadlela[a]*

[a]*School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, KwaZulu-Natal, South Africa*

[b]*Food Security and Safety Niche Area, Faculty of Natural and Agricultural Sciences, North- West University, Private Bag X2046, Mmabatho, North West Province South Africa*

[c]*Laboratory of Growth Regulators, Faculty of Science, Palacký University & Institute of Experimental Botany AS CR, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*

[d]*Ecology Area, University Pablo de Olavide, Carretera de Utrera Km 1, 41013 Sevilla, Spain*

***Correspondence:** anathimagadlela@icloud.com

Author contributions:

Ntuthuko S. Zungu conducted the study, analyzed results and wrote the manuscript. Adeyemi Aremu and Jiri Gruz were involved in the analysis of cytokinins and phenolic acids. Thandeka Khoza, María Pérez-Fernández were involved in the editing the manuscript. Anathi Magadlela was the study leader, planned and funded the experiments and analysis. He was involved in all data collection, analysis and interpretation as well as the manuscript write-up.

Student: …….

Supervisor: Anathi Magadlela ……..

ACKNOWLEDGEMENTS

I would like to extend my gratitude and appreciation to my supervisors, Dr A. Magadlela, Dr T. Khoza and Dr P. Mathabe, for giving me the opportunity to conduct this study, and for the educational guidance that they provided throughout the duration of the study.

I wish to express my sincere thanks to Dr T. Khoza and her students (Lab 39) for the encouragement and unending support extended to me. I further place on record my sincere gratitude all my friends and colleagues who, directly and/or indirectly, had a hand in the completion of the study and my overall wellbeing.

Last but not least, I would like to express my love to all my family members, especially to my parents, grandmother, and my sons. You have always been, and still are my motivation. To Londeka goes my love and gratitude. Thank you for making this journey possible and worth while.

This study was funded by the National Research Foundation (NRF) Thuthuka grant (113576).

TABLE OF CONTENTS

SUMMARY ……..ii
PREFACE iv
DECLARATION 1: PLAGIARISM ……....v
DECLARATION 1: PUBLICATIONS ……....vi
ACKNOWLEDGEMENTS ……..viii
LIST OF FIGURES ……...xiii
LIST OF TABLES ……..xvi
LIST OF ABBREVIATIONS ……...xvii
CHAPTER 1 ……..1

General introduction ……1
2.1. General introduction ……2
2.1.1. Bio-fertilizers ……....2
2.1.2. Symbiotic N_2 fixation ……....2
2.1.3. *Pisum sativum* L. ……3
2.1.4. Phytohormornes role in stress tolerance ……3
2.1.5. Research questions ……5
2.2. References ……6
CHAPTER 2 ……11

Literature review ……11
1. Introduction ……...12
1.1.Family leguminosea ……..12
1.2.Biological nitrogen fixation ……..13
1.2.1. Nodulation and types of nodules ……..14
1.3.Carbon and energy costs during N-fixation ……..16
1.4.Environmental stresses that affect symbiotic nitrogen fixation ……17
1.5.Causes and effects of soil phosphorus deficiency on N_2 fixation ……18
1.6.The use of crops as forage ……19
1.7.Study species ……20

1.8.References ……24

CHAPTER 3 ……32

Soil geochemistry and microflora ……...32

Graphical abstract ……..33

Abstract ……34

3.1. Introduction ……...35

3.2. Materials and methods ……...37

3.2.1. Total soil nutrient analysis and spore count ……37

3.2.1.1. Soil site description ……37

3.2.2. Soil fungal spores ……...38

3.2.2.1. Extraction, purification and enumeration ……...38

3.2.2.2. Fungal DNA extraction ……...38

3.2.2.3. DNA amplification, sequencing and identification ……38

3.2.3. Soil-borne bacteria ……39

3.2.3.1. Isolation and enumeration ……...39

3.2.3.2. Bacterial DNA extraction ……...39

3.2.3.3. DNA amplification, sequencing and identification ……39

3.2.4. Measurement of biodiversity ……..40

3.2.5. Soil enzyme activity ……...40

3.2.6. Data analysis……...42

3.3. Results ……...42

3.3.1. Soil geochemistry ……42

3.3.1.1. Soil colour and spore count ……42

3.3.1.2. Primary soil nutrients ……..42

3.3.1.3. Intermediate soil nutrients ……..43

3.3.1.4. Soil micronutrients ……..…43

3.3.1.5. Soil relative acidity and C:N ratios ……43

3.3.2. Functional microbial diversity ……43

3.3.3. Endophytic microorganisms ……...44

3.3.3.1. Bacteria ……..44

3.3.3.2. Fungi ……..45
3.3.4. Soil enzyme activities ……45
3.3.4.1. Carbon cycling enzymes ……45
3.3.4.2. Organic matter and lignin degrading enzymes ……45
3.3.4.3. Nitrogen and phosphate cycling enzymes ……..45
3.4. Discussion ……..46
3.5. Conclusion ……49
3.6. Acknowledgements ……...50
3.7. References ……..50
Figures and tables ……..……..57
CHAPTER 4 ……68

Plant growth and nutrition ……68
Graphical abstract ……..69
Abstract ……70
4.1. Introduction ……...71
4.2. Materials and methods ……...73
4.2.1. Soil collection sites ……73
4.2.2. Soil geochemistry and AM fungi analysis ……73
4.2.3. Seed germination, bacterial inoculation and growth ……..73
4.2.4. Plant nutrient analysis ……74
4.2.5. Growth calculations ……...74
4.2.6. Bacterial isolation, colony PCR and identification ……75
4.2.7. Cytokinin and phenolic acids quantification ……..76
4.2.8. Statistical analysis ……..77
4.3. Results ……...77
4.3.1. Geochemistry and microflora ……77
4.3.2. Seedling emergence and growth kinetics ……78
4.3.3. Endophytic bacteria identification ……79
4.3.4. Phenolic acids concentration ……..79
4.3.5. Cytokinin concentration ……79
4.4. Discussion ……..80

4.5. Conclusion …….83
4.6. Acknowledgements ……...83
4.7. References …….83
Figures and tables ……91
CHAPTER 5 ……....97
General discussion ……...97
5.1.General discussion ……....98
5.2.Future work ……100
5.3.References ……...101
APPENDIX A: Biomes of South Africa…..……...104

LIST OF FIGURES

CHAPTER 2

Figure 2.1.: Overview of Biological Nitrogen Fixation. Adapted from Herridge *et al.,* 2008. **14**

Figure 2.2.: Diagram illustrating the (A) determinate and the (B) indeterminate nodules. Both types of nodules have the NC – nodule cortex NE – nodule endodermis VB – vascular bundle and NP– nodule parenchyma. The determinate nodule has the S – senescence zone and the NF – nitrogen fixation zone. The indeterminate nodule has I – active meristem II – infection zone II-III – interzone (intermediate) III – nitrogen fixation zone IV – senescent zone A – invasion zone B – pre-fixing zone C – efficient zone D – inefficient zone. **15**

Figure 2.3.: Tripartite symbiotic association nutrient uptake pathways. Solar radiation is converted by plants into photosynthates that are transported to the *Rhizobia* and *Arbuscular Mycorrhiza*. In exchange, the microorganisms provide the plant with essential nutrients. **16**

Figure 2.4.: The generic stressor-induced response in plants. The four phases of plant stress response are the Alarm, Resistance, Exhaustion and Regeneration phase. Plant stress response is initiated when a biotic/abiotic stressor is induced. The plant goes from the No Stress to the Alarm phase, which is characterized by stress sensing and response signaling. This triggers the Resistance phase in which the plant acclimatize and adapts to the stressor imposed. This is achieved by increasing levels of repair and protection to maintain cell viability. If the stressor persists, the plant enters the Exhaustion phase. In this phase, the plant experiences chronic damage and loss of viability as a result of increased rate of cell death. However, if the stressor is removed, the plant enters the Regeneration phase. During this phase, the plant achieves new standards in signaling and regulation to compensate for the loss caused by the stressor. Adapted from Lichtenthaler, 1998. **18**

Figure 2.5.: *Pisum sativum* L. (A) intercropped with vetch and (B) grazed by pigs. **23**

Figure 2.6.: Factors influencing plant growth and development. The current study is focused on the factors outlined in red. **24**

CHAPTER 3

Figure 3.1: Soil colour variation between the four KZN sites. A: Hluhluwe (dark brown) B: Izingolweni (red-brown) C: Bergville (brown) D: Ashburton (black). **57**

Figure 3.2: Arbuscular mycorrhizal spore count. Soil samples were processed using wet sieving and decanting technique followed by sucrose centrifugation and filtrate. Arbuscular mycorrhizal spores were observed and enumerated microscopically. Spore viability not confirmed. Results are mean ± SE, n = 5. Means with the same letter indicate that no significant differences were detected after a one-way ANOVA ($p \leq 0.05$). **57**

Figure 3.3: Concentration of soil primary nutrients. A: Phosphorus (P), B: Nitrogen (N) and C: Potassium (K). Results are mean ± SE. Different letters on top of the columns indicate significant differences after one-way ANOVA ($p \leq 0.05$; n = 5) **58**

Figure 3.4: Concentration of soil intermediate nutrients. Soil samples were sent to CEDARA College of Agriculture, (South Africa) for total soil nutrient analysis. A: Calcium (Ca) and B: Magnesium (Mg). Results are mean ± SE. Different letters on top of the columns indicate significant differences after one-way ANOVA ($p \leq 0.05$; n = 5). **59**

Figure 3.5: Concentration of soil micronutrients. Soil samples were sent to CEDARA College of Agriculture, (South Africa) for total soil nutrient analysis. A: Zinc (Zn), B: Manganese (Mn) and C: Copper (Cu). Results are mean ± SE. Different letters on top of the columns indicate significant differences after one-way ANOVA ($p \leq 0.05$; n = 5). **60**

Figure 3.6: Soil relative acidity. Soil samples were sent to CEDARA College of Agriculture, (South Africa) for total soil nutrient analysis. A: pH, B: Exchange acidity and C: Total cation. Results are mean ± SE. Different letters on top of the columns indicate significant differences after one-way ANOVA ($p \leq 0.05$; n = 5). **61**

Figure 3.7: Percentage bacterial strain relative abundance. **62**

Figure 3.8: Percentage fungal strain relative abundance. **63**

CHAPTER 4

Figure 4.1.: Forage pea (*Pisum sativum* L.) seedlings after (A) four and (B) seven days of growth. **91**

LIST OF TABLES

CHAPTER 2

Table 2.1.: Relative seed size of legume plant genera. **20**

Table 2.2.: Intercropping research in temperate regions. **21**

Table 2.3.: South African major field pea production regions. **23**

CHAPTER 3

Table 3.1: Organic carbon to nitrogen ratio from soil treatments. **64**

Table 3.2: Functional Microbial diversity index of the soil samples. **64**

Table 3.3: Endophytic strains isolated from the soil samples. **65**

Table 3.4: Soil carbon cycling enzyme activities in $nmolh^{-1}g^{-1}$ of soil **66**

Table 3.5: Soil organic matter and lignin degrading enzyme activities in $nmolh^{-1}g^{-1}$ of soil **66**

Table 3.6: Soil nitrogen and phosphate cycling enzyme activities in $nmolh^{-1}g^{-1}$ of soil **66**

Table S1: Values of the Biological Index for Soil Fertility based on dehydrogenase and catalase activities **67**

CHAPTER 4

Table 4.1.: KwaZulu-Natal soil geochemistry and microflora data. **91**

Table 4.2.: Relative seedling emergence comparison between soil sites. **92**

Table 4.3.: Growth kinetic of *Pisum sativum* L. grown in KwaZulu-Natal soils. **93**

Table 4.4.: Identities of root nodule inhabiting microorganisms. **94**

Table 4.5.: Concentration of phenolic acids in *Pisum sativum* L. grown in KwaZulu-Natal soils. **95**

Table 4.6.: Concentration of cytokinis in *Pisum sativum* L. grown in KwaZulu-Natal soils. **96**

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylate
Al	Aluminium
AM	Arbuscular mycorrhiza
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BIF	Biological index for soil fertility
BNF	Biological nitrogen fixation
BR	Brassinosteroids
C	Carbon
Ca	Calcium
Ca	Calcium
CFU	Colony forming unit (s)
CK	Cytokinins
cZ	*cis*-Zeatin
DHZR	Dihydrozeatin riboside
DNA	Deoxyribonucleic acid
ET	Ethylene
Fe	Iron
GA	Gibberellins
Hsp	Heat shock protein
IAA	Indole-3-acetic acid
ILDIS	International legume database
iP	Isopentenyladenine
ISR	Induced systematic resistance
JA	Jasmonate
K	Potassium
KZN	KwaZulu-Natal
Mg	Magnesium

Mn	Manganese
Mo	Molybdenum
N	Nitrogen
N_2	Dinitrogen
NDFA	Nitrogen derived from atmosphere
NH_3	Ammonia
NH_4^+	Ammonium
NO_3^-	Nitrate
O	Oxygen
-OG	-*O*-glucoside
P	Phosphorus
PCR	Polymerase chain reaction
PO_4	Phosphate
Pro	Proline
-ROG	-Riboside *O*-glucoside
SA	South Africa
SNAR	Specific nitrogen assimilation rate
SNUR	Specific nitrogen utilization rate
SPUR	Specific phosphorus utilization rate
tZ	*trans*-Zeatin
Z	Zeatin
ZR	Zeatin riboside

Chapter 1

General introduction

1.1. General introduction

1.1.1. Bio-fertilizers

The term bio-fertilizer refers to the use of microorganisms to enhance soil fertility and/or promote plant growth (Mishra and Dash, 2014). Bio-fertilizers usually work through the colonization of the plant rhizosphere to provide the host plant with the necessary nutrients for growth and development (Wu *et al.,* 2005). There are currently three main groups of bio-fertilizers, which are bacteria, cyanobacteria and fungi (Wu *et al.,* 2005; Mazid and Khan, 2015). These microorganisms increase plant nutrient availability through different processes such as nitrogen fixation, secreting plant growth promoting substances like indole-acetic acid, phosphate solubilization, and the release of scavenging molecules like siderophores which chelate and transport iron (Wu *et al.,* 2005; Wani *et al*., 2013; Mazid and Khan, 2015). Bio-fertilizers such as *Rhizobium, Mycorrhizal* fungi, *Azospirilium* and *Azotobacter* have been historically used to promote growth and yield potential of a variety of field crops, including *Pisum sativum* (pea), *Triticum* (wheat), Zea *mays* (maize) and *Saccharum officinarum* (sugarcane) (Awasthi *et al*., 2011; Ei-Lattief, 2016; Celador-Lera *et al.,* 2018). The more recently used bio-fertilizers include *Paenibacillus*, which not only employ the aforementioned processes, but also promote plant growth through bio-control agent (insecticides) production and inducing systematic resistance (ISR) in the host plant (Grady *et al.,* 2016). All of the plant growth promoting microorganisms can either be free-living in soil or plant-associated in a symbiotic relationship with the host (Glick, 1995).

1.1.2. Symbiotic N_2 fixation

One of the extensively studied symbiotic N_2 fixation is between legumes and *Rhizobia.* Both the host plant and bacteria can survive independently but unlike *Azobacter*, *Rhizobia* are unable to independently fix N_2 (Prell and Poole, 2006; Ei-Lattief, 2016). In the absence of the bacteroids (nodules), plants can assimilate N from the soil but the symbiosis allows the plant to access vast N reservoir in the atmosphere (Prell and Poole, 2006). However, the symbiotic N fixation can impose a metabolic burden to the host, as it requires a significant amount of energy (i.e. ATP) and carbon (Schulze, 2004). Phosphorus is a crucial component of the energy molecule ATP, and if limited can negatively affect nodulation, protein synthesis, respiration, growth, bio-energetic processes and N nutrient acquisition and metabolism (Schulze, 2004; Sulieman and Tran, 2015). There are other nutrients that impair symbiotic N_2 fixation like molybdenum (Mo) that are required

by the nitrogenase enzyme for efficient functioning, and iron (Fe) which is a component of leghemoglobin (Allen *et al.*, 1994; Mendonça *et al*., 1999). However, endophytic plant growth-promoting bacteria can also play a role in plant nutrient acquisition and growth.

Apart from N_2 fixation, endophytic bacteria such as *Bacillus* produce indole-3-acetic acid (IAA), siderophores, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and solubilize P and K (Glick, 2014). Different soil ecosystems have characteristic soil types, microbial population and nutrient status (Costa *et al.,* 2006). It is therefore imperative to evaluate the effects of such soil ecosystems on plant growth carbon/energy costs, protein expression profile, and N source preference and phytohormone production during symbiotic N fixation.

1.1.3. *Pisum sativum* L.

Pisum sativum L. is one of the three species under the genus *Pisum* as per the International Legume Database (ILDIS, 2018). The other two species are *P. abyssinicum* and *P. fulvum* and all three species are under the family Leguminosae (www.ildis.org). *P. sativum* L. is referred to as a winter legume as it grows optimally during the cool season (May – July), and does not grow well under elevated temperatures of the summer season (Cowie *et al.*, 1990). Furthermore, *P. sativum* L. is adapted to most soil types and is considered as a fast-growing legume that is grown across the world for various reasons, including fodder, manure and as a rotation crop (Nikolopoulou *et al.*, 2007). *P. sativum* L. is known to be nodulated by *Rhizobium leguminosarum bv. viciae* but can also establish a tripartite relationship with Arbuscular mycorrhizal fungi (Evans *et al*., 1996; Schulze, 2004). The amount of fixed N is moderate, ranging from 166 to 286 kg/ha (Kumar and Goh, 2000). When the crops are cultivated, they act as fertilizer enriching the soil through the break down and release of proteins, carbon and other components of the plant (Piotrowska and Wilczewski, 2012). The ability of *P. sativum* L. to self-pollinate allows for easy experimentation since pure gene lines are easily obtained.

1.1.4. Phytohormones role in stress tolerance

Abiotic stresses pose a major threat to crop yield across the world (Egamberdieva *et al*., 2017). The prevention of such stresses could assist in the alleviation of crop loss to provide sufficient food and feed for the ever-increasing population. On the other hand, global warming and climate change are gradually increasing the degree of stresses that affect agricultural land (Ahuja *et al.*,

2010). Plants are well adapted to a multitude of environments (Miura and Tada, 2014) and evaluating the adaptation events at a molecular level could be useful in sustainable agriculture. Plant phytohormones have been associated with plant growth and development, defense and to some degree, stress tolerance (Egamberdieva *et al*., 2017).

Phytohormones that have been reported to be responsible for plant defense include salicylic acid (SA), jasmonates (JAs) and ethylene (ET), while auxins (IAAs), brassinosteroids (BRs), cytokinins and gibberellins (GAs) have been associated with plant growth and development (Egamberdieva *et al*., 2017). Evidence suggests that abscisic acid (ABA) plays a major role in plant stress tolerance (Kohli *et al*., 2013). Extensive work has been conducted on the role of ABA in inducing and/or mediating signaling events that lead to enhanced systematic plant stress tolerance responses. However, phytohormones can play multi-facetted roles with the possibility of crosstalk during signaling events (Kohli *et al*., 2013; Egamberdieva *et al*., 2017).

Indole-3-acetic acid (IAA) that promotes cell growth and differentiation (Kende and Zeevaart, 1997) is a great example of this. IAA concentration decreased in rice plants exposed to salinity stress but tolerance was achieved through increased biosynthesis of other phytohormones (Iqbal and Ashraf, 2013). Possible crosstalk was demonstrated by Iqbal and Ashraf (2007) through the IAA-induced biosynthesis of salicylic acid (SA) in plants under salt stress. It is evident that phytohormones have a collective role in plant stress tolerance. For instance, ABA enhances water and nutrient uptake during stress conditions by promoting a deeper, more complex root system (Shrivastava and Kumar, 2015). However, like SA, ABA also assists in the up-regulation of the antioxidant system through nitric oxide synthesis and osmolyte accumulation (Uchida et al., 2002; Zhou *et al*., 2005).

One of the most prominent osmolytes involved in stress tolerance is proline (Pro) (Per *et al*., 2017). Certain phytohormones (e.g. GA) not only increase Pro accumulation, but also macro and/or micronutrients (Tuna *et al.,* 2008). Apart from the aforementioned survival strategies, plants also synthesize thiol-containing compounds like glutathione (GSH) (Anjum *et al*., 2014). GSH is a strong antioxidant that regulates metabolic events such as the chelation of toxic metals and protein protection from oxidative stress (Hossain *et al*., 2012). Root-associated microorganisms have also been reported to confer stress tolerance to plants through the production of phytohormones (Ruiz-Lozano *et al*., 2012).

The plant growth-promoting microorganisms alleviate plant stress through the regulation of the nutritional and hormonal homeostasis, which in turn leads to induced systemic responses (Ruiz-Lozano *et al*., 2012; Egamberdieva *et al.,* 2017). Root-associated *Rhizobia, Bacillus, Actinobacteria* and fungal species have been reported to produce phytohormones like IAA, ABA, and CK (Egamberdieva *et al.,* 2017). The production of such phytohormones leads to increased plant growth and alteration of the root morphology. The endogenous and exogenous production of such phytohormones also plays a role in plant protein expression (Ruiz-Lozano *et al*., 2012).

1.2.Research questions

Does varying nutrient status in grassland and savanna ecosystem soils in KwaZulu-Natal, South Africa affect the microbe symbiosis, nutrient acquisition, cytokinin and phenolic acids in *Pisum sativum* L.?

Aims and Objectives:

The study was aimed at evaluating the effects of varying nutrients in soil collected from four different geographical sites of KwaZulu-Natal grasslands and savannas on the plant-microbe symbiosis, N nutrition, phenolic cacids, cytokinins and growth of *Pisum sativum* L. under greenhouse conditions.

The objectives of the study are to:

- To examine the nutrient concentrations, pH, microbial composition and enzyme activities in soils collected from different geographic areas in KwaZulu-Natal grassland and savannah ecosystems.
- To identify nodule and root colonizing symbionts during soil nutrient stress in *Pisum sativum* L.
- To evaluate how varying nutrient availability affects the N nutrition and carbon growth costs in *Pisum sativum* L.
- To identify and quantify stress-induced phytohormones and phytochemicals during varying nutrient availability in *Pisum sativum* L.

Hypothesis:

Varying nutrient availability in KwaZulu-Natal grassland and savanna soils affect plant-microbe symbiosis, nutrition, growth and biosynthesis of cytokinin and phenolic acids in *Pisum sativum* L..

1.3.References

2. Ahuja, I., de Vos, R.C., Bones, A.M. and Hall, R.D., 2010. Plant molecular stress responses face climate change. *Trends in Plant Science*, 15(12), pp.664-674.
3. Allen, R.M., Chatterjee, R., Madden, M.S., Ludden, P.W. and Shah, V.K., 1994. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Critical Reviews in Biotechnology*, 14(3), pp.225-249.
4. Amores-Sánchez, M.I. and Medina, M.Á., 1999. Glutamine, as a precursor of glutathione, and oxidative stress. *Molecular Genetics and Metabolism*, 67(2), pp.100-105
5. Anjum, N.A., Aref, I.M., Duarte, A.C., Pereira, E., Ahmad, I. and Iqbal, M., 2014. Glutathione and proline can coordinately make plants withstand the joint attack of metal (loid) and salinity stresses. *Frontiers in Plant Science*, 5, p.662.
6. Awasthi, R., Tewari, R. and Nayyar, H., 2011. Synergy between plants and P-solubilizing microbes in soils: effects on growth and physiology of crops. *International Research Journal of Microbiology*, 2(12), pp.484-503.
7. Bouche, N. and Fromm, H., 2004. GABA in plants: just a metabolite?. *Trends in Plant Science*, 9(3), pp.110-115.
8. Celador-Lera, L., Jiménez-Gómez, A., Menéndez, E. and Rivas, R., 2018. Biofertilizers based on bacterial endophytes isolated from cereals: potential solution to enhance these crops. In *Role of Rhizospheric Microbes in Soil* (pp. 175-203). *Springer*, Singapore.
9. Costa, R., Götz, M., Mrotzek, N., Lottmann, J., Berg, G. and Smalla, K., 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiology Ecology*, 56(2), pp.236-249.
10. Cowie, A.L., Jessop, R.S. and MacLeod, D.A., 1990. Effect of soil nitrate on the growth and nodulation of winter crop legumes. *Australian Journal of Experimental Agriculture*, 30(5), pp.651-654.

11. De Gara, L., de Pinto, M.C. and Tommasi, F., 2003. The antioxidant systems vis-à-vis reactive oxygen species during plant–pathogen interaction. *Plant Physiology and Biochemistry*, 41(10), pp.863-870.
12. Egamberdieva, D., Wirth, S.J., Alqarawi, A.A., Abd_Allah, E.F. and Hashem, A., 2017. Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness. *Frontiers in Microbiology*, 8, pp.2104.
13. Ei-Lattief, E.A., 2016. Use of Azospirillum and Azobacter bacteria as biofertilizers in cereal crops: A review. *International Journal of Research in Engineering and Applied Sciences (IJREAS)*, 6(7), pp.36-44.
14. Evans, J., Gregory, A., Dobrowolski, N., Morris, S.G., O'Connor, G.E. and Wallace, C., 1996. Nodulation of field-grown Pisum sativum and Vicia faba: competitiveness of inoculant strains of Rhizobium *leguminosarum bv. viciae* determined by an indirect, competitive ELISA method. *Soil Biology and Biochemistry*, 28(2), pp.247-255.
15. Glick, B.R., 1995. The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology*, 41(2), pp.109-117.
16. Glick, B.R., 2014. Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiological Research*, 169(1), pp.30-39.
17. Grady, E.N., MacDonald, J., Liu, L., Richman, A. and Yuan, Z.C., 2016. Current knowledge and perspectives of Paenibacillus: a review. *Microbial Cell Factories*, 15(1), pp.203.
18. Hossain, M.A., Piyatida, P., da Silva, J.A.T. and Fujita, M., 2012. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. *Journal of Botany*, 2012.
19. Iqbal, M. and Ashraf, M., 2007. Seed treatment with auxins modulates growth and ion partitioning in salt-stressed wheat plants. *Journal of Integrative Plant Biology*, 49(7), pp.1003-1015.
20. Iqbal, M. and Ashraf, M., 2013. Gibberellic acid mediated induction of salt tolerance in wheat plants: Growth, ionic partitioning, photosynthesis, yield and hormonal homeostasis. *Environmental and Experimental Botany*, 86, pp.76-85.

21. Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D. and Bohnert, H.J., 2001. Gene expression profiles during the initial phase of salt stress in rice. *The Plant Cell*, 13(4), pp.889-905.
22. Kende, H. and Zeevaart, J., 1997. The five" Classical" plant hormones. *The plant cell*, 9(7), pp.1197.
23. Kohli, A., Sreenivasulu, N., Lakshmanan, P. and Kumar, P.P., 2013. The phytohormone crosstalk paradigm takes center stage in understanding how plants respond to abiotic stresses. *Plant Cell Reports*, 32(7), pp.945-957.
24. Liu, H., Sultan, M.A.R.F., li Liu, X., Zhang, J., Yu, F. and xian Zhao, H., 2015. Physiological and comparative proteomic analysis reveals different drought responses in roots and leaves of drought-tolerant wild wheat (*Triticum boeoticum*). PLoS One, 10(4), pp.0121852.
25. Matés, J.M., Pérez-Gómez, C., de Castro, I.N., Asenjo, M. and Márquez, J., 2002. Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. *The International Journal of Biochemistry & Cell Biology*, 34(5), pp.439-458.
26. Mazid, M. and Khan, T.A., 2015. Future of bio-fertilizers in Indian agriculture: an overview. *International Journal of Agricultural and Food Research*, 3(3).
27. Mendonça, E.H., Mazzafera, P. and Schiavinato, M.A., 1999. Purification of leghemoglobin from nodules of Crotalaria infected with Rhizobium. *Phytochemistry*, 50(2), pp.313-316.
28. Mishra, P. and Dash, D., 2014. Rejuvenation of biofertilizer for sustainable agriculture and economic development. *Consilience*, (11), pp.41-61.
29. Miura, K. and Tada, Y., 2014. Regulation of water, salinity, and cold stress responses by salicylic acid. *Frontiers in Plant Science*, 5, p.4.
30. Nikolopoulou, D., Grigorakis, K., Stasini, M., Alexis, M.N. and Iliadis, K., 2007. Differences in chemical composition of field pea (Pisum sativum) cultivars: Effects of cultivation area and year. *Food Chemistry*, 103(3), pp.847-852.
31. Per, T.S., Khan, N.A., Reddy, P.S., Masood, A., Hasanuzzaman, M., Khan, M.I.R. and Anjum, N.A., 2017. Approaches in modulating proline metabolism in plants for salt and

drought stress tolerance: Phytohormones, mineral nutrients and transgenics. *Plant Physiology and Biochemistry*, 115, pp.126-140.

32. Piotrowska, A. and Wilczewski, E., 2012. Effects of catch crops cultivated for green manure and mineral nitrogen fertilization on soil enzyme activities and chemical properties. *Geoderma*, 189, pp.72-80.
33. Prell, J. and Poole, P., 2006. Metabolic changes of rhizobia in legume nodules. *Trends in Microbiology*, 14(4), pp.161-168.
34. Renault, H., El Amrani, A., Palanivelu, R., Updegraff, E.P., Yu, A., Renou, J.P., Preuss, D., Bouchereau, A. and Deleu, C., 2011. GABA accumulation causes cell elongation defects and a decrease in expression of genes encoding secreted and cell wall-related proteins in Arabidopsis thaliana. *Plant and Cell Physiology*, 52(5), pp.894-908.
35. Ruiz-Lozano, J.M., Porcel, R., Azcón, C. and Aroca, R., 2012. Regulation by arbuscular mycorrhizae of the integrated physiological response to salinity in plants: new challenges in physiological and molecular studies. *Journal of Experimental Botany*, 63(11), pp.4033-4044.
36. Schulze, J., 2004. How are nitrogen fixation rates regulated in legumes?. *Journal of Plant Nutrition and Soil Science*, 167(2), pp.125-137.
37. Shrivastava, P. and Kumar, R., 2015. Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi Journal of Biological Sciences*, 22(2), pp.123-131.
38. Sulieman, S. and Tran, L.S.P., 2015. Phosphorus homeostasis in legume nodules as an adaptive strategy to phosphorus deficiency. *Plant Science*, 239, pp.36-43.
39. Tuna, A.L., Kaya, C., Dikilitas, M. and Higgs, D., 2008. The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. *Environmental and Experimental Botany*, 62(1), pp.1-9.
40. Uchida, A., Jagendorf, A.T., Hibino, T., Takabe, T. and Takabe, T., 2002. Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. *Plant Science*, 163(3), pp.515-523.

41. Wang, W., Vinocur, B., Shoseyov, O. and Altman, A., 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science*, 9(5), pp.244-252.
42. Wani, S.A., Chand, S. and Ali, T., 2013. Potential use of *Azotobacter chroococcum* in crop production: an overview. *Current Agriculture Research Journal*, 1(1), pp.35-38.
43. Wu, S.C., Cao, Z.H., Li, Z.G., Cheung, K.C. and Wong, M.H., 2005. Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. *Geoderma*, 125(1-2), pp.155-166.
44. www.ildis.org/
45. Zhou, B., Guo, Z., Xing, J. and Huang, B., 2005. Nitric oxide is involved in abscisic acid-induced antioxidant activities in *Stylosanthes guianensis*. *Journal of Experimental Botany*, 56(422), pp.3223-3228.

Chapter 2

Literature review

2. Introduction

2.1. Family Leguminosae (Fabaceae)

The Leguminosae or Fabaceae commonly referred to as the pea family is one of the largest land plant families of angiosperms (Hickey and King, 1988; Wojciechowski, 2003; Pennington *et al*., 2004). The pea family is usually placed in the order Fabales (Bello *et al*., 2009) and is of utmost importance agriculturally and economically due to its wide distribution and other attributes, such as biological nitrogen fixation (BNF) through symbiotic association with soil micro-organisms (Ellison *et al.,* 2006). Based on species number, Fabaceae which consists of ~720 genera and ~19 000 known species is ranked third after Orchidaceae and Asteraceae (Wojciechowski, 2003; Ellison *et al.,* 2006). Most taxonomic systems identify two monophyletic (*Mimosoideae* and *Papilionoideae*) and one-paraphyletic (*Caesalpilionoideae*) subfamilies that constitute the Fabaceae family (Wojciechowski, 2003; Pennington *et al*., 2004; Ellison *et al.,* 2006).

The Fabaceae family has a worldwide distribution and is commonly distributed in the tropical, temperate and dry forest regions in Africa and America (Crews, 1999; Doyle and Luckow, 2003). The use of members of the Fabaceae family has been coupled to human evolution as the family diversified about 56-66 million years ago during the first geologic epoch of the Paleogene (Poth *et al*., 2011). Members of the Fabaceae family with agricultural and economical importance include *Pisum sativum* (pea), *Glycine max* (soybean), *Arachis hypogaea* (peanut) and *Phaseolus* (beans) (Werner, 2005; Singh *et al.,* 2007).

The ability of Fabaceae family to undertake biological nitrogen (N) fixation through mutual symbiosis with *Rhizobium* bacteria has equipped farmers and gardeners with cheaper alternatives to expensive chemical fertilizers (Anil *et al.,* 1998). This ability makes Fabaceae important in the land-based N-cycle and has led to increased use of plants such as legumes to replenish soil with low N content (crop rotation) and increase crop yield through intercropping (Balsdon *et al*., 1997; Anil *et al.,* 1998).

2.2.Biological nitrogen (N) fixation

Biological N fixation is a process in which various N-fixing microorganisms reduce molecular dinitrogen (N_2) in the atmosphere into ammonia (NH_3) in a reaction catalyzed by an oxygen-sensitive enzyme called nitrogenase (Robson and Postgate, 1980; Werner, 2005).

$$N_2 + 16ATP + 16H_2O + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i + 8H^+$$

The microorganisms capable of fixing molecular nitrogen are called diazotrophs (Dixon and Wheeler, 1986) and are present in both the agricultural and natural systems as either free-living or plant-associated bacteria (Figure 2.1.) (Zahran, 1999; Herridge *et al.,* 2008). These bacteria are classified under the genus *Rhizobium* or the genus *Bradyrhizobium* (Zahran, 1999; Herridge *et al.,* 2008). Plants are unable to convert N_2 into the usable form as they lack the nitrogenase enzyme (Robson and Postgate, 1980; Werner, 2005). However they have overcome this by establishing a symbiotic association with rhizobia, in return the plants provide the rhizobia with carbon as organic acids and protection by housing the rhizobia in the root nodules (Robson and Postgate, 1980; Werner, 2005).

The symbiotic association is established by the rhizobia in soils infecting the plant root hair inducing nodulation (Gage, 2004; Werner, 2005). Certain *Rhizobium* strains have been reported to infect multiple plant species (Zahran, 1999; Anil *et al.,* 1998). In the legume-rhizobia symbiosis, the bacterium is unable to fix N_2 independently (De Varennes and Goss, 2007). However, both entities can live independent of each other (De Varennes and Goss, 2007). The symbiotic association can also be tripartite by including *Arbuscular Mycorrhizal* fungi, which increase the plant root surface area to optimize nutrient (e.g. phosphorus) and water uptake (Dodd, 2000; Johansson *et al*., 2004). Phosphorus (P) is a major nutrient element in plants, even more in legume plants as it plays a role in cellular energy metabolism, promotes root growth and nodule development (Uchida, 2000; Khan *et al*., 2010).

To better understand the effects of essential nutrients like P on plant growth and stress tolerance, it is imperative to assess more than just the interaction between the host and a single nodulating microbial species. With more evidence suggesting that the nodule microbiome is a complex,

perhaps even coordinated community with multiple beneficial cross talks and features (Kohli *et al*., 2013). The best approach towards sustainable agriculture is to understand the roles of the phytomicrobiome as a collective, including endophytic, free-living and nodule-associated bacteria. Research has shown that non-rhizobial bacteria are capable of not only inhabiting root nodules but can also be potential N-fixers that induce nodulation in certain plant species (Mishra *et al*., 2009). Nevertheless, certain microbial species (e.g. Bacillus) promote nodulation and N availability when co-inoculated with rhizobial strains, but fail to independently induce nodulation (Rajendran *et al*., 2008).

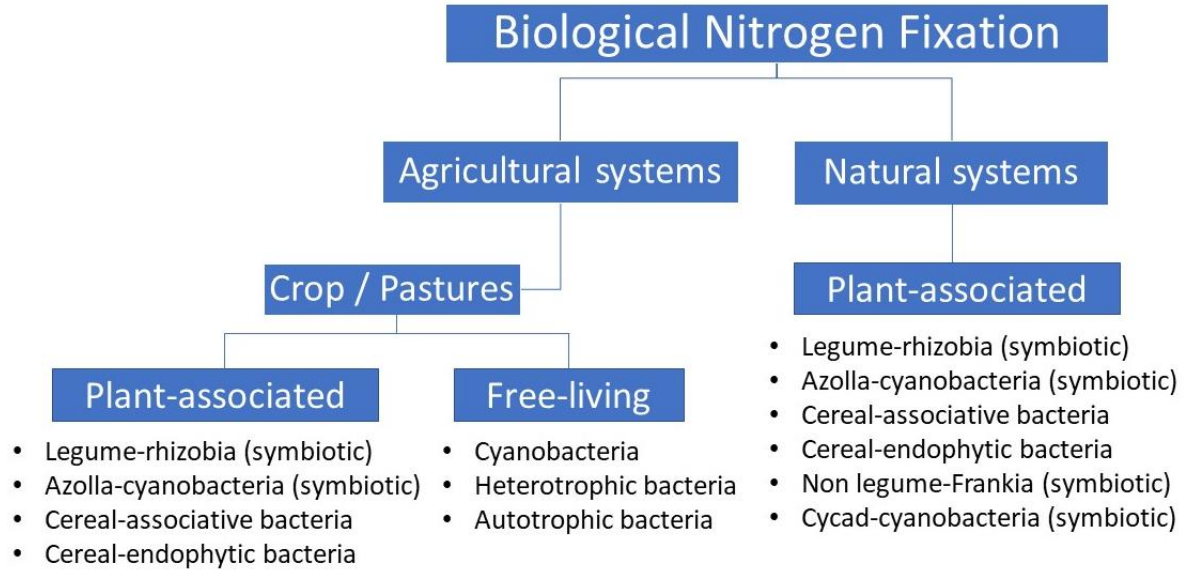


Figure 2.1.: Overview of Biological Nitrogen Fixation. Adapted from Herridge *et al.*, 2008.

2.2.1. Nodulation and types of nodules

Two main types of root nodules have been identified that are associated with nitrogen-fixing bacteria, namely the indeterminate nodules and the determinate nodules (Ferguson *et al.*, 2010). Determinate nodules are mostly found on tropical legumes such as *Glycine* and *Vigna* and are characterized by their spherical shape and loss of meristematic activity succeeding initiation, therefore making nodule growth reliant on cell expansion (Schultze and Kondorosi, 1998; D'haeseleer *et al*., 2010). On the contrary, indeterminate nodules are found on both the tropical and temperate legumes such as *Pisum* and *Medicago* and are characterized by their cylindrical shape and persistent meristematic activity after initiation, thus nodule growth is dependent on the production of new cells (Schultze and Kondorosi, 1998; D'haeseleer *et al*., 2010). This has led

to indeterminate nodules having zones of various stages of maturity (Figure 2.2.). Indeterminate nodules tend to produce and export amide N products while the determinate nodules produce ureide N products for plant use (Vance *et al*., 1979; Schulze and Kondorosi, 1998).

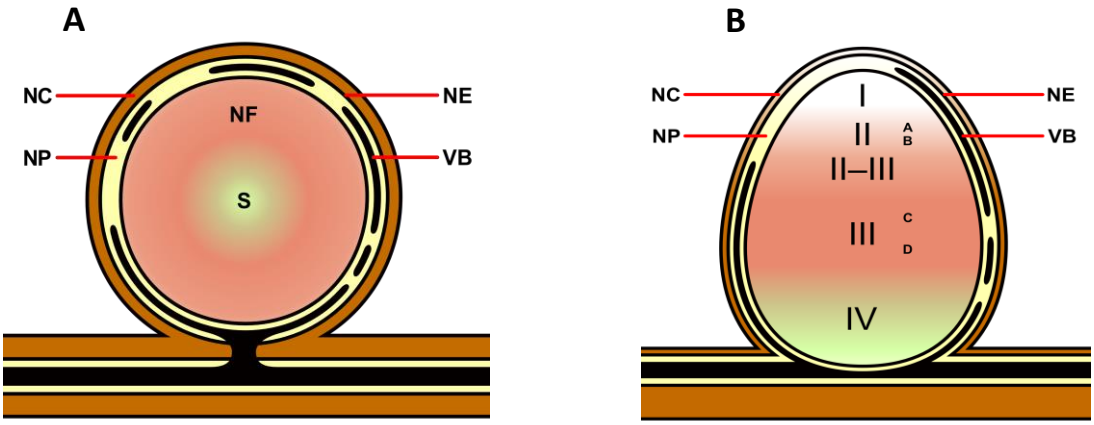


Figure 2.2.: Diagram illustrating the (A) determinate and the (B) indeterminate nodules. Both types of nodules have the **NC** – nodule cortex **NE** – nodule endodermis **VB** – vascular bundle and **NP**– nodule parenchyma. The determinate nodule has the **S** – senescence zone and the **NF** – nitrogen fixation zone. The indeterminate nodule has **I** – active meristem **II** – infection zone **II-III** – interzone (intermediate) **III** – nitrogen fixation zone **IV** – senescent zone **A** – invasion zone **B** – pre-fixing zone **C** – efficient zone **D** – inefficientzone.

Nodulation is initiated by the release of flavonoids by the legume plant roots (Subramanian *et al*., 2007). Specific rhizobial bacteria in the surrounding inoculated soil sense these flavonoids and this results in the expression of nod genes and root hair colonization (Vasse *et al*., 1990; Kanamori *et al*., 2006). Upon attachment to the root hair, the rhizobial bacteria release nod factors that cause the root hair to curl in such a manner that encapsulates the rhizobial bacteria (Kanamori *et al*., 2006). The rhizobial bacteria undergo cell division to form a micro-colony that travels down the infection thread and into the nodule cells cytoplasm, where they differentiate into nitrogen-fixing bacteroids (Kanamori *et al*., 2006). Nodulation is dependent on external factors such as soil acidity and internal factors such as auto-regulation (Schultze and Kondorosi, 1998; Kanamori *et al*., 2006). On average, effective N fixation and nodule development occur four weeks after seed germination. It is well accepted that symbiotic N_2 fixation imposes a metabolic burden on the host plant, as the reaction to reduce atmospheric N_2 into NH_3 is an energy-consuming reaction (Warembourg and Roumet, 1989).

2.3.Carbon (C) and energy cost during N-fixation

Lambers and De Visser (1984) approximated the cost N-fixation to be 6 mg of C per mg of N reduced while recent studies suggest that the cost is dependent on plant and/or bacterial species involved (Valentine *et al.*, 2010). During symbiotic N_2 fixation, host plant photosynthates along with 8 adenosine triphosphates (ATPs) are required to produce a single ammonium (NH_4^+) molecule that serves as an N source to the plant (Valentine *et al.*, 2010).

$$N_2 + 16ATP + 16H_2O + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i + 8H^+$$

During the tripartite symbiotic association, the carbon energy cost is even greater as more plant photosynthates are required by the AM and rhizobia (De Varennes and Goss, 2007). There is no evidence suggesting that N_2 fixation is detrimental to plant productivity and yield potential, and this raises the notion that C costs of N_2 fixation are not coupled to the yield potential, but this is yet to be confirmed (Vance and Heichel, 1991).

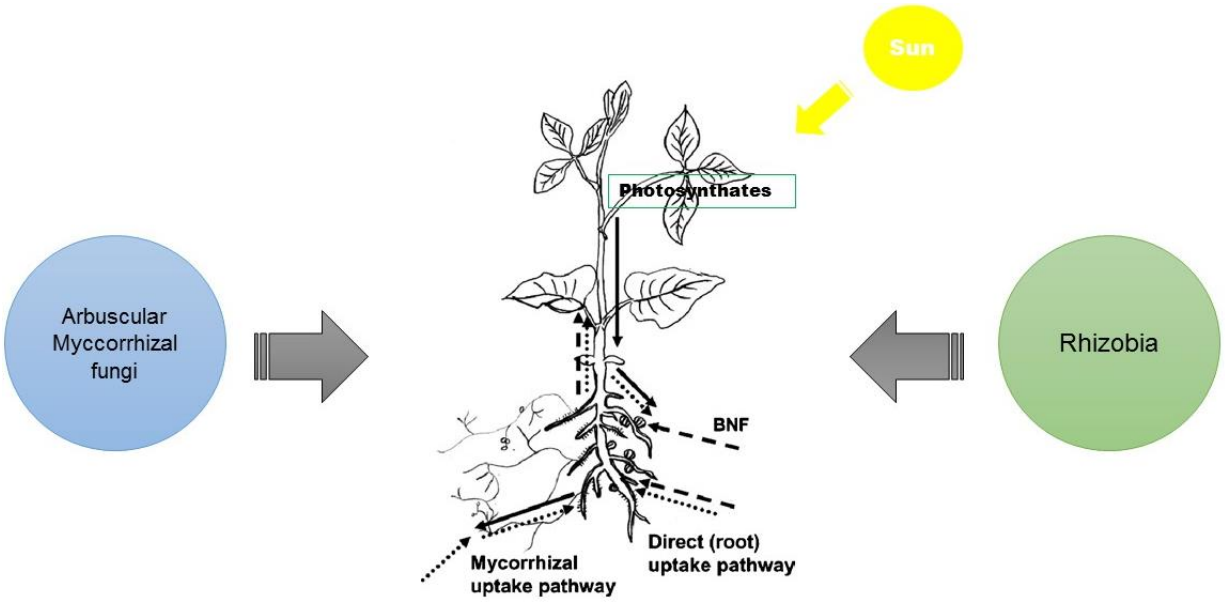


Figure 2.3.: Tripartite symbiotic association nutrient uptake pathways. Solar radiation is converted by plants into photosynthates that are transported to the *Rhizobia* and *Arbuscular Mycorrhiza.* In exchange, the microorganisms provide the plant with essential nutrients.

2.4.Environmental stresses that affect symbiotic nitrogen fixation

In most organisms (including legume plants) exposure to stress leads to either a non-specific general response or a stressor-induced response to help in the restitution or regeneration phase upon alleviation of the stressor (Lichtenthaler, 1998). The effects of physical stress such as salinity, nutrient deficiency, acidity (pH), drought and heat have been extensively studied and found to elicit plant response in terms of signal transduction, biophysical characteristics, energy metabolism, and overall growth and development (Kasuga *et al.*, 1999; Pugnaire and Luque, 2001). Extensive work in grassland and savannah ecosystems has been based on the effects of drought and salinity stress (Munns, 2002; Chaves *et al*., 2009; Jouyban, 2012). It should be noted that plants rarely grow under optimum conditions and this has led to the notion that stress (biotic or abiotic) might play a key role in plant biological diversity (Wang *et al.,* 2006). Four distinct phases of stress response have been identified (Figure 2.4.) based on stresses that are either for plant growth (eu-stress) or against it (dis-stress) (Lichtenthaler, 1998).

When it comes to symbiotic N-fixation, most of the aforementioned stresses usually have a direct effect on the bacteria and not the host plant (O'Hara, 2001; Glick, 2012). By impairing the bacterial growth and physiology, these factors subsequently affect the host plant N acquisition (O'Hara, 2001; Glick, 2012). A notable factor is that acid soil complexes are characterized by high aluminium (Al) and manganese (Mn) that sequester P, which is an important nutrient that promotes nodulation and N fixation (O'Hara, 2001; Glick, 2012). Symbiotic N fixation is also affected by the limitation of other nutrients such as iron, calcium and molybdenum (O'Hara, 2001; Glick, 2012).

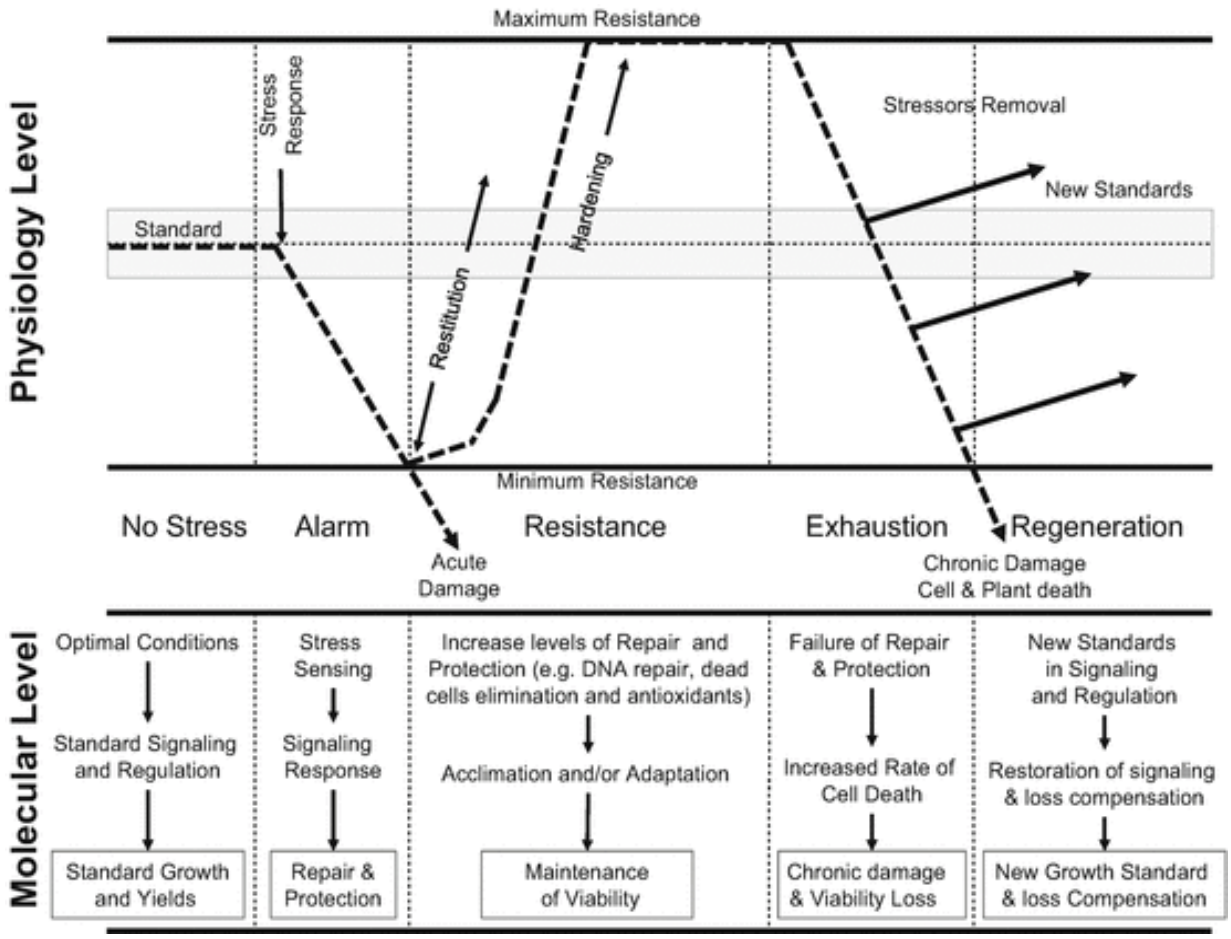


Figure 2.4.: The generic stressor-induced response in plants. The four phases of plant stress response are the Alarm, Resistance, Exhaustion and Regeneration phase. Plant stress response is initiated when a biotic/abiotic stressor is induced. The plant goes from the No Stress to the Alarm phase, which is characterized by stress sensing and response signaling. This triggers the Resistance phase in which the plant acclimatize and adapts to the stressor imposed. This is achieved by increasing levels of repair and protection to maintain cell viability. If the stressor persists, the plant enters the Exhaustion phase. In this phase, the plant experiences chronic damage and loss of viability as a result of increased rate of cell death. However, if the stressor is removed, the plant enters the Regeneration phase. During this phase, the plant achieves new standards in signaling and regulation to compensate for the loss caused by the stressor. Adapted from Lichtenthaler, 1998.

2.5.Causes and effects of soil phosphorus (P) deficiency on N_2 fixation

Symbiotic N_2 fixation has enabled legumes to be self-sufficient by acquiring N through N_2 reduction into NH_3 by *Rhizobium and Bradyrhizobium* bacteria that rely on host plant photosynthetic energy (Okon and I'iardy, 2012). P is regarded as the main carrier molecule for photosynthesis-produced energy that is used for plant growth and reproduction (Shenoy and

Kalagudi, 2005; Blankenship *et al*., 2011). However, P is not only present in ATP but is also a structural component of DNA and RNA which are involved in the genetic make-up and protein synthesis of all living organisms (La Roche *et al.,* 1993). P is also involved in catalysis, post-translational modification such as phosphorylation, activation/deactivation of certain proteins through phospho-transferase and kinase (La Roche *et al.,* 1993; Dyhrman, 2016).

The readily available form of P for plants is the phosphate form (PO_4), with di-hydrogen phosphate ($H_2PO_4^-$) being the predominant soluble P ion in soils (Sims and Pierzynski, 2005; Yadav and Verma, 2012). Sufficient P levels not only promote plant root growth but also cold tolerance, early maturity and nodulation in certain legumes (Grant *et al*., 2001; Zaidi *et al.,* 2009). However, soil P is one of the most limiting nutrients for most forage and crop plants (Khan *et al.,* 2010). The low P availability is usually linked to aluminium (Al^{3+}) toxicity that is coupled to low soil pH (Bose *et al.,* 2013). The process in which soil P is rendered unavailable to plants is called P fixation by cations such as aluminium (Al^{3+}), iron (Fe^{+3}, Fe^{+2}), calcium (Ca^{+2}), magnesium (Mg^{+2}) and manganese (Mn^{+2}), which limits and/or reduces P mobility and solubility thus affecting P acquisition by plants (Reeve and Sumner, 1970; Reyaz, 2013).

P deficiency has a direct effect on the host plant as it stunts growth, reduces leaf area and the number of leaves thus minimizing photosynthetic ATP and other photosynthates (Khan *et al.,* 2010). This has an indirect effect on N_2 fixation as the microbial activities in the root nodules are dependent on these photosynthates to function (Denison and Kiers, 2011). Timely visual diagnosis of P deficiency in plants is hard to achieve but certain plants (e.g. maize) show purple discoloration due to anthocyanin synthesis that is triggered by low P (McCauley *et al.,* 2009). Besides flower coloration, anthocyanin have been reported to have antioxidant activity and radiation protection that reduces the rate of cell death in plants (Kano *et al.,* 2005). This makes anthocyanin production one of the many possible plant survival strategies that need to be investigated.

2.6.The use of crops as forage

Numerous varieties of field-crops such as cereals and leguminous plants have been successfully used as either forage or silage for over a decade (Hill and Leaver, 1990). The importance of such

field-crops has increased over the years, not only in the westernized countries but globally (Phipps, 1994). The increase is coupled with the field-crops potential to supply ruminant animals (such as livestock) with an adequate amount of energy-rich fodder (Hill and Leaver, 1990; Phipps, 1994). The use of field legumes (or intercropping) as forage has been favored over the use of cereals because they require minimal protein supplementation to promote milk or meat production (Phipps, 1994; Anil *et al.,* 1998). The family *Fabaceae* that includes peas has been used in crop rotations due to its various advantages that range from N-fixing ability to the reduction of soil erosion (Anil *et al*., 1998). Legume plant genera can be divided into two groups according to seed size (Table 2.1.) (McCartney and Fraser, 2010) and used as an intercrop for forage (Table 2.2.) but the yield is dependent on external factors such as stress (Fujita *et al.*, 1992). Comparison of the survival strategies of the different genera has the potential to enable the selection of advantageous traits for sustainable agriculture.

Table 2.1.: Relative seed size of legume plant genera

Group	Genus	Seed size
One	*Medicago*	Small / Medium
One	*Trifolium*	Small / Medium
One	*Melilotus*	Small / Medium
One	*Lens*	Small / Medium
Two	*Lupinus*	Large
Two	*Pisum**	Large
Two	*Phaseolus*	Large
Two	*Lathyrus*	Large

The table is an example of the division of legume plant genera based on seed size. *Genus of interest. Adapted from McCartney and Fraser (2010).

Table 2.2.: Intercropping research in temperate regions

Intercrop		Country	Authors	Purpose
Oats				
	Faba bean	Canada	Jedel and Helm (1993)	Forage
	Peas (*Pisum sativa*)	Australia	Mason and Pritchard (1987)	Forage
		North	Chapko *et al.* (1991)	Forage
	Vetch (*Vicia sativa*)	America	Moreira (1989)	Forage
		Portugal	Papastylianou (1990)	Forage
		Cyprus		
Barley				
	Kale	U.K.	Moorby *et al*. (1998)	Forage
	Peas	N. America	Jedel and Helm (1993)	Forage
	Persian clover (*Trifolium resupinatum*)	Canada	Stout *et al*. (1997)	Forage
Wheat				
	White clover (*Trifolium repens)*	U.K.	Balsdon *et al.* (1997)	Forage
	Peas	Canada	Walton (1975)	Forage
	Soyabean (*Glycine max*)	Canada	Walton (1975)	Forage

Table shows intercropping research conducted globally in which the field crops were to be used as animal fodder. Adapted from Anil *et al*., 1998.

2.7.Study species

Pisum sativum L. is an annual winter legume that is grown in most parts of the world, especially in farms and/or grassland ecosystems (Ceyhan and Avci, 2005). *P. sativum* L. is viewed as an essential plant for effective soil management that is coupled to soil tillage, crop rotation and the addition of green manures to form part of the terrestrial nutrient cycle (Cousin, 1997). Green manures (e.g. *P. sativum*) is broken down by soil microorganisms into nutrients that are released to enrich the soil and made readily available to succeeding plants (Cherr *et al*., 2006). This has an overall turnover increase of soil C and N content since the proteins/amino acids in the plant are converted into nitrates (NO_3^-) post-harvest (Midmore *et al*., 2000). Field pea has been found

to grow best in well-drained soils with a pH range of 6.0 to 7.0, with adequate moisture due to the plant being drought intolerant (Miles and Sonde, 2003).

The pea plant has fibrous, shallow roots that are the site of nodulation, which occurs when the soil population of rhizobia is above the optimal nodulation threshold (~100/g of soil) at soil pH ≤ 6.6 (Singleton and Tavares, 1986; Rice *et al.,* 2000). The efficiency of nodulation and nodule activity is also governed by temperature (high temperature = low nodulation), *Rhizobia* viability in the soil type and duration of host plant absence (Zahran, 1999). However, the plants are more susceptible to heat and drought stress during the flowering stage which usually occurs ~50 days after sowing (Guilioni *et al*., 1997; Morrison and Stewart, 2002). The fibrous root system also helps anchor the forage crop in a manner that minimizes soil erosion (https://www.nda.agric.za/docs/erosion/erosion.htm). Soil type also has an effect on the yield, with dark brown and black soils being identified as the ideal soil type to obtain better crop yield (https://www3.epa.gov/npdes/pubs/cafo_permitmanual_appendixa.pdf). There are white-flowered varieties of field pea such as Whistler pea that have less anthocyanin content than their purple-flowered counterparts but both varieties are known for vining and climbing, with vines reaching six inches (Grotewold, 2006; Moreau *et al*., 2012; https://www.daff.gov.za). Field pea is known for having great grazability as a lone crop and as an intercrop e.g. with oat or vetch (https://www.pivotandgrow.com).

The high protein, low fat content and various applications of pea are the basis of its lucrativeness (Janzen *et al*., 2014). Profits can be generated through retail as animal feed, human food and seeds for farming. The major production countries of field pea include Canada, China, the Soviet Union (Russia) and India (Janzen *et al*., 2014). However, in South Africa the major production areas exclude the KwaZulu-Natal (KZN) province (Table 2.3.). KZN is a relatively mountainous province that is located on the southeastern side of South Africa (Appendix A: Figure S2). Limitation of essential soil macronutrients, like in most South African regions, has been reported in KZN grasslands and savannahs (Craine *et al.,* 2008). Therefore, the assessment of the effects of KZN soil geochemistry and microflora will determine whether forage pea maybe adapted to the nutrient-poor soils in grassland and savannah ecosystems and be used by subsistence and commercial farmers as green manure or as feed.

Figure 2.5.: *Pisum sativum* L. (A) intercropped with vetch and (B) grazed by pigs.

Table 2.3.: South African major field pea production regions

Province	District	Town
Western Cape	Eden	George
Mpumalanga	Gert Sibande	Secunda
	Makaligwa	Ermelo
	Nkangala	Middleburg
		Delmas
Free State	Thabo Mofutsanyana	Bethlehem
		Witsieshoek
		Harrismith
		Ventersdorp
		Klerksdorp
North West	Bojanala Platinum	Rustenburg
		Moretele
		Koster
		Brits
	Dr Kenneth Kaunda	Potchefstroom

*Information retrieved from https://www.daff.gov.za

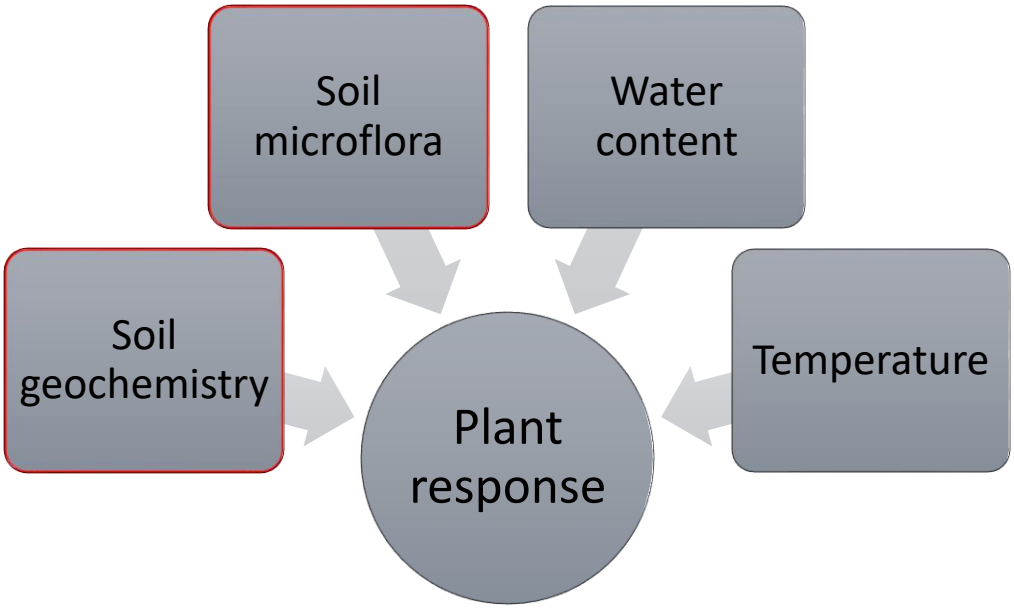


Figure 2.6.: Factors influencing plant growth and development. The current study is focused on the factors outlined in red.

2.8.References

1. Anil, L., Park, J., Phipps, R.H. and Miller, F.A., 1998. Temperate intercropping of cereals for forage: a review of the potential for growth and utilization with particular reference to the UK. *Grass and Forage Science*, 53(4), pp.301-317.
2. Balsdon, S.L., Martyn, T.M., Clements, R.O. and George, S., 1997. The potential of a clover: cereal bi-cropping system to produce high quality whole-crop silage. *Fifth Research Conference British Grassland Society*, 8-10 September 1997, Seale Hayne Faculty of Agriculture, Food and Land Use, University of Plymouth, UK. pp. 85-86.
3. Bello, M.A., Bruneau, A., Forest, F. and Hawkins, J.A., 2009. Elusive Relationships Within Order Fabales: Phylogenetic Analyses Using matK and rbcL Sequence Data1. *Systematic Botany*, 34(1), pp.102-114.
4. Blankenship, R.E., Tiede, D.M., Barber, J., Brudvig, G.W., Fleming, G., Ghirardi, M., Gunner, M.R., Junge, W., Kramer, D.M., Melis, A. and Moore, T.A., 2011. Comparing photosynthetic and photovoltaic efficiencies and recognizing the potential for improvement. *Science*, 332(6031), pp.805-809.

5. Bose, J., Babourina, O., Shabala, S. and Rengel, Z., 2013. Low-pH and aluminum resistance in *Arabidopsis correlates* with high cytosolic magnesium content and increased magnesium uptake by plant roots. *Plant and Cell Physiology*, 54(7), pp.1093-1104.
6. Ceyhan, E. and Avci, M.A., 2005. Combining ability and heterosis for grain yield and some yield components in pea (*Pisum sativum* L.). *Pakistan Journal of Biological Sciences*, 8(10), pp.1447-1452.
7. Chapko, L.B., Brinkman, M.A. and Albrecht, K.A., 1991. Oat, oat-pea, barley, and barley-pea for forage yield, forage quality, and alfalfa establishment. *Journal of Production Agriculture*, 4(4), pp.486-491.
8. Chaves, M.M., Flexas, J. and Pinheiro, C., 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany*, 103(4), pp.551-560.
9. Cherr, C.M., Scholberg, J.M.S. and McSorley, R., 2006. Green manure approaches to crop production. *Agronomy Journal*, 98(2), pp.302-319.
10. Cousin, R., 1997. Peas (*Pisum sativum* L.). *Field Crops Research*, 53(1-3), pp.111-130.
11. Crews, T.E., 1999. The presence of nitrogen fixing legumes in terrestrial communities: Evolutionary vs ecological considerations. In *New Perspectives on Nitrogen Cycling in the Temperate and Tropical Americas* (pp. 233-246). *Springer*, Dordrecht.
12. D'haeseleer, K., Goormachtig, S. and Holsters, M., 2010. Legume nodule development. In *Plant Developmental Biology-Biotechnological Perspectives* (pp. 91-136). *Springer* Berlin Heidelberg.
13. De Varennes, A. and Goss, M.J., 2007. The tripartite symbiosis between legumes, rhizobia and indigenous *mycorrhizal fungi* is more efficient in undisturbed soil. *Soil Biology and Biochemistry*, 39(10), pp.2603-2607.
14. Denison, R.F. and Kiers, E.T., 2011. Life histories of symbiotic rhizobia and mycorrhizal fungi. *Current Biology*, 21(18), pp.775-785.
15. Dixon, R.O.D. and Wheeler, C.T., 1986. Nitrogen fixation in plants. Blackie and son, *Glasgow* (UK). 1986.
16. Dodd, J.C., 2000. The role of arbuscular mycorrhizal fungi in agro-and natural ecosystems. *Outlook on Agriculture*, 29(1), pp.55-62.

17. Doyle, J.J. and Luckow, M.A., 2003. The rest of the iceberg. Legume diversity and evolution in a phylogenetic context. *Plant Physiology*, 131(3), pp.900-910.
18. Dyhrman, S.T., 2016. Nutrients and their acquisition: phosphorus physiology in microalgae. In *The physiology of microalgae* (pp. 155-183). *Springer*, Cham.
19. Ellison, N.W., Liston, A., Steiner, J.J., Williams, W.M. and Taylor, N.L., 2006. Molecular phylogenetics of the clover genus (Trifolium—Leguminosae). *Molecular phylogenetics and evolution*, 39(3), pp.688-705.
20. Ferguson, B.J., Indrasumunar, A., Hayashi, S., Lin, M.H., Lin, Y.H., Reid, D.E. and Gresshoff, P.M., 2010. Molecular analysis of legume nodule development and autoregulation. *Journal of Integrative Plant Biology*, 52(1), pp.61-76.
21. Fujita, K., Ofosu-Budu, K.G. and Ogata, S., 1992. Biological nitrogen fixation in mixed legume-cereal cropping systems. *Plant and Soil*, 141(1-2), pp.155-175.
22. Gage, D.J., 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews*, 68(2), pp.280-300.
23. Glick, B.R., 2012. Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*
24. Grant, C.A., Flaten, D.N., Tomasiewicz, D.J. and Sheppard, S.C., 2001. The importance of early season phosphorus nutrition. *Canadian Journal of Plant Science*, 81(2), pp.211-224.
25. Guilioni, L., Wery, J. and Tardieu, F., 1997. Heat stress-induced abortion of buds and flowers in pea: is sensitivity linked to organ age or to relations between reproductive organs?. *Annals of Botany*, 80(2), pp.159-168.
26. Herridge, D.F., Peoples, M.B. and Boddey, R.M., 2008. Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil*, 311(1-2), pp.1-18.
27. Hickey, M. and King, C., 1988. 100 families of flowering plants. Cambridge University Press.
28. Hill, J. and Leaver, J.D., 1990. Utilisation of Whole Crop Wheat by dairy cattle. In: Pollott G.E (ed.) Milk and Meat from Forage Crops. Occasional Symposium No 24, *British Grassland Society,* pp. 99-102.
29. https://www.nda.agric.za/docs/erosion/erosion.htm

30. Jedel, P.E. and Helm, J.H., 1993. Forage potential of pulse-cereal mixtures in central Alberta. *Canadian Journal of Plant Science*, 73(2), pp.437-444.
31. Johansson, J.F., Paul, L.R. and Finlay, R.D., 2004. Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. *FEMS Microbiology Ccology*, 48(1), pp.1-13.
32. Jouyban, Z., 2012. The effects of salt stress on plant growth. *Technical Journal of Engineering and Applied Sciences*, 2(1), pp.7-10.
33. Kanamori, N., Madsen, L.H., Radutoiu, S., Frantescu, M., Quistgaard, E.M., Miwa, H., Downie, J.A., James, E.K., Felle, H.H., Haaning, L.L. and Jensen, T.H., 2006. A nucleoporin is required for induction of Ca2+ spiking in legume nodule development and essential for rhizobial and fungal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, 103(2), pp.359-364.
34. Kano, M., Takayanagi, T., Harada, K., Makino, K. and Ishikawa, F., 2005. Antioxidative activity of anthocyanins from purple sweet potato, *Ipomoera batatas* cultivar Ayamurasaki. *Bioscience, Biotechnology, and Biochemistry*, 69(5), pp.979-988.
35. Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K., 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotechnology*, 17(3), pp.287.
36. Khan, M.S., Zaidi, A., Ahemad, M., Oves, M. and Wani, P.A., 2010. Plant growth promotion by phosphate solubilizing fungi–current perspective. *Archives of Agronomy and Soil Science*, 56(1), pp.73-98.
37. Kohli, A., Sreenivasulu, N., Lakshmanan, P. and Kumar, P.P., 2013. The phytohormone crosstalk paradigm takes center stage in understanding how plants respond to abiotic stresses. *Plant Cell Reports*, 32(7), pp.945-957.
38. La Roche, J., Geider, R.J., Graziano, L.M., Murray, H. and Lewis, K., 1993. Induction of Specific Proteins in Eukaryotic Algae Grown Under Iron-, Phosphorus-, or Nitrogen-Deficient Conditions 1. *Journal of Phycology*, 29(6), pp.767-777.
39. Lambers, H. and De Visser, R., 1984. Energy metabolism in nodulated roots. In *Advances in Nitrogen Fixation Research* (pp. 453-460). *Springer*, Dordrecht.
40. Lichtenthaler, H.K., 1998. The stress concept in plants: an introduction. *Annals of the New York Academy of Sciences*, 851(1), pp.187-198.

41. Mason, W.K. and Pritchard, K.E., 1987. Intercropping in a temperate environment for irrigated fodder production. *Field Crops Research*, 16(3), pp.243-253.
42. McCartney, D. and Fraser, J., 2010. The potential role of annual forage legumes in Canada: A review. *Canadian Journal of Plant Science*, 90(4), pp.403-420.
43. McCauley, A., Jones, C. and Jacobsen, J., 2009. Plant nutrient functions and deficiency and toxicity symptoms. *Nutrient Management Module*, 9, pp.1-16.
44. Midmore, D.J., Ladha, J.K., Olk, D.C. and Schmidhalter, U., 2000. Legume decomposition and nitrogen release when applied as green manures to tropical vegetable production systems. *Agronomy Journal*, 92(2), pp.253-260.
45. Miles, C.A. and Sonde, M., 2003. Pea shoots. Washington State University Cooperative Extension.
46. Mishra, P.K., Mishra, S., Selvakumar, G., Bisht, J.K., Kundu, S. and Gupta, H.S., 2009. Coinoculation of *Bacillus thuringeinsis*-KR1 with *Rhizobium leguminosarum* enhances plant growth and nodulation of pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.). *World Journal of Microbiology and Biotechnology*, 25(5), pp.753-761.
47. Moreau, C., Ambrose, M.J., Turner, L., Hill, L., Ellis, T.N. and Hofer, J.M., 2012. The b gene of pea encodes a defective flavonoid 3′, 5′-hydroxylase, and confers pink flower color. *Plant Physiology*, 159(2), pp.759-768.
48. Moreira, N., 1989. The effect of seed rate and nitrogen fertilizer on the yield and nutritive value of oat-vetch mixtures. *The Journal of Agricultural Science*, 112(1), pp.57-66.
49. Morrison, M.J. and Stewart, D.W., 2002. Heat stress during flowering in summer Brassica. *Crop science*, 42(3), pp.797-803.
50. Munns, R., 2002. Comparative physiology of salt and water stress. *Plant, Cell & Environment*, 25(2), pp.239-250.
51. O'Hara, G.W., 2001. Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. *Australian Journal of Experimental Agriculture*, 41(3), pp.417-433.
52. Okon, Y. and I'IARDY, R.W.F., 2012. Developments in Basic and Applied Biological Nitrogen Fixationl. Plant Physiology 8: A Treatise: Nitrogen Metabolism, 8, pp.5.

53. Papastylianou, I., 1990. Response of pure stands and mixtures of cereals and legumes to nitrogen fertilization and residual effect on subsequent barley. *The Journal of Agricultural Science*, 115(1), pp.15-22.
54. Pennington, R.T., Cronk, Q.C. and Richardson, J.A., 2004. Introduction and synthesis: plant phylogeny and the origin of major biomes. Philosophical Transactions of the Royal Society B: *Biological Sciences*, 359(1450), pp.1455-1464.
55. Phipps, R.H., 1994. Complementary forages for milk production. *Recent Advances in Animal Nutrition*.
56. Poth, A.G., Colgrave, M.L., Philip, R., Kerenga, B., Daly, N.L., Anderson, M.A. and Craik, D.J., 2011. Discovery of cyclotides in the fabaceae plant family provides new insights into the cyclization, evolution, and distribution of circular proteins. *ACS Chemical Biology*, 6(4), pp.345-355.
57. Pugnaire, F.I. and Luque, M.T., 2001. Changes in plant interactions along a gradient of environmental stress. Oikos, 93(1), pp.42-49.
58. Rajendran, G., Sing, F., Desai, A.J. and Archana, G., 2008. Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. *Bioresource Technology*, 99(11), pp.4544-4550.
59. Reeve, N.G. and Sumner, M.E., 1970. Effects of aluminum toxicity and phosphorus fixation on crop growth on oxisols in natal 1. *Soil Science Society of America Journal*, 34(2), pp.263-267.
60. Reyaz, R., 2013. Molecular analysis of Rhizobium isolates of *Cajanus cajan* (L.) Millsp. collected from strongly acidic soil regimes by employing 2-D Electrophoresis (Doctoral dissertation, Birsa Agricultural University, Kanke, Ranchi, Jharkhand).
61. Rice, W.A., Clayton, G.W., Olsen, P.E. and Lupwayi, N.Z., 2000. Rhizobial inoculant formulations and soil pH influence field pea nodulation and nitrogen fixation. *Canadian Journal of Soil Science*, 80(3), pp.395-400.
62. Robson, R.L. and Postgate, J.R., 1980. Oxygen and hydrogen in biological nitrogen fixation. *Annual Reviews in Microbiology*, 34(1), pp.183-207.
63. Schultze, M. and Kondorosi, A., 1998. Regulation of symbiotic root nodule development. *Annual Review of Genetics*, 32(1), pp.33-57.

64. Shenoy, V.V. and Kalagudi, G.M., 2005. Enhancing plant phosphorus use efficiency for sustainable cropping. *Biotechnology Advances*, 23(7-8), pp.501-513.
65. Sims, J.T. and Pierzynski, G.M., 2005. Chemistry of phosphorus in soils. Chemical processes in soils (chemicalprocess), pp.151-192.
66. Singh, R.J., Chung, G.H. and Nelson, R.L., 2007. Landmark research in legumes. *Genome*, 50(6), pp.525-537.
67. Singleton, P.W. and Tavares, J.W., 1986. Inoculation response of legumes in relation to the number and effectiveness of indigenous Rhizobium populations. *Applied Environmental Microbiology*, 51(5), pp.1013-1018.
68. Stout, D.G., Brooke, B., Hall, J.W. and Thompson, D.J., 1997. Forage yield and quality from intercropped barley, annual ryegrass and different annual legumes. *Grass and forage Science*, 52(3), pp.298-308.
69. Subramanian, S., Stacey, G. and Yu, O., 2007. Distinct, crucial roles of flavonoids during legume nodulation. *Trends in Plant Science*, 12(7), pp.282-285.
70. Valentine, A.J., Benedito, V.A. and Kang, Y., 2010. Legume nitrogen fixation and soil abiotic stress: from physiology to genomics and beyond. *Annual Plant Reviews* Volume 42: Nitrogen Metabolism in Plants in the Post-Genomic Era, pp.207-248.
71. Vance, C.P. and Heichel, G.H., 1991. Carbon in N2 fixation: limitation or exquisite adaptation. *Annual Review of Plant Biology*, 42(1), pp.373-390.
72. Vance, C.P., Heichel, G.H., Barnes, D.K., Bryan, J.W. and Johnson, L.E., 1979. Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. *Plant Physiology*, 64(1), pp.1-8.
73. Vasse, J., De Billy, F., Camut, S. and Truchet, G., 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *Journal of Bacteriology*, 172(8), pp.4295-4306.
74. Walton, P.D., 1975. Annual forages seeding rates and mixtures for central Alberta. *Canadian Journal of Plant Science*, 55(4), pp.987-993.
75. Wang, B.C., Zhou, J., Wang, Y.C., Zhu, L.C. and Teixeira da Silva, J.A., 2006. Physical stress and plant growth. Floriculture, ornamental and plant biotechnology: advances and topical issues, 3, pp.68-85.

76. Warembourg, F.R. and Roumet, C., 1989. Why and how to estimate the cost of symbiotic N_2 fixation? A progressive approach based on the use of ^{14}C and ^{l5}N isotopes. In *Ecology of arable land—perspectives and challenges* (pp. 31-41). *Springer*, Dordrecht.
77. Werner, D., 2005. Production and biological nitrogen fixation of tropical legumes. In *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment* (pp. 1-13). *Springer*, Dordrecht.
78. Wojciechowski, M.F., 2003. Reconstructing the phylogeny of legumes (Leguminosae): an early 21st century perspective. *Advances in Legume Systematics*, part, 10, pp.5-35.
79. Yadav, B.K. and Verma, A., 2012. Phosphate solubilization and mobilization in soil through microorganisms under arid ecosystems. In *The functioning of ecosystems*. InTech.
80. Zahran, H.H., 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews*, 63(4), pp.968-989.
81. Zaidi, A., Khan, M., Ahemad, M. and Oves, M., 2009. Plant growth promotion by phosphate solubilizing bacteria. *Acta Microbiologica et Immunologica Hungarica*, 56(3), pp.263-284.
82. https://www3.epa.gov/npdes/pubs/cafo_permitmanual_appendixa.pdf
83. Grotewold, E., 2006. The genetics and biochemistry of floral pigments. Annual Reviews in Plant Biology, 57, pp.761-780.
84. https://www.daff.gov.za/Daffweb3/Portals/0/Brochures%20and%20Production%20guidelines/Field%20Pea%20Production%20Guideline.pdf
85. https://www.pivotandgrow.com/resources/production/green-manures/module-2-green-manure-profiles/
86. Janzen, J., Brester, G. and Smith, V., 2014. Dry peas: trends in production, trade, and price. *Agricultural Marketing Policy Center*, briefing, 57.
87. Craine, J.M., Morrow, C. and Stock, W.D., 2008. Nutrient concentration ratios and co-limitation in South African grasslands. *New Phytologist*, 179(3), pp.829-836.
88. Sithole, N., Pérez-Fernández, M. and Magadlela, A., 2019. Nutritional status of soils from KwaZulu-Natal modulate symbiotic interactions and plant performance in *Vigna unguiculata* L.(Walp). *Applied Soil Ecology*, 142, pp.1-7.

Chapter 3

Soil geochemistry & microflora

Soil nutrition, microbial composition and associated soil enzyme activities in KwaZulu-Natal grasslands and savannah ecosystems soils

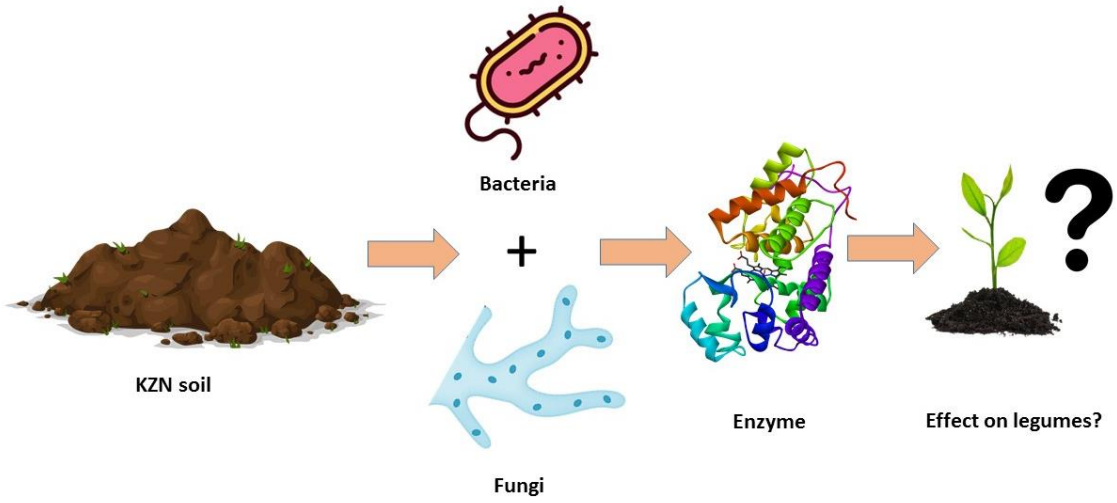
Ntuthuko S. Zungu[a], Samson O. Egbewale[b], Ademola O. Olarinan[ab], María Pérez-Fernández[c]*, Anathi Magadlela[a]

[a]*School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, KwaZulu-Natal, South Africa*

[b]*School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa*

[c]*Ecology Area, University Pablo de Olavide, Carretera de Utrera Km 1, 41013 Sevilla, Spain*

***Correspondence:** María Pérez-Fernández **email:** maperfer@upo.es



Graphical abstract of the research conducted and presented in this write-up.

Abstract

Successful agriculture is dependent on soil quality and nutrition. Soil is the primary source of nutrients that are assimilated by plant root-systems to promote plant growth and development. The availability of these soil nutrients is regulated by factors such as pH, microbial composition and soil nutrient enzyme activities in ecosystem soils. This study aim was to determine the nutrition, microbe composition, and soil enzyme activities in four soils (from Hluhluwe, Izingolweni, Bergville, and Ashnurton) from different KwaZulu-Natal (KZN) grassland and savannah ecosystems. The four sites were found to differ significantly in all four aspects. Microorganisms identified in the soils were from the families *Bacillaceae*, *Hypocreaceae*, *Mucoraceae*, and *Nectriaceae*. Bacterial and fungal genera identified amongst these soils showed varying diversity and species richness. Bergville soils had the lowest pH, cation exchange capacity, micro, and macro nutrient concentrations. Furthermore, Bergville soil showed reduced asparaginase and lignin peroxidase activities and had the lowest dehydrogenase activities. Therefore Bergville soils had the most limiting geochemical properties which may affect the growth of grassland and savannah ecosystem vegetation and sustainable agricultural practices.

Key words: Successful agriculture, soil quality, grassland and savannah ecosystems, microbial composition.

3.1. Introduction

The poor nutrient status of soils across the African continent has posed a serious threat to plant growth and sustainable agricultural practices (Goldman, 1995; Henao and Baanante, 1999). Due to the ever-increasing population worldwide with stronger demand for agricultural products, there is a need to increase either the soil fertility of the already present agricultural land or expansion by identifying regions that are suitable for agricultural practices. African savannahs and grasslands represent putative crop areas, however, studies on geochemistry of most South African ecosystems are reported to be nutrient-poor and relatively acidic (Nandwa 2001; Mafongoya *et al*., 2006). The decreased pH in these soils leads to decreased cation-exchange capacity, indicating the overall decrease in plant essential soil nutrients such as potassium (K^+), calcium (Ca^{2+}) and ammonium (NH_4^+) (Aprile and Lorandi, 2012). These acidic conditions also lead to the sequestration of soil nutrients like phosphorus (P), which is rendered insoluble through binding with cations (Sharma *et al.,* 2013). However, soil amelioration or even soil resetting can be achieved by the enhancement of soil-borne microorganisms, as some of them have been reported to solubilize these bound and insoluble nutrients, thus increasing their availability to plants (Latha et al., 2011; Pérez-García et al., 2011).

The soil microbiota consists of N_2-fixing *Rhizobia, Ectomycorrhiza, Arbuscular Mycorrhiza, Pseudomonas, Ochrobactrum, Bacillus, Paenibacillus, Klebsiella, Lysinobacillus,* and *Actinomycetes* among others (Martínez-Hidalgo and Hirsch, 2017). All of these microorganisms are potential plant growth promoters (Martínez-Hidalgo and Hirsch, 2017). For instance, *Bacillus* has been isolated from root nodules and shown to solubilize phosphate and synthesize hydrolytic enzymes, polyamines, and lipopeptides (Maymon *et al*., 2015). On the other hand, *Ectomycorrhiza and Arbuscular Mycorrhiza* increase plant below ground surface area to maximize nutrient uptake (Wardle *et al.,* 2004). While some *Pseudomonas, Ochrobactrum, and Klebsiella* species have the ability to fix nitrogen through associative symbiotic and/or endophytic relationship in combination with certain non-legume plants (Franche *et al*., 2009). The rate of nitrogen fixation is dependent on many external factors that include competition amongst the soil microbial species, soil moisture, temperature, plant sanction for specific bacteria and pH (Nandwa 2001; Stopnisek *et al*., 2014). The combined activity of the microbiota and the environment might be responsible for the

increased soil enzyme activity that is characteristic in most soils. Furthermore, soil quality can be determined by measuring several enzymatic activities as a surrogate of the microbial diversity in them. Soil enzymes originate from a variety of organisms and are of importance for the decomposition of many labile organic substrates, activating biogeochemical cycling. Their activity reflects the functional diversity and activity of the microorganisms involved in decomposition processes (Sinsabaugh *et al*., 2008) at the time that they guarantee the correct soil functioning. Soil microorganisms produce extracellular enzymes that hydrolyze and transform polymeric compounds into readily available nutrients that can be assimilated by plants and microorganisms (Lucas *et al.,* 2008). These extracellular enzymes are responsible for the mineralization and cycling of terrestrial nitrogen (N), phosphate (P) and carbon (C). Furthermore, these enzymes play a role in preventing oxidative degradation caused by reactive oxygen species (Nanda *et al*., 2010). Catalase is one of the enzymes known to be indicative of soil oxidative stress tolerance and is known to reduce hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) (Angaji *et al.,* 2012). Enzymes involved in C cycling include β-D- Cellobiohydrolase, dehydrogenase, and β-D-Glucosidase. They achieve this by releasing saccharides from glycosides and catalysis of the degradation of cellulose and cellotetraose into cellobiose that can be further transformed into glucose (Henriksson *et al*., 1998). Apart from catalase, there are other important oxidoreductase enzymes like laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP), which protect against oxidative stress (Lundell *et al*., 2010). Their primary functions include lignin transformation and H_2O_2 reduction into H_2O. On the other hand, enzymes like asparaginase and β-D- Glucosaminidase convert asparagine into aspartic acid and ammonia (NH_3), and hydrolyze chito-oligosaccharides (Hill *et al*., 1967; Mega *et al.,* 1972). This has an overall effect on N mineralization and increased N assimilation in plants. While phosphatases are responsible for P cycling and mineralization through the hydrolysis of phosphoric acid monoester to produce a phosphate anion (Turner *et al.,* 2002). Plants growing in grassland and savannah ecosystem soils are subjected to these collective effects of soil geochemistry and microflora composition might employ different survival strategies and show varying growth patterns.

Therefore the aim of this study was to determine soil nutrition and chemistry, soil enzyme activities and microbiota in soils collected different locations with varying altitudes in KwaZulu-Natal (KZN) province, South Africa. The specific objective was to determine the range of potential

activity of ten extracellular enzymes of South African soils and how these vary according to the soil properties and microbial diversity.

3.2. Materials and methods

3.2.1. Total soil nutrient analysis and spore count

Soil samples collected from four different locations with varying altitudes in KwaZulu-Natal (KZN) province, South Africa were sent to the KwaZulu-Natal Department of Agriculture and Rural Development's Analytical Services Unit at Cedara College of Agriculture, South Africa, for total soil nutrient and cation concentrations, cation exchange acidity and pH analysis. The four soil collection sites were: Ashburton, midland KZN (elevation 670m; 29.3855º S; 30.2642º E), Hluhluwe, northern KZN (elevation 100m; 28.058ºS; 32.124º E), Bergville, mountainous KZN (elevation 1640m; 28.3414º S; 29.417º E) and Izingolweni, southern KZN (elevation 450m; 30.4332° S; 30.610º E) (supplementary figure 1). Arbuscular mycorrhizal spore count analysis was conducted according to Smith and Dickson (1991) at Rhodes University, South Africa.

3.2.1.1.Soil site description

Hluhluwe is an area situated between iSimangaliso Wetland Park and Hluhluwe-iMfolozi Park on the banks of Hluhluwe River. The climate in the area is warm and temperate, with annual average temperature and precipitation of 21.3ºC and 894 mm, respectively. This site is classified as having the Zululand Lowveld (SVI23) vegetation type with *Hyparrhenia*, *Acacia* and *Dichrostachys cinerea* as indicator species (Rutherford *et al*., 2006).

Izingolweni is an area situated inland of Port Shepstone. This area is subjected to an annual average precipitate and temperature of 450 mm and 17.0 °C respectively. This site is classified as having the Eastern Valley Bushveld (SVs6) vegetation type with *Euphorbia* and *Aloe* as indicator species (Rutherford *et al*., 2006).

Bergville is situated in the hills of Drakensberg Mountain. This site is subjected to annual average precipitation and temperature of 684 mm and 13°C respectively. This site is classified as having the KwaZulu-Natal Highland Thornveld (Gs6) vegetation type with *Hyparrhenia hirta* and *Acacia sieberiana var woodii* as indicator species (Rutherford *et al*., 2006).

Ashburton is situated next to the South African National road (N3), approximately 12 km east of Pietermaritzburg. This site is subjected to an average annual precipitation of 691 mm and mean annual temperatures of 18.8 °C. Moreover, the land is mostly non-rocky which makes it suitable for annual cropping and dominated by bushland vegetation pattern. This site is classified as having the Midlands Mistbelt Grassland (Gs9) vegetation type with *Themeda triandra* as an indicator species (Rutherford *et al*., 2006).

Soil fungal spores

3.2.1.2.Extraction, purification and enumeration

Fungal spores were isolated using a modified procedure described by Gerdeman and Nicolson (1963). For each soil sample, 10g/100ml dH_2O was decanted through tri-layered sieves of 500 mm, 100 µm and 50 µm mesh sizes. The residues (spores) were later suspended in 100 mL of water in a 250 mL beaker for further purification. The residual spore fraction was re-suspended in 100 ml H_2O and purified using a modified procedure described by Daniel and Skipper (1982). The spore suspensions (10 ml/50 ml Falcon tube) were injected with 25 ml of a 70% sucrose solution (w/v) and centrifuged at 130 xg for 5 minutes. Spores collected from the interface were washed for 2 minutes on 32 µm sieves with H_2O and transferred to petri dishes for enumeration. Spores were enumerated at 40x magnification under a stereomicroscope and enumeration was based on parameters described by the international culture collection of vesicular and arbuscular mycorrhizal fungi (INVAM) (https://invam.wvu.edu/methods/spores/enumeration-of-spores).

3.2.1.3.Fungal DNA extraction

All the different extracted spores were grown on PDA dishes. Approximately, 10 mm of fungal mycelia were transferred from a fresh dish to a 1.5 ml sterile Eppendorf tube and its total genomic DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrepTM kit (Zymo Research-USA) in accordance to the manufacturer's instructions.

3.2.1.4.DNA amplification, sequencing and identification

The extracted fungal DNA was amplified using the ITS4 (5′-T TCCTCCGCTTATTGATATGC -3′) and ITS5 (5′- GGAAGTAAAAGTCGTAACAAGG -3′) primers in the PCR. The PCR mixtures consisted of 10 µl DNA, 5 µl of 10 X reaction buffer, 2 µl 25mM $MgCl_2$, 2.5 µl of each

primer, 0.25 µl of Taq DNA polymerase, 1 µl of 10mM dNTP and volume made up to 50 µl with MilliQ H_2O. A T100 Thermal Cycler (Biorad, USA) was used for amplification with the initial denaturation at 95 °C for 2 min, 25 cycles of denaturation at 95 °C for 30 sec, annealing at 53 °C for 45 sec and elongation at 72 °C for 8 min with a final elongation at 72 °C for 8 min. The PCR products were resolved on 1.0% (w/v) agarose gels (Seakem) and visualized after staining with ethidium bromide (0.5 µg/ml) using the Chemigenius Bio-imaging System (Syngiene, England). Positive amplicons (~600 bp) were excised and sequenced at Inqaba Biotech Pty. Ltd., South Africa and the sequences were compared against the GenBank database. Homologues were identified using the BLASTN program at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.2. Soil-borne bacteria

3.2.2.1.Isolation and enumeration

A three-fold serial dilution was conducted on 10 g of soil samples and 100 µl of each dilution was used to inoculate nutrient agar (NA) plates. The inoculated plates were incubated at 37 °C for 18 hours and distinct bacterial colonies were enumerated using the colony-forming unit method (CFU) (Goldman and Green, 2008).

3.2.2.2.Bacterial DNA extraction

Bacterial DNA was extracted using a modified boiling procedure described by Akinbowale *et al.* (2007). Bacterial colonies (≤ 5) picked off NA plates were suspended in 70 µl MilliQ H_2O, boiled in a water bath at 100 °C for 10 min and placed on ice for 5 min. The suspension was centrifuged at 13817 xg in a micro-centrifuge (Spectrafuge 16M, Labnet) for 5 minutes and the supernatant (~50 µl) was transferred to sterile Eppendorf tubes.

3.2.2.3.DNA amplification, sequencing and identification

The extracted bacterial DNA was amplified using the 63F (5'- CAGGCCTAACACATGCAAGTC -3′) and 1387R (5′- GGGCGGTGTGTACAAGGC -3′) primers in the PCR. The PCR mixtures consisted of 10 µl DNA, 5 µl of 10 X reaction buffer, 2 µl 25mM MgCl2, 2.5 µl of each primer, 0.25 µl of Taq DNA polymerase, 1 µl of 10mM dNTP and volume made up to 50 µl with MilliQ H2O. A T100 Thermal Cycler (Biorad, USA) was used for amplification with the initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 92 °C for 30 sec, annealing at 56 °C

for 45 sec and elongation at 75 °C for 45 sec. with a final elongation at 75 °C for 10 min. The PCR products were resolved on 1.0% (w/v) agarose gels (Seakem) and visualized after staining with ethidium bromide (0.5 µg/ml) using the Chemigenius Bio-imaging System (Syngiene, England). Positive amplicons (~1324 bp) were excised and sequenced at Inqaba Biotech Pty. Ltd., South Africa and the sequences were compared against the GenBank database. Homologues were identified using the BLASTN program at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.3. Measurement of microbial diversity

The biodiversity measurements were based on Total CFU/ml, Total spore/g, Percentage relative abundance, relative density, isolation frequency, Shannon-Wiener index of diversity (H), Simpson index of dominance (D), Evenness (E), $R_{margalef}$ and E_{pielou}. W

3.2.4. Soil enzyme activity

Soil β-Glucosidase and β-Cellobiohydrolase activity were determined according to Jackson *et al.* (2013). Briefly, fluorogenic 4-Methylumbelliferyl (MUB)-linked substrates were used for the colorimetric quantification. 4-MUB-β-glucopyranoside and 4-MU-β-D-cellobioside fluorogenic substrates were used to assay β-Glucosidase and β-Cellobiohydrolase, respectively. Five gram soil samples were homogenized at low speed in 50 ml milliQ H_2O for 2 hours at 4°C. The supernatants were transferred into black 96-well microplates prior to adding the respective substrates. The sample run consisted of 200 µl soil aliquot plus 50 µl substrate and incubated alongside reference standards (200 µl buffer + 50 µl standard), quench standards (200 µl soil aliquot + 50 µl standard), sample controls (200 µl soil aliquot + 50µl buffer), negative controls (200 µl buffer + 50µl substrate) and blanks (250 µl buffer). The reaction was stopped with 0.5 M NaOH after a 2 hour incubation period at 30°C. Thereafter, fluorescent absorbance was measured at 450 nm on a Glomax Multi Plus microplate reader (BioTek, USA) and the results expressed as nmol $h^{-1}g^{-1}$ from one unit enzyme activity of 0.001 nmol h^{-1} g^{-1} of the reaction mixture.

Soil Catalase (E.C. 1.11.1.6) activity was determined according to the modified method of Orta-Zavalza *et al.* (2014). The method is based on H_2O_2 decomposition were 10 ml soil suspension is mixed with 790 µl of potassium phosphate buffer (50 mM, pH 7.0) and catalysed with 100 µl of a 30 mM (v/v) H_2O_2 solution. The reaction mixture was incubated at 30°C for 3 minutes before

measuring absorbance at 240 nm on an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA) (ε=34.9 cm^{-1} M^{-1}) of 0.1 nmol h^{-1} g^{-1} of the reaction mixture.

Soil dehydrogenase activity was determined according to Ezirim *et al.* (2017). Briefly, a 5g soil sample was homogenized in 10 ml of 0.2% (w/v) triphenyl tetrazolium chloride (TTC) and 10 ml of 0.1 M Tris buffer (pH=7.6) prior to incubation at 37°C for 6 hours. The reduced reaction product, triphenyl formazan (RTF) was extracted using methanol (10 ml) through centrifugation for 5 min at 4000 xg. The absorbance was measured at 485 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA) (ε=15,433 cm^{-1} M^{-1}) of 0.1 nmol h^{-1} g^{-1} of the reaction mixture.

Laccase (E.C. 1.10.3.2) activity was measured according to Patel *et al*. (2009) through the oxidation of 2, 2- Azino-bis-3-ethyl-benzthiozoline-6-sulphonic acid (ABTS). Briefly, the soil suspension (100 µl) was mixed with 800 µl of 20 mM Na- Acetate buffer (pH 4.5) and 100 µl of 50 mM ABTS. The reaction was terminated with 40 µl of a 20% (v/v) trichloroacetic acid (TCA) solution after a 15 minute incubation period at 30°C. The absorbance was measured at 420 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA) (ε=34.9 cm−1 M−1) of 0.1 nmol h−1 g−1 of the reaction mixture.

Lignin peroxidase (E.C. 1.11.1.14) activity was determined using a modified method described by Agrawal and Shahi (2017). Briefly, the soil suspension (83.5 µl) was mixed with 749.5 µl of 125 mM Na-tartrate buffer (pH 3.0) and 0.16 mM azure B solution. The reaction was initiated by adding 83.5 µl of 2 mM H_2O_2 (pH 3.0) before incubating for 15 minutes at 30°C. The absorbance was measured at 651 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA) (ε=85,000 cm−1 M−1) of 0.1 nmol h−1 g−1 of the reaction mixture.

Manganese peroxidase (E.C. 1.11.1.13) activity was determined according to Patel *et al.* (2009). The method is based on the oxidation of 2, 6- dimethoxy phenol (DMP) to produce a yellow-brown coloured product. Briefly, the reaction mixture contained 83.5 µl of soil suspension, 749.5 µl of 50 mM Na-tartrate buffer (pH 4.0) and 2 mM of 2, 6-DMP. The enzymatic reaction was initiated by adding 83.5 µl of 0.4 mM H_2O_2 (pH 4.0) before incubating for 15 minutes at 30°C. The absorbance was measured at 469 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA) (ε=27,500 cm^{-1} M^{-1}) of 0.1 nmol h^{-1} g^{-1} of the reaction mixture.

L-Asparaginase (E.C. 3.5.1.1) activity determined according to Shirfrin *et al*. (1974) by measuring the NH_3 released from *L*-Asparaginase hydrolysis. The soil suspension (50 µl) was mixed with 800 µl of 10 mM L-Asparaginase and 100 µl of 50 mM Tris buffer (pH 8.6). The mixture was incubated for 15 minutes at 37°C and the reaction was stopped by adding 50 µl of 1.5M TCA. Thereafter, the reaction mixture was centrifuged at 10000 xg for 2 minutes before transferring 20 µl of the supernatant into 4.3 ml of milliQ H_2O. The enzymatic reaction was initiated by adding 50 µl of Nessler's reagent and incubating for 15 min at 30°C. The absorbance was measured at 436 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA). One unit of enzyme activity was expressed as an increase in absorbance of 0.1 unit min^{-1} L^{-1} of the reaction mix using NH_3 as standard.

Soil β-Glucosaminidase and β-phosphatase activity were determined according to Jackson *et al.* (2013) by using 4-MUB-N-acetyl-β-D-Glucosaminide and 4-MUB-Phosphate as the fluorogenic substrates, respectively.

3.2.5. Data analyses

IBM Statistics 24 was used to test for differences in soil spore counts macro, intermediate and micro nutrients, as well as pH, exchange activity, and total cation in the four study sites soil of KwaZulu-Natal (KZN), using one- way analysis of variance (ANOVA). Where the ANOVA showed significant differences between treatments, a Bonferroni's post hoc test was used to separate the means (≤ 0.05).

3.3. Results

3.3.1. Soil geochemistry

3.3.1.1.Soil colour and spore count

Ashburton soil (Figure 3.1A) had a black appearance while soils from Hluhluwe (Figure 3.1B), Bergville (Figure 3.1C) and Izingolweni (Figure 3.1D) had black/dark brown, brown and red-brown colour, respectively. Microscopic enumeration of Arbuscular mycorrhizal spores showed no significant difference ($p>0.05$) between Izingolweni (11.6 spores/100g), Bergville (15.8 spores/100g of soil) and Ashburton (13.4 spores/100g), while Hluhluwe (5.2 spores/100g) was significantly different ($p<0.05$) from the other sites (Figure 3.2).

3.3.1.2.Primary soil nutrients

Soil primary nutrients include phosphorus (P), nitrogen (N) and potassium (K). Hluhluwe had the highest N and K concentration but had the least P concentration, while Bergville had the highest P concentration (Figure 3.3). Ashburton and Izingolweni had the least N and K concentration, respectively (Figure 3.3).

3.3.1.3.Intermediate soil nutrients

Soil intermediate nutrients include calcium (Ca), magnesium (Mg) and sulfur (S). Hluhluwe had the highest concentration of both calcium (58.06 µmol) and magnesium (42.27 µmol), followed by Izingolweni (Ca = 31.54 µmol; Mg = 19.48 µmol), Ashburton (Ca = 19.32 µmol; Mg = 11.03 µmol) and Bergville (Ca = 7.77 µmol; Mg = 3.41 µmol) (Figure 3.4).

3.3.1.4.Soil micronutrients

Soil micronutrients include zinc (Zn), manganese (Mn) and copper (Cu). Ashburton had the highest zinc (0.024 µmol) and manganese (0.24 µmol) concentration, while the other three sites showed similar Zn (0.009 µmol) concentration with Hluhluwe having the most Cu (0.29 µmol) and the least Mn (0.04 µmol), and Bergville having the least Cu (0.01 µmol) concentration (Figure 3.5).

3.3.1.5.Soil relative acidity and C: N ratios

Ashburton (pH 5.07) and Hluhluwe (pH 4.96) had the highest active acidity/pH, followed by Izingolweni (pH 4.63) and Bergville (pH 4.06) (Figure 3.6). On the other hand, Bergville had the highest exchange acidity (1.496 cmol/L) while the other three sites had similar exchange acidity ~0.006 cmol/L (Figure 3.6). Hluhluwe had the highest concentration of total cations (20.014 cmol/L) followed by Izingolweni (11.370 cmol/L), Ashburton (6.736 cmol/L) and Bergville (4.180 cmol/L) (Figure 3.6). Hluhluwe and Izingolweni had similar C: N ratios (15:1) while Bergville had 14:1 and Ashburton had 11:1 (Table 3.1).

3.3.2. Functional microbial diversity

Microbial diversity differed in quantity and quality from soil to soil. Hluhluwe soil had the second highest number of viable bacterial cells ($2.04x10^5$ CFU/ml) and had six different species that had relatively moderate diversity (H=1.180; λ=0.622) and evenness (E_{pielou}=0.659) (Table 3.2). Izingolweni soil had the least number of viable bacterial cells ($1.57x10^4$ CFU/ml) and had the same number of species as Hluhluwe (Table 3.2). However, Izingolweni soil was second to Hluhluwe in terms of diversity (H=0.959; λ=0.563) and evenness (E_{pielou}=0.535) (Table 3.2). Bergville soil had the second least number of viable bacterial cells ($5.88x10^4$ CFU/ml) and had the least number of species (Richness=5) (Table 3.2). There was also minimal diversity (H=0.224; λ=0.096) and evenness (E_{pielou}=0.139) amongst the species (Table 3.2). While Ashburton soil had the most number of viable bacterial cells ($3.31x10^5$ CFU/ml) and had the most number of species (Richness= 7) (Table 3.2), there was minimal diversity (H=0.462; λ=0.222) and evenness (E_{pielou}=0.238) amongst the species (Table 3.2).

The fungal (AMF) microflora, Hluhluwe soil had the most spores ($2.65x10^3$ spores/g) and fungal species (Richness=7) (Table 3.2). The Hluhluwe soil fungal community showed a high diversity (H=1.594; λ=0.769) and evenness (E_{pielou}=0.819) (Table 3.2). Bergville soil was second to Hluhluwe with $2.24x10^3$ spores/g and three species that had moderate diversity (H=0.884; λ=0.521) and evenness (E_{pielou}=0.804) (Table 3.2). Izingolweni soil was third with $6.89x10^2$ spores/g and two species that had minimal diversity (H=0.385; λ=0.226) and moderate evenness (E_{pielou}=0.556) (Table 3.2). Ashburton soil had the least fungal spores ($6.76x10^2$ spores/g) and two species that had minimal diversity (H=0.471; λ=0.295) and moderate evenness (E_{pielou}=0.679) (Table 3.2).

3.3.3. Endophytic microorganisms

3.3.3.1.Bacteria

A total of 21 endophytic microbial strains (bacteria and fungi) were isolated from the soil samples and identified using BLASTN. The bacterial strains belonged to the family *Bacilaceae* (Figure 3.7) while fungal strains belonged to the family Nectriaceae, Mucoraceae, and Hypocreaceae (Figure 3.8). The percentage relative abundance was determined to better understand the microbial composition of each soil site. Hluhluwe soil had a bacterial composition of 49.7% *Bacillus thuringiensis*, 35.3% *Lysinibacillus xylanilyticus*, 0.4% *Bacillus cereus*, and 4.9% of each of *Bacillus mycoides, Bacillus aryabhattai* and *Bacillus huizhouensis* strains. Izingolweni soil had

47.8% *B. thuringiensis*, 45.4% *L. xylanilyticus*, 4.8% *B. cereus*, 0.8% *B. methylotrophicus* and 0.6% of each of *L. boronitolerans* and *B. huizhouensis* strains. Bergville soil had 95% *L. xylanilyticus*, 4.4% *B. thuringiensis*, 0.3% *B. cereus* and 0.2% of each of *B. simplex* and *B. arbutinivorans* strains. Ashburton soil had 87.4% *L. xylanilyticus*, 10% *B. thuringiensis*, 1.5% *B. mycoides* and 0.3% of each of *B. aryabhattai*, *B. methylotrophicus* and *B. arbutinivorans* strains (Figure 3.7).

3.3.3.2.Fungi

Hluhluwe soil had a fungal composition of 32.3% *Mucor velutinosus*, 26.9% *Gibberella intermedia*, 18.3% *Fusarium sinensis*, 11.9% *Fusarium oxysporum*, 8.1% *Trichoderma spirale*, 1.2% *Trichoderma koningiopsis*, and 1.2% *Trichoderma gamsii* (Figure 3.8). Izingolweni soil had 87.1% *Amylomyces rouxii* and 12.9% *Rhizopus stolonifera*. Bergville soil had 63.5% *Fusarium conctricum*, 24.9% *Fusarium oxysporum* and 11.6% *Amylomyces rouxii* (Figure 3.8). Ashburton soil had 82% *Rhizopus stolonifera* and 18% *Fusarium conctricum* (Figure 3.8).

3.3.4. Soil enzyme activities

3.3.4.1.Carbon cycling enzymes

Nutrient cycling enzyme activities were assessed to determine factors influencing soil nutrient availability. Hluhluwe soil had the highest glucosidase, cellobiohydrolase and catalase activity. Izingolweni, Bergville and Ashburton soils had the second, third and fourth highest carbon cycling enzyme activities, respectively (Table 3.4).

3.3.4.2.Organic matter and lignin degrading enzymes

Izingolweni soil had the highest dehydrogenase, lignin peroxidase and manganese peroxidase activity, but had the lowest laccase activity (Table 3.5). Hluhluwe soil had the second highest laccase and Mn-peroxidase activity but had the second lowest dehydrogenase activity (Table 3.5). Bergville soil had the third highest laccase and Mn-peroxidase activity but had the lowest dehydrogenase activity. Lignin peroxidase activity was not detected in soils from both Hluhluwe and Bergville (Table 3.5). Ashburton soil had the second highest lignin peroxidase activity, third highest dehydrogenase and laccase activity, but had the lowest Mn-peroxidase activity (Table 3.5).

3.3.4.3.Nitrogen and phosphate cycling enzymes

Hluhluwe soil had the highest asparaginase and phosphatase activity but had the third highest glucosaminidase activity (Table 3.6). Izingolweni soil had the second highest glucosaminidase and asparaginase activity, and the third highest phosphatase activity (Table 3.6). Bergville soil had the highest glucosaminidase activity and the lowest phosphate and asparaginase activity (Table 3.5). Ashburton soil had the second, third and fourth highest phosphatase, asparaginase and glucosaminidase activity, respectively (Table 3.6).

3.4.Discussion

This study was aimed at assessing the properties of KZN soils to infer their potential effects on the growth and development of grassland and savannah ecosystem plants and to evaluate their potentiality for sustainable agriculture. There are many different factors that determine soil potential and fertility. Soil colour is one of those factors but it only provides insight on the possible composition and oxidative state of the soil (Osunade, 1992). Black/dark-brown colour of Ashburton and Hluhluwe soil could be indicative of high humus content and/or the presence of manganese (Mn) oxide (Schulze et al., 1993; Rabenhorst and Parikh, 2000). The different phases of organic matter degradation usually impart brown to black colours to soil (Tate *et al.*, 1990), while Mn-oxides have characteristic dark colours (Lindbo *et al*., 2010). On another note, red-brown colour of Ezingolweni soil could be indicative of good drainage and the presence of iron oxides (Schwertmann, 1993). Lighter brown coloured soils like Bergville soil may be indicative of the presence of varying contents of organic matter (Konen *et al.,* 2003). However, it is difficult to draw definite conclusions from soil colour observations since it is a qualitative analysis. The use of a more quantitative method like soil nutrient testing increases confidence in conclusions drawn in most studies (Smethurst, 2000).

Soil nutrient analysis of macronutrients revealed that Hluhluwe, Ashburton and Izingolweni had the lowest P, N and K concentrations, respectively. This suggests that plants growing in Hluhluwe soils would have diminished root growth and development due to low nutrient availability, together with the reduced soil moisture due to the scarce annual precipitation. In addition, microbial identification did not reveal P solubilizing microorganisms thus reducing the amounts of this nutrient for plant growth (Williamson *et al*., 2001). However, the low N concentration in

Ashburton soil might have minimal effects on plant growth since some plants have the potential to be self-sufficient when it comes to N acquisition (Drew *et al.*, 2012). N acquisition in these soils might happen thanks to endophytic bacteria as they are well represented in this soil (Figure 3.7). On the contrary to plants growing in Ashburton, Izingolweni grown plants are expected to have reduced growth due to low K concentration (Cakmak, 2005). Fast-growing plants including legumes such as *P. sativum* L. require high levels of K, which is essential for plant growth and development (Uchida, 2000). Potassium is required for the activation of enzymes, photosynthesis, protein synthesis and photosynthate transportation (Wang *et al*., 2013). The latter being important to establish and maintain symbiosis with plant growth-promoting microorganisms. This means Ashburton and Izingolweni soil growing plants might have reduced growth rates and biomass accumulation as this soils have reduced concentrations of both K and N. The presence of plants in these soils indicates that the reduction of those nutrients is compensated by the presence of active soil microbial consortia aiding the plants via associative N fixation (Figure 3.7) (Latha *et al*., 2011).

Analysis of intermediate nutrients revealed that Bergville had the lowest calcium (Ca^{2+}) and magnesium (Mg^{2+}) concentrations. Calcium plays a role in the regulation of plant cell wall rigidity and construction (Uchida, 2000). This means Bergville soil-grown plants might have stunted growth as a result of poor germination and weak stems (Bonilla *et al.*, 2004). Magnesium is present in the center of the chlorophyll porphyrin ring and is also involved in ATP production (Nelson and Ben-Shem, 2004). Plants deficient in Mg^{2+} have decreased photosynthetic rates and most likely in anabolic reactions that rely on energy from ATP hydrolysis (Candan and Tarhan, 2003). Micronutrient deficiencies have also been reported to have adverse effects on plant growth and nutrition (Uchida, 2000). For instance, zinc (Zn) deficiency mostly affects growth, hormone production and flowering (Hafeez *et al.,* 2013). While copper (Cu) and manganese (Mn) deficiencies affect protein synthesis, chlorophyll production, respiration, and leads to delayed maturity (Uchida, 2000). However, it is important to determine a plant's nutrient sufficiency range before deducing that a particular nutrient is deficient (Baldock and Schulte, 1996).

The identification of soils well suited for a particular plant's growth requires the determination of the plant's optimum growth conditions, the soil's nutritional status, and the interactions that plants

can establish with soil microorganisms. Some legumes and grasses are known to thrive in well-drained, slightly acidic soils with a pH range of 5.5 to 6.8 (McCauley *et al.*, 2009; www.agric.wa.gov.au/field-peas) and the same can be said for microorganisms (Carney and Matson, 2005). However, all the soils analysed in the present study were below this pH range. Bergville soils had the lowest pH and total cations, whilst having the highest exchange acidity. Under acidic conditions such as those in Bergville soils, base cations (like Mg^{2+}, Ca^{2+}, K^{+} and Na^{+}) are removed from soil clay particles (Zhang *et al*., 2007). These base cations are replaced by acidic cations such as Al^{3+}, H^{+} and Mn^{2+} (Larssen and Carmichael, 2000; Zhang *et al*., 2007). This promotes P-fixation, leaching of essential base cations and leads to an overall decrease in total exchangeable cations (Qian and Cai, 2007). The decrease in base saturation leads to elevated exchange acidity and decreased cation exchange capacity (Zhang *et al*., 2007). Furthermore, low pH soil decreases plant uptake of K^{+}, Ca^{2+} and Mg^{2+} since Mn^{2+} and H^{+} outcompetes these ions for apoplast loading (Ferguson *et al*., 2013). In addition, low pH hinders the development of profuse microbial communities (Rousk *et al*., 2010) which results in the observed low values of richness and diversity in our soils.

Soil microflora is known to have a significant effect on soil fertility and plant growth. Numerous microorganisms have been reported to have plant growth-promoting capabilities and are essential in nutrient cycling. Members of the bacterial family *Bacillaceae,* including ones in Table 3.3 and Figure 3.7, have been reported to have such capabilities. For instance, *B. thuringiensis, B. cereus, B. aryabhattai, B. simplex* and *B. methylotrophicus* promote plant growth by improving nutrient availability by solubilizing P and K, regulating phytohormones, enhancing N fixation, preventing pathogens and producing abscisic acid (ABA), indole acetic acid (IAA), cytokinins and gibberellic acid (Vendan *et al*., 2010; Pérez-García *et al*., 2011; Kavamura *et al*., 2013; Pérez-Fernández and Valentine, 2017), while other members like *Lysinibacillus xylanilyticus* degrade complex molecules such as hemicellulose into simpler molecules (Chantarasiri *et al.*, 2017). Furthermore, most *Bacillaceae* members are catalase, amylase and nitrate reduction positive (Logan and De Vos, 2009). Given their ubiquity in soil, their catalase activity assists in oxidative stress tolerance through the reduction of H_2O_2 into H_2O and O_2. Another bacterial enzyme that helps improve soil nutrient availability is asparaginase (Joy, 1988). It converts asparagine into aspartic acid and ammonia (NH_3), which is used by plants as an N source.

Although we cannot directly relate the presence of soil bacteria with particular enzyme activities, we can nevertheless partly relate those activities with the soil nutritional status to depict the better scenarios for plant growth. In our study, the soil with the greatest asparaginase activity is Hluhluwe that is at the same time the one with the highest level of total N. Considering the combined dehydrogenase and catalase activities, it is possible to estimate the biological index of soil fertility (Stefanic, 1984). According to it (Table S1) Izingolweni soil is the one with the greatest fertility (59.75) that coincides with their balanced levels of all nutrients, only showing relatively reduced concentrations of K. On the other hand, Bergville soils have the lowest fertility which can be attributed to the low levels of meso and micro nutrients. Soils from Hlulhuwe and Ashburton had intermediate values of fertility and similar between them. These two soils do not follow a homogeneous pattern of nutrient distribution, with the former showing P, Zn and Cu deficiencies; the later has balanced levels of macronutrients and a clear imbalance for the remaining elements.

The different soil sites in this study had the presence of fungal species that were mostly saprophytes (Figure 3.8). These species employ different strategies to improve soil fertility but some are known pathogens of certain plant species (Michielse and Rep, 2009). They serve a common role in soil organic matter and lignin degradation through extracellular enzymes (Novotný *et al.*, 2004; Jastrow *et al.*, 2007). Laccases and Mn-peroxidases are the enzymes commonly produced by fungi, while lignin peroxidase is also produced by bacteria (Theuerl and Buscot, 2010). Apart from degrading herbaceous and/or woody material, members of the fungal families present in the different soils have other beneficial abilities. For instance, *Gibberella intermedia* have the ability to promote seedling growth through the production of gibberellin (Tsavkelova *et al.*, 2008). While *Amylomyces rouxii* and *Rhizopus stolonifer* produce different kinds of glucosidases and esterases that improve carbon turnover (Ogundero 1988; Anal, 2019). Members of *Trichoderma* are able to produce ammonia, and other fungi solubilize phosphates and micronutrients and are currently commercialized as crop enhancers to replace chemical fertilizers (Ousley *et al*., 1994; Altomare *et al*., 1999). The phosphate-solubilizing ability is most likely a result of phosphatase activity. Their chitinase activity, which is also present in some bacteria, assists in N and C cycling in terrestrial ecosystems (Lorito *et al.*, 1993).

Based on the microbial and enzyme activity data, Bergville soil is estimated to provide plants with the least nutrition. Not only did Bergville soil have no lignin peroxidase and asparaginase activity,

but it also had low dehydrogenase activity. Dehydrogenase is an intracellular enzyme that is present in all microorganisms and can be used as a basis to determine overall microbial activity (Wolińska and Stępniewska, 2012).

3.5.Conclusions

Grassland and savanna ecosystem soil may support the establishment and growth of these ecosystem plants and sustainable agriculture practices. Given that these different soils have different diversity of microbes and various soil enzymes that can solubilize bound nutrients. However, external inputs such as liming of soils in accordance with plant sufficiency range might be required to maximize yield potential.

3.6.Acknowledgements

The authors would like to thank the National Research Foundation, South Africa, for funding this work (NRF grant no. UID 113576). We acknowledge the Environmental Microbiology/Biotechnology research lab at the School of Life Sciences, University of KwaZulu-Natal, Durban, for their research facilities.

3.7.References

1. Agrawal, N. and Shahi, S.K., 2017. Degradation of polycyclic aromatic hydrocarbon (pyrene) using novel fungal strain *Coriolopsis byrsina* strain APC5. *International Biodeterioration & Biodegradation*, 122, pp.69-81.
2. Altomare, C., Norvell, W.A., Björkman, T. and Harman, G.E., 1999. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus **Trichoderma harzianum** Rifai 1295-22. *Applied Environmental Microbiology*, 65(7), pp.2926-2933.
3. Anal, A.K., 2019. Quality ingredients and safety concerns for traditional fermented foods and beverages from Asia: A review. *Fermentation*, 5(1), pp.8.
4. Angaji, S.A., Mousavi, S.F. and Babapour, E., 2012. Antioxidants: A few key points. *Annals of Biological Research*, 3(8), pp.3968-77.

5. Aprile, F. and Lorandi, R., 2012. Evaluation of cation exchange capacity (CEC) in tropical soils using four different analytical methods. *Journal of Agricultural Science*, 4(6), pp.278.
6. Baldock, J.O. and Schulte, E.E., 1996. Plant analysis with standardized scores combines DRIS and sufficiency range approaches for corn. *Agronomy Journal*, 88(3), pp.448-456.
7. Bonilla, I., El-Hamdaoui, A. and Bolaños, L., 2004. Boron and calcium increase *Pisum sativum* seed germination and seedling development under salt stress. *Plant and Soil*, 267(1-2), pp.97-107.
8. Cakmak, I., 2005. The role of potassium in alleviating detrimental effects of abiotic stresses in plants. *Journal of Plant Nutrition and Soil Science*, 168(4), pp.521-530.
9. Candan, N. and Tarhan, L., 2003. Relationship among chlorophyll-carotenoid content, antioxidant enzyme activities and lipid peroxidation levels by Mg2+ deficiency in the *Mentha pulegium* leaves. *Plant Physiology and Biochemistry*, 41(1), pp.35-40.
10. Carney, K.M., Matson, P.A. 2005. Plant communities, soil microorganisms and soil carbon cycing:Does altering the world belowground matter to ecosystem functioning? *Ecosystems* 8:928-940.
11. Chantarasiri, A., Boontanom, P. and Nuiplot, N.O., 2017. Isolation and characterization of Lysinibacillus sphaericus BR2308 from coastal wetland in Thailand for the biodegradation of lignin. *Aquaculture, Aquarium, Conservation & Legislation*, 10(2), pp.200-209.
12. Daniels, B.A. and Skipper, H.D., 1982. Methods for the recovery and quantitative estimation of propagules from soil. In: Schneck NC. (eds.) Methods and principles of mycorrhizal research. *American Phytopathological Society*, pp.244.
13. Drew, E., Herridge, D., Ballard, R., O'Hara, G., Deaker, R., Denton, M., Yates, R., Gemell, G., Hartley, E., Phillips, L. and Seymour, N., 2012. Inoculating legumes: a practical guide. *Grains Research and Development Corporation*.
14. Ezirim, C.Y., Chikezie, P.C., Iheanacho, K.M. and Nwachukwu, N.R., 2017. Comparative Activities of Soil Enzymes from Polluted Sites in Egbema, Imo State, Nigeria.
15. Ferguson, B., Lin, M.H. and Gresshoff, P.M., 2013. Regulation of legume nodulation by acidic growth conditions. *Plant signaling & behavior*, 8(3), pp.e23426.
16. Franche, C., Lindström, K. and Elmerich, C., 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant and Soil*, 321(1-2), pp.35-59.

17. Gerdemann, J.W. and Nicolson, T.H., 1963. Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. *Transactions of the British Mycological society*, 46(2), pp.235-244.
18. Goldman, A., 1995. Threats to sustainability in African agriculture: searching for appropriate paradigms. *Human Ecology*, 23(3), pp.291-334.
19. Goldman, E., Green, L.H. 2008. Practical Handbook of Microbiology, Second Edition. USA: CRC Press, *Taylor and Francis Group*. pp. 864. ISBN 978-0-8493-9365-5.
20. Hafeez, B., Khanif, Y.M. and Saleem, M., 2013. Role of zinc in plant nutrition-a review. *American Journal of Experimental Agriculture*, 3(2), pp.374.
21. Henao, J. and Baanante, C.A., 1999. Estimating rates of nutrient depletion in soils of agricultural lands of Africa. Muscle Shoals: *International Fertilizer Development Center*.
22. Henriksson, G., Sild, V., Szabó, I.J., Pettersson, G. and Johansson, G., 1998. Substrate specificity of cellobiose dehydrogenase from *Phanerochaete chrysosporium*. Biochimica et Biophysica Acta (BBA)-*Protein Structure and Molecular Enzymology*, 1383(1), pp.48-54.
23. Hill, J.M., Roberts, J., Loeb, E., Khan, A., MacLellan, A. and Hill, R.W., 1967. L-asparaginase therapy for leukemia and other malignant neoplasms: remission in human leukemia. *Jama*, 202(9), pp.882-888.
24. Jackson, C.R., Tyler, H.L. and Millar, J.J., 2013. Determination of microbial extracellular enzyme activity in waters, soils, and sediments using high throughput microplate assays. *Journal of Visualized Experiments*, (80), pp.e50399.
25. Jastrow, J.D., Amonette, J.E. and Bailey, V.L., 2007. Mechanisms controlling soil carbon turnover and their potential application for enhancing carbon sequestration. *Climatic Change*, 80(1-2), pp.5-23.
26. Johannes Rousk, J., Bååth, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, JG., Knight, R. and Noah Fierer 2010. Soil bacteria and fungal communities across a pH gradient in an arable soil. *The ISME Journal* 4:1340-1351.
27. Joy, K.W., 1988. Ammonia, glutamine, and asparagine: a carbon–nitrogen interface. *Canadian Journal of Botany*, 66(10), pp.2103-2109.
28. Kavamura, V.N., Santos, S.N., da Silva, J.L., Parma, M.M., Ávila, L.A., Visconti, A., Zucchi, T.D., Taketani, R.G., Andreote, F.D. and de Melo, I.S., 2013. Screening of

Brazilian cacti rhizobacteria for plant growth promotion under drought. *Microbiological Research*, 168(4), pp.183-191.

29. Konen, M.E., Burras, C.L. and Sandor, J.A., 2003. Organic carbon, texture, and quantitative color measurement relationships for cultivated soils in north central Iowa. *Soil Science Society of America Journal*, 67(6), pp.1823-1830.
30. Larssen, T. and Carmichael, G.R., 2000. Acid rain and acidification in China: the importance of base cation deposition. *Environmental Pollution*, 110(1), pp.89-102.
31. Latha P, Anand T, Prakasam V, Jonathan EI, Paramathma M, Samiyappan R. 2011. Combining *Pseudomonas*, *Bacillus* and *Trichoderma* strains with organic amendments and micronutrient to enhance suppression of collar and root rot disease in physic nut. *Applied Soil Ecology*, 49, pp. 215–223.
32. Lindbo, D.L., Stolt, M.H. and Vepraskas, M.J., 2010. Redoximorphic features. In *Interpretation of Micromorphological Features of Soils and Regoliths* (pp. 129-147). Elsevier.
33. Logan, N.A. and De Vos, P.A.U.L., 2009. Family *Bacillaceae*. Bergey's Manual of Systematic Bacteriology, 3, pp.20.
34. Lorito, M., Harman, G.E., Hayes, C.K., Broadway, R.M., Tronsmo, A., Woo, S.L. and Di Pietro, A., 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology*, 83(3), pp.302-307.
35. Lucas, N., Bienaime, C., Belloy, C., Queneudec, M., Silvestre, F. and Nava-Saucedo, J.E., 2008. Polymer biodegradation: Mechanisms and estimation techniques–A review. *Chemosphere*, 73(4), pp.429-442.
36. Lundell, T.K., Mäkelä, M.R. and Hildén, K., 2010. Lignin-modifying enzymes in filamentous basidiomycetes–ecological, functional and phylogenetic review. *Journal of Basic Microbiology*, 50(1), pp.5-20.
37. Mafongoya, P.L., Bationo, A., Kihara, J. and Waswa, B.S., 2006. Appropriate technologies to replenish soil fertility in southern Africa. *Nutrient Cycling in Agroecosystems*, 76(2-3), pp.137-151.
38. Martínez-Hidalgo, P. and Hirsch, A.M., 2017. The nodule microbiome: N2-fixing rhizobia do not live alone. *Phytobiomes*, 1(2), pp.70-82.

39. Maymon, M., Martínez-Hidalgo, P., Tran, S.S., Ice, T., Craemer, K., Anbarchian, T., Sung, T., Hwang, L.H., Chou, M., Fujishige, N.A. and Villella, W., 2015. Mining the phytomicrobiome to understand how bacterial coinoculations enhance plant growth. *Frontiers in Plant Science*, 6, pp.784.
40. McCauley, A., Jones, C. and Jacobsen, J., 2009. Soil pH and organic matter. *Nutrient Management Module*, 8(2), pp.1-12.
41. Mega, T., Ikenaka, T. and Matsushima, Y., 1972. Studies on N-Acetyl-β-D-glucosaminidase of Aspergillus oryzae: II. Substrate Specificity of the Enzyme. *The Journal of Biochemistry*, 71(1), pp.107-114.
42. Michielse, C.B. and Rep, M., 2009. Pathogen profile update: *Fusarium oxysporum*. *Molecular Plant Pathology*, 10(3), pp.311-324.
43. Nanda, A.K., Andrio, E., Marino, D., Pauly, N. and Dunand, C., 2010. Reactive oxygen species during plant-microorganism early interactions. *Journal of Integrative Plant Biology*, 52(2), pp.195-204.
44. Nandwa, S.M., 2001. Soil organic carbon (SOC) management for sustainable productivity of cropping and agro-forestry systems in Eastern and Southern Africa. In *Managing Organic matter in tropical soils*: Scope and limitations (pp. 143-158). *Springer*, Dordrecht.
45. Nelson, N. and Ben-Shem, A., 2004. The complex architecture of oxygenic photosynthesis. *Nature Reviews Molecular Cell Biology*, 5(12), pp.971.
46. Novotný, Č., Svobodová, K., Erbanová, P., Cajthaml, T., Kasinath, A., Lang, E. and Šašek, V., 2004. Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. *Soil Biology and Biochemistry*, 36(10), pp.1545-1551.
47. Ogundero, V.W., 1988. Pectin esterase production by *Rhizopus stolonifer* from post-harvest soft rots of potato tubers in Nigeria and its activity. *Food/Nahrung*, 32(1), pp.59-65.
48. Orta-Zavalza, E., Briones-Martin-del-Campo, M., Castano, I. and De Las Penas, A., 2014. Catalase activity assay in *Candida glabrata*. *Bio-protocol*, 4(6), pp.1072.
49. Osunade, M.A., 1992. The significance of colour in indigenous soil studies. *International Journal of Environmental Studies*, 40(2-3), pp.185-193.
50. Ousley, M.A., Lynch, J.M. and Whipps, J.M., 1994. Potential of *Trichoderma* spp. as consistent plant growth stimulators. *Biology and Fertility of Soils*, 17(2), pp.85-90.

51. Patel, H., Gupte, A. and Gupte, S., 2009. Biodegradation of fluoranthene by basidiomycetes fungal isolate *Pleurotus ostreatus* HP-1. *Applied Biochemistry and Biotechnology*, 157(3), pp.367.
52. Pérez-Fernández, M. and Valentine, A., 2017. Enhanced plant performance in *Cicer arietinum* L. due to the addition of a combination of plant growth-promoting bacteria. *Agriculture* 7(40) doi:10.3390/agriculture7050040
53. Pérez-García, A., Romero, D. and De Vicente, A., 2011. Plant protection and growth stimulation by microorganisms: biotechnological applications of *Bacilli* in agriculture. *Current Opinion in Biotechnology*, 22(2), pp.187-193.
54. Qian, C. and Cai, Z., 2007. Leaching of nitrogen from subtropical soils as affected by nitrification potential and base cations. *Plant and Soil*, 300(1-2), pp.197-205.
55. Rabenhorst, M.C. and Parikh, S., 2000. Propensity of soils to develop redoximorphic color changes. *Soil Science Society of America Journal*, 64(5), pp.1904-1910.
56. Rutherford, M.C., Mucina, L. and Powrie, L.W., 2006. Biomes and bioregions of southern Africa. *The vegetation of South Africa, Lesotho and Swaziland*, 19, pp.30-51.
57. Schulze, D.G., Nagel, J.L., Van Scoyoc, G.E., Henderson, T.L., Baumgardner, M.F. and Stott, D.E., 1993. Significance of organic matter in determining soil colors. *Soil Color*, pp.71-90.
58. Schwertmann, U., 1993. Relations between iron oxides, soil color, and soil formation. *Soil Color*, pp.51-69.
59. Sharma, S.B., Sayyed, R.Z., Trivedi, M.H. and Gobi, T.A., 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*, 2(1), pp.587.
60. Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. *Ecol. Lett*. 11, pp. 1252–1264.
61. Smethurst, P.J., 2000. Soil solution and other soil analyses as indicators of nutrient supply: a review. *Forest Ecology and Management*, 138(1-3), pp.397-411.

62. Smith, S.E. and Dickson, S., 1991. Quantification of active vesicular-arbuscular mycorrhizal infection using image analysis and other techniques. *Functional Plant Biology*, 18(6), pp.637-648.
63. Stefanic, 1984. Researches concerning a biological index of soil fertility. In Nemes, M.P., Kiss, S., Papacotea, P., Stefanic, C., Rusan, M. (Eds). Fifth Symposium on Soil Biology. *Rumanian National Society of Soil Science Bucharest*, pp. 33-45
64. Stopnisek, N., Bodenhausen, N., Frey, B., Fierer, N., Eberl, L. and Weisskopf, L., 2014. Genus-wide acid tolerance accounts for the biogeographical distribution of soil Burkholderia populations. *Environmental Microbiology*, 16(6), pp.1503-1512.
65. Theuerl, S. and Buscot, F., 2010. Laccases: toward disentangling their diversity and functions in relation to soil organic matter cycling. *Biology and Fertility of Soils*, 46(3), pp.215-225.
66. Tsavkelova, E.A., Bömke, C., Netrusov, A.I., Weiner, J. and Tudzynski, B., 2008. Production of gibberellic acids by an orchid-associated *Fusarium proliferatum* strain. *Fungal Genetics and Biology*, 45(10), pp.1393-1403.
67. Turner, B.L., Baxter, R. and Whitton, B.A., 2002. Seasonal phosphatase activity in three characteristic soils of the English uplands polluted by long-term atmospheric nitrogen deposition. *Environmental Pollution*, 120(2), pp.313-317.
68. Uchida, R., 2000. Essential nutrients for plant growth: nutrient functions and deficiency symptoms. *Plant Nutrient Management in Hawaii's Soils*, pp.31-55.
69. Vendan, R.T., Yu, Y.J., Lee, S.H. and Rhee, Y.H., 2010. Diversity of endophytic bacteria in ginseng and their potential for plant growth promotion. *The Journal of Microbiology*, 48(5), pp.559-565.
70. Wang, M., Zheng, Q., Shen, Q. and Guo, S., 2013. The critical role of potassium in plant stress response. *International Journal of Molecular Sciences*, 14(4), pp.7370-7390.
71. Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., Van Der Putten, W.H. and Wall, D.H., 2004. Ecological linkages between aboveground and belowground biota. *Science*, 304(5677), pp.1629-1633.
72. Williamson, L.C., Ribrioux, S.P., Fitter, A.H. and Leyser, H.O., 2001. Phosphate availability regulates root system architecture in *Arabidopsis*. *Plant Physiology*, 126(2), pp.875-882.

73. Wolińska, A. and Stępniewska, Z., 2012. Dehydrogenase activity in the soil environment. *Dehydrogenases*, pp.183-210.
74. Zhang, J.E., Ouyang, Y. and Ling, D.J., 2007. Impacts of simulated acid rain on cation leaching from the Latosol in south China. *Chemosphere*, 67(11), pp.2131-2137.

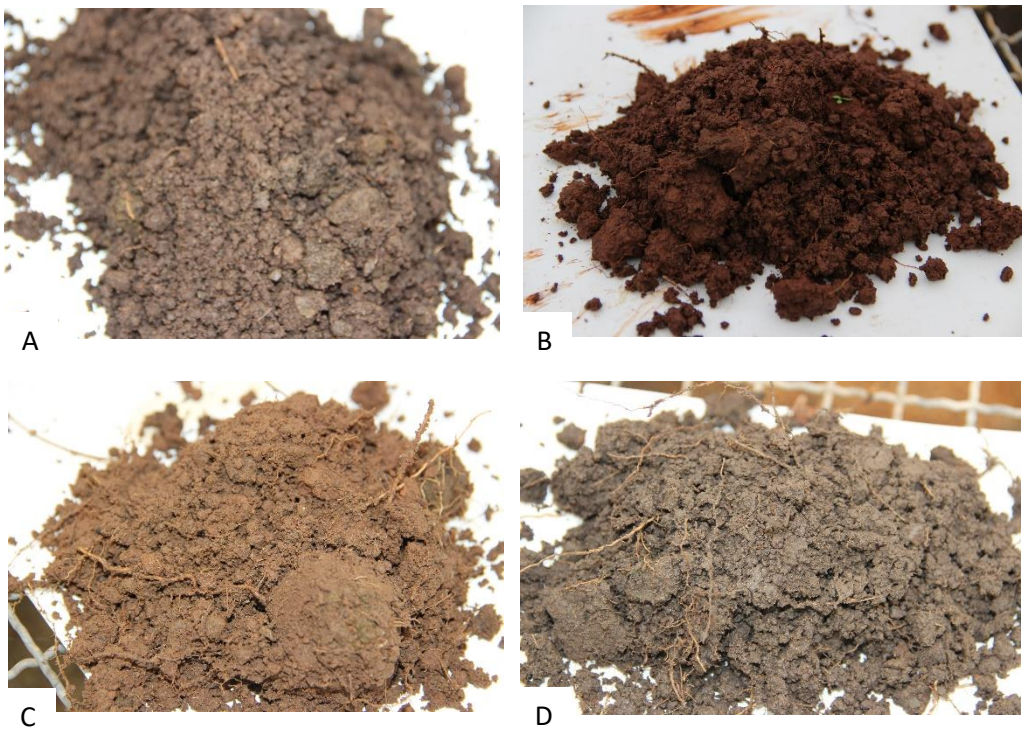


Figure 3.1: Soil colour variation between the four KZN sites. **A:** Hluhluwe (dark brown) **B:** Izingolweni (red-brown) **C:** Bergville (brown) **D:** Ashburton (black).

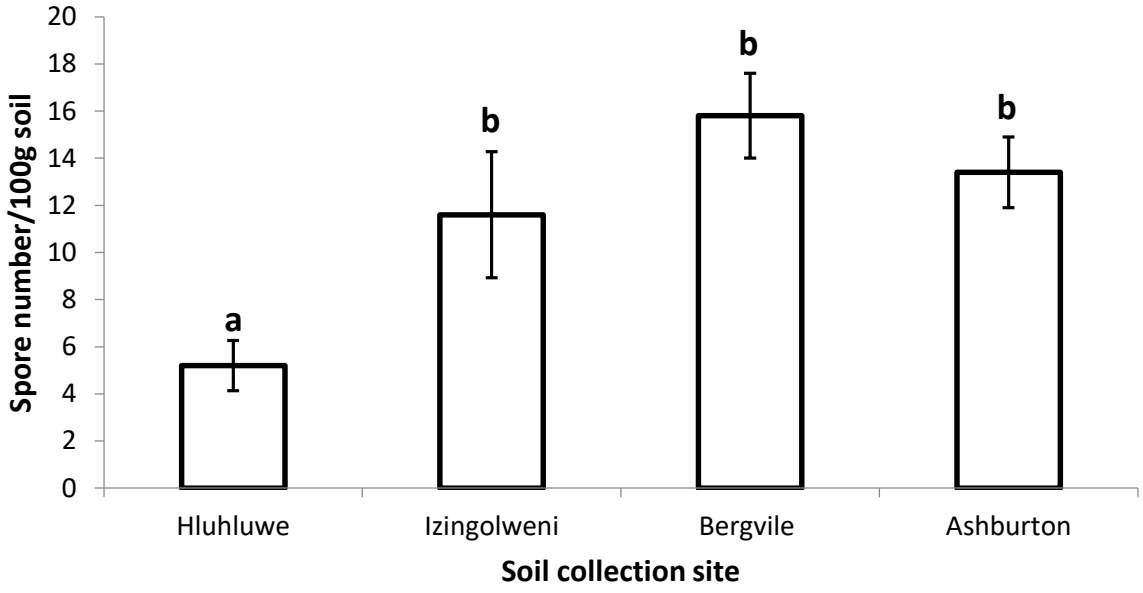


Figure 3.2: Arbuscular mycorrhizal spore count. Soil samples were processed using wet sieving and decanting technique followed by sucrose centrifugation and filtrate. Arbuscular mycorrhizal spores were observed and enumerated microscopically. Spore viability not confirmed. Results are mean ± SE, n = 5. Means with the same letter indicate that no significant differences were detected after a one-way ANOVA ($p \leq 0.05$).

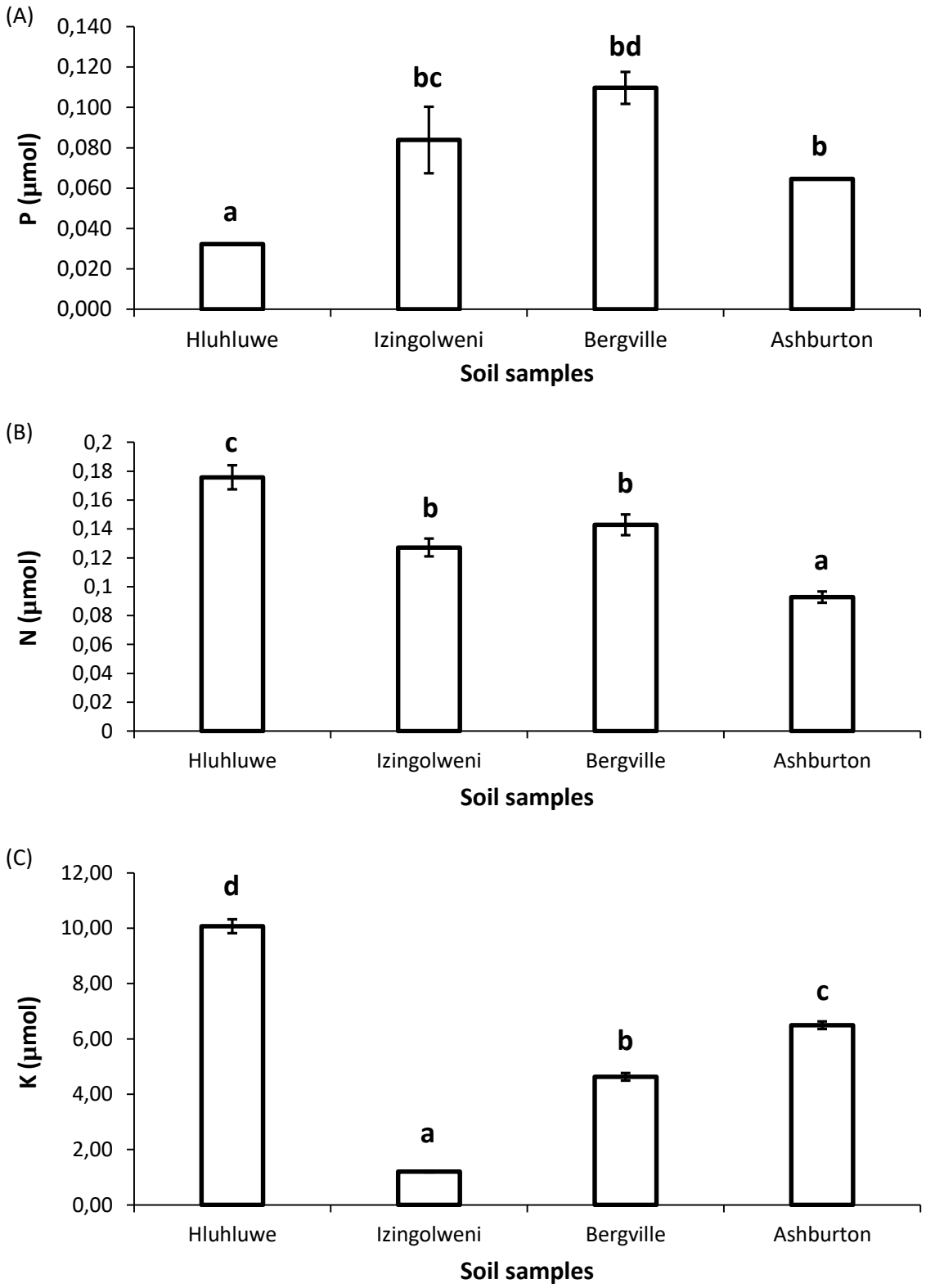


Figure 3.3: Concentration of soil primary nutrients. A: Phosphorus (P), **B:** Nitrogen (N) and **C:** Potassium (K). Results are mean ± SE. Different letters on top of the columns indicate significant differences after one-way ANOVA ($p \leq 0.05$; n = 5).

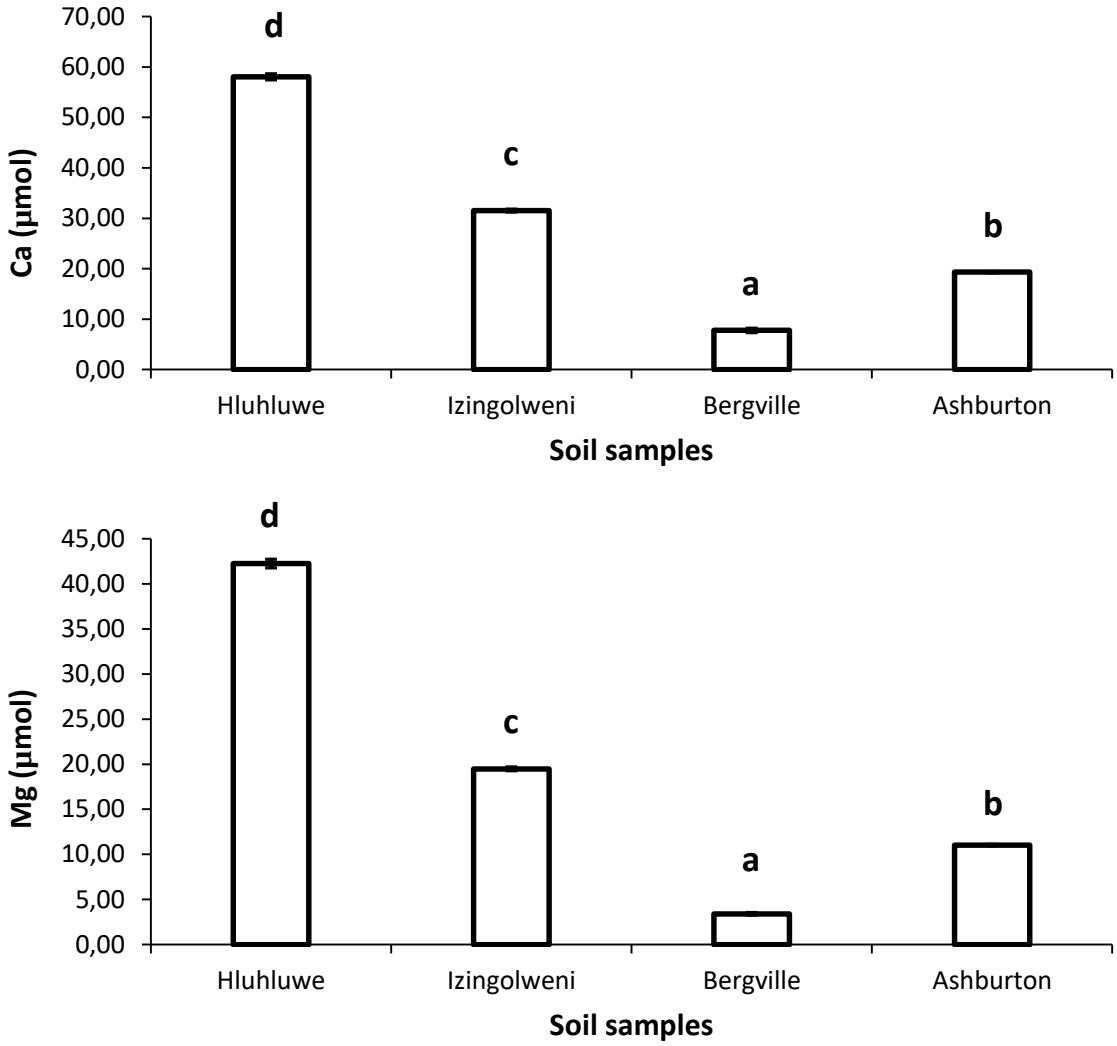


Figure 3.4: Concentration of soil intermediate nutrients. Soil samples were sent to CEDARA College of Agriculture, (South Africa) for total soil nutrient analysis. **A:** Calcium (Ca) and **B:** Magnesium (Mg). Results are mean ± SE. Different letters on top of the columns indicate significant differences after one-way ANOVA ($p \leq 0.05$; n = 5).

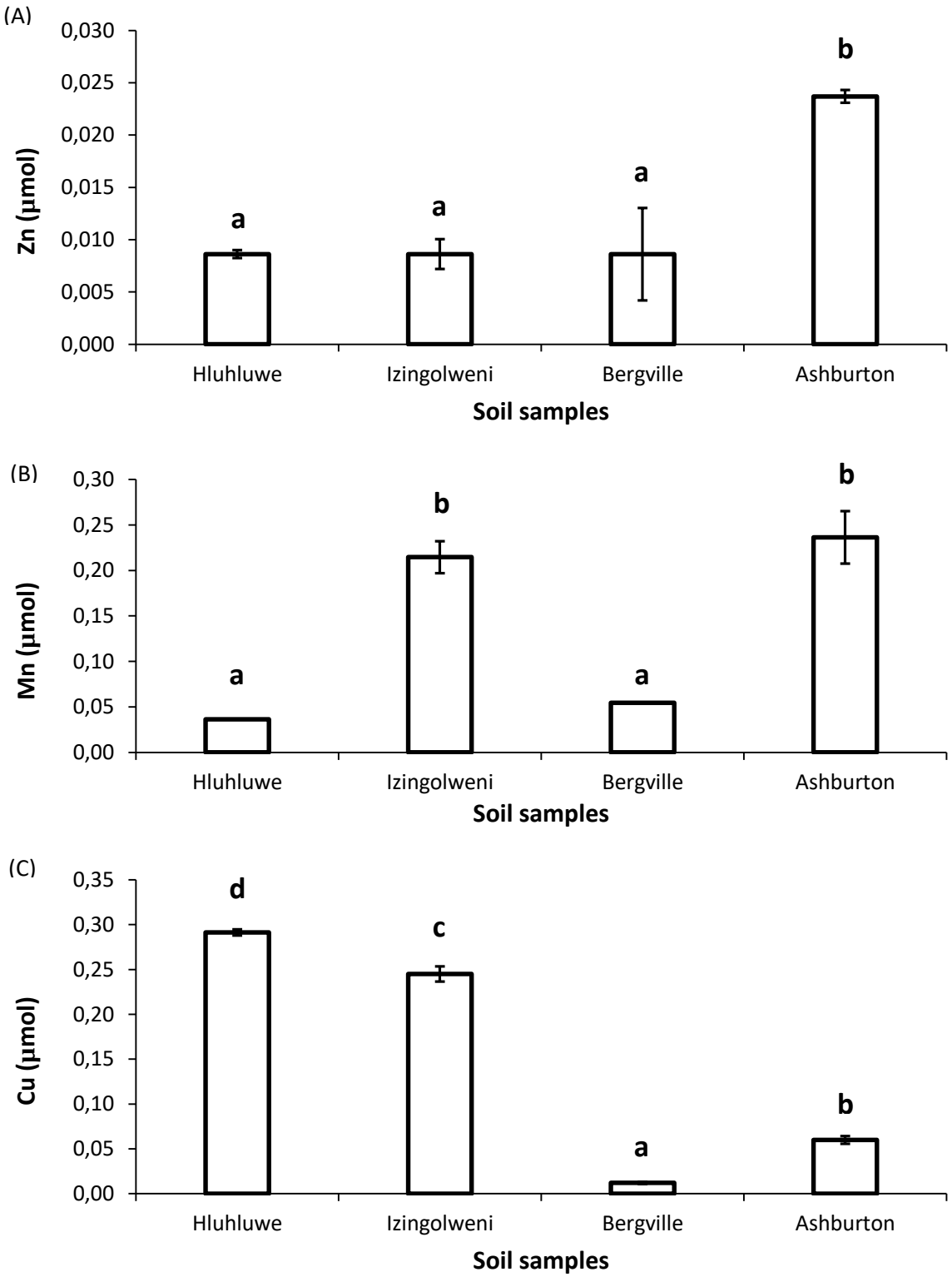


Figure 3.5: Concentration of soil micronutrients. Soil samples were sent to CEDARA College of Agriculture, (South Africa) for total soil nutrient analysis. **A:** Zinc (Zn), **B:** Manganese (Mn) and **C:** Copper (Cu). Results are mean ± SE. Different letters on top of the columns indicate significant differences after one-way ANOVA ($p \leq 0.05$; n = 5).

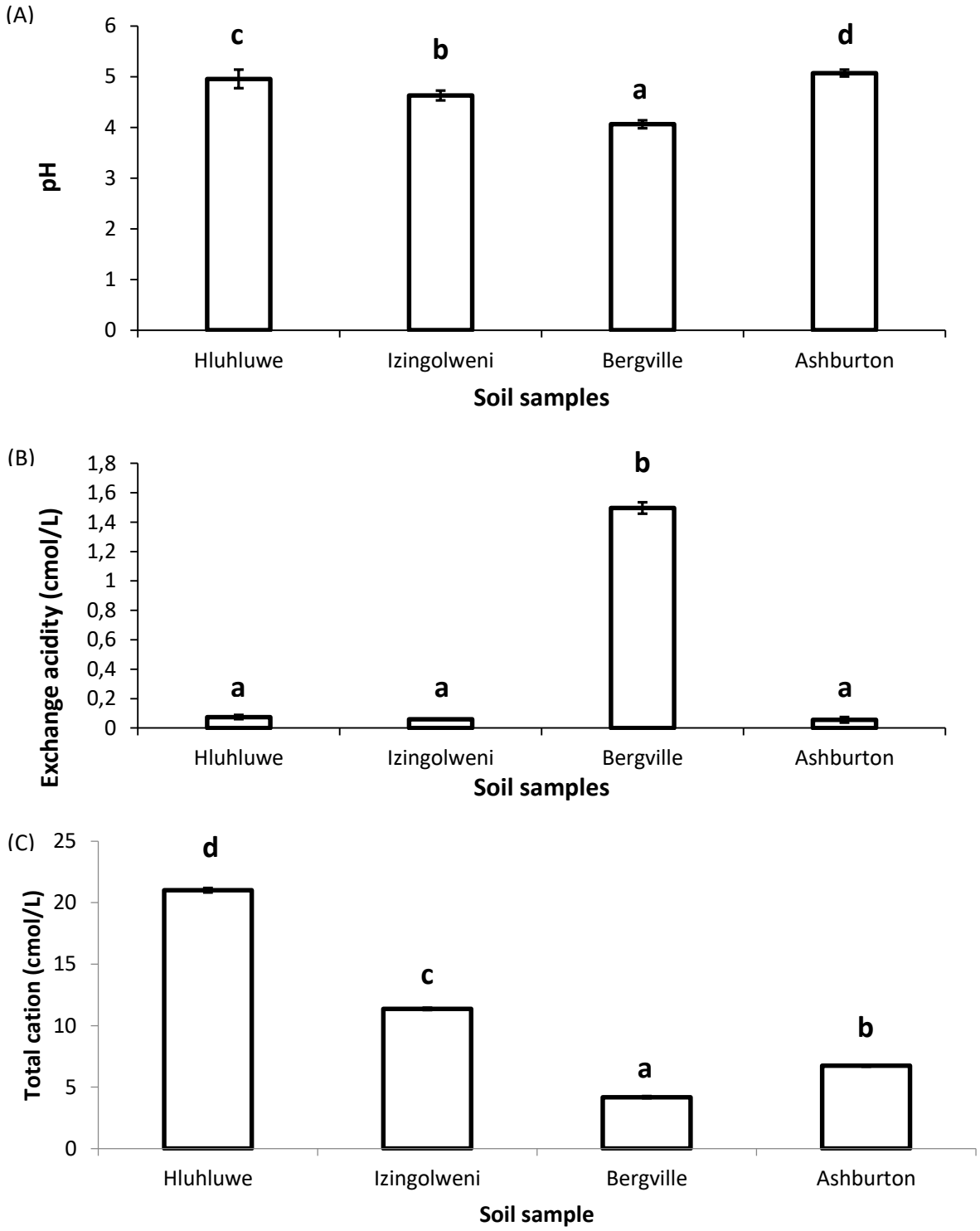
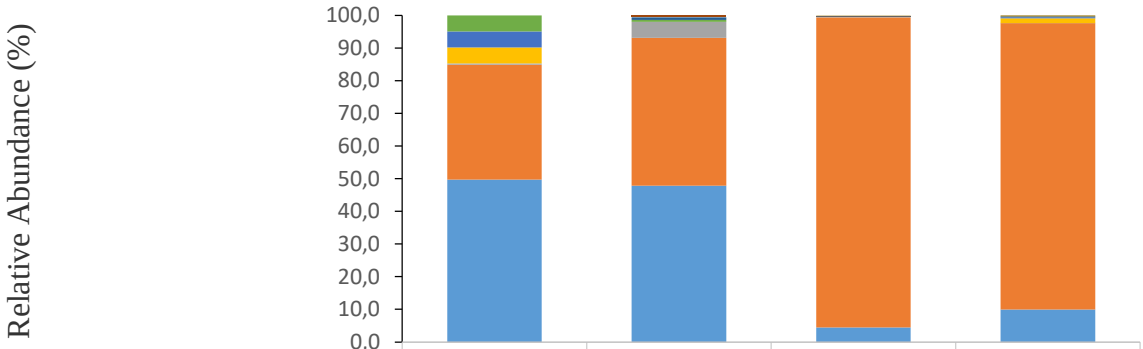
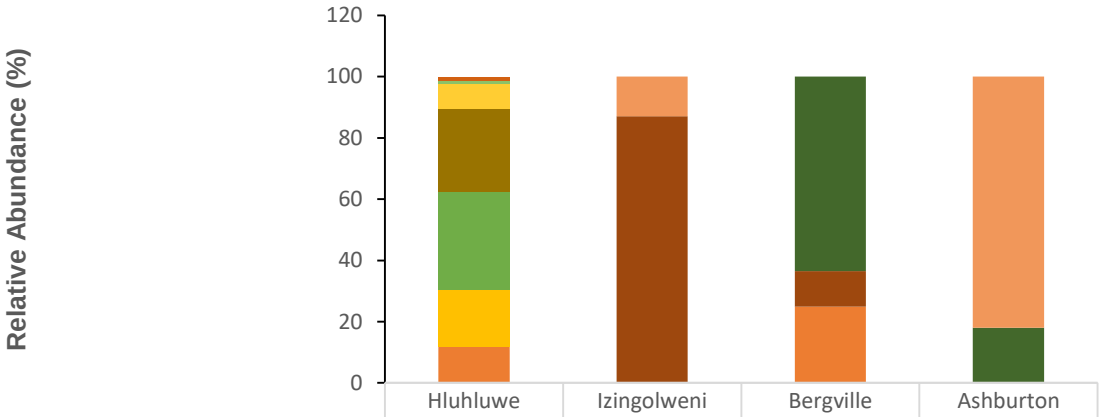


Figure 3.6: Soil relative acidity. Soil samples were sent to CEDARA College of Agriculture, (South Africa) for total soil nutrient analysis. **A:** pH, **B:** Exchange acidity and **C:** Total cation. Results are mean ± SE. Different letters on top of the columns indicate significant differences after one-way ANOVA ($p \leq 0.05$; n = 5).



	Hluhluwe	Izingolweni	Bergville	Ashburton
Bacillus arbutinivorans strain 1404		0,0	0,2	0,3
Bacillus simplex strain P49_BA1H		0,0	0,2	0,0
Lysinibacillus boronitolerans strain MGB4045		0,6	0,0	0,0
Bacillus methylotrophicus strain HB5		0,7	0,0	0,3
Bacillus huizhouensis strain CA98	4,9	0,6	0,0	0,0
Bacillus aryabhattai strain SO4H51	4,9	0,0	0,0	0,3
Bacillus mycoides strain NSII-15	4,9	0,0	0,0	1,5
Bacillus cereus strain RM	0,4	4,8	0,3	0,0
Lysinibacillus xylanilyticus strain MAHUQ-40	35,2	45,3	95,0	87,6
Bacillus thuringiensis strain Eca8	49,7	47,8	4,4	10,0

Figure 3.7: Percentage bacterial strain relative abundance.



	Hluhluwe	Izingolweni	Bergville	Ashburton
Trichoderma gamsii isolate P2-18	1,2			
Trichoderma koningiopsis isolate	1,2			
Trichoderma spirale strain TU Graz 11TSM4	8,1			
Rhizopus stolonifer strain APBSDSF79		12,9		82
Fusarium concntricum isolate F1			63,5	18
Gibberella intermedia isolate F22	26,9			
Amylomyces rouxii CBS 416.77		87,1	11,6	
Mucor velutinosus strain Beca_7a	32,3			
Fusarium sinensis isolate RM63	18,3			
Fusarium oxysporum strain Strain LL-5	11,9		24,9	

Figure 3.8: Percentage fungal strain relative abundance.

Table 3.1: Organic carbon to nitrogen ratio from soil treatments.

Soil site	Organic C (%)	N (%)	C:N ratio	Effect on soil N
Hluhluwe	3.62	0.246	15:1	Mineralization
Izingolweni	2.68	0.178	15:1	Mineralization
Bergville	2.82	0.200	14:1	Mineralization
Ashburton	1.4	0.130	11:1	Mineralization

Table 3.2: Functional Microbial diversity index of the soil samples.

Phylum	Site	Total CFU/mL	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					
Fungi (AMF)	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 3.3: Endophytic strains isolated from the soil samples.

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

Table 3.4: Soil carbon cycling enzyme activities in $nmolh^{-1}g^{-1}$ of soil

Site	β-Glucosidase	β-Cellobiohydrolase	Catalase
Hluhluwe	0.911 ± 0.113^{a}	0.190 ± 0.023^{a}	90.9 ± 25.1^{a}
Izingolweni	0.546 ± 0.095^{b}	0.178 ± 0.006^{a}	64.3 ± 11.9^{a}
Bergville	0.356 ± 0.042^{b}	0.072 ± 0.003^{b}	61.8 ± 10.6^{a}
Ashburton	0.335 ± 0.012^{b}	0.072 ± 0.006^{b}	56.1 ± 3.01^{a}
LSD ($_{0.05}$)	0.231	0.036	44.7

Values in the same column with different letters as superscripts are significantly different by least significant difference ($p\leq0.05$).

Table 3.5: Soil organic matter and lignin degrading enzyme activities in $nmolh^{-1}g^{-1}$ of soil

Site	Dehydrogenase	Laccase	Lignin Peroxidase	Manganese Peroxidase
Hlulhuwe	$36,7\pm16,8^{b}$	$86,12\pm15,1^{a}$	ND	$220,9\pm57,2^{ab}$
Izingolweni	$123,9\pm41,4^{a}$	$28,5\pm29,5^{a}$	$105,7\pm49,1^{a}$	$338,8\pm121,7^{b}$
Bergville	$19,9\pm3,17^{b}$	$62,5\pm10,4^{a}$	ND	$185,2\pm18,1^{ab}$
Ashburton	$60,4\pm10,9^{b}$	$50,7\pm21,9^{a}$	$40,9\pm66,3^{a}$	$50,3\pm68,3^{b}$
LSD ($_{0.05}$)	69,12	61,6	125,5	227,7

ND: Not detected, Values in the same column with different letters as superscripts are significantly different by least significant difference ($p\leq0.05$)

Table 3.6: Soil nitrogen and phosphate cycling enzyme activities in $nmolh^{-1}g^{-1}$ of soil

Site	β-Glucosaminidase	*L*-Asparaginase	β-Phosphatase
Hlulhuwe	$0,637\pm0,007^{c}$	$574,5\pm12,6^{a}$	$1,149\pm0,05^{a}$
Izingolweni	$0,734\pm0,011^{b}$	$66,2\pm4,51^{b}$	$0,797\pm0,012^{b}$
Bergville	$0,981\pm0,016^{a}$	ND	$0,686\pm0,020^{c}$
Ashburton	$0,567\pm0,034^{d}$	$15,5\pm0,201^{c}$	$0,806\pm0,046^{b}$
LSD ($_{0.05}$)	0,059	515,7	0,103

ND: Not detected, Values in the same column with different letters as superscripts are significantly different by least significant difference ($p\leq0.05$)

Table S1: Values of the Biological Index for Soil Fertility based on dehydrogenase and catalase activities

Site	Dehydrogenase	Catalase	BIF
Hlulhuwe	36.7	90.9	24.54
Izingolweni	123.9	64.3	59.75
Bergville	19.9	61.8	9.22
Ashburton	60.4	56.1	25.41

Biological Index of soil Fertility **(**Stefanic, 1984):

$$BIF = (1.5\ DH\ k\ 100\ CA)/2$$

Where DH is dehydrogenase

CA Catalase

k proportionality Coefficient (0.01)

Figure S1.: Map of KwaZulu Natal province illustrating soil collection sites for the present study. Hluhluwe, Northern KZN (28°0′58″S 32°12′4″, Altitude ~100 m); Izingolweni, Southern KZN (30°43′32″S 30°6′10″E, Altitude ~450 m); Bergville, Mountainous KZN (28°34′14″S 29°4′17″E, Altitude ~1040 m); Ashburton, Midland KZN (29°38′55″S 30°26′42″E, Altitude ~670 m). Image retrieved from Sithole *et al*. (2019).

Chapter 4

Plant growth & nutrition

Pisum sativum L. can acclimatize to nutrient deficient soils from KwaZulu- Natal grasslands and savanna ecosystems, South Africa.

Ntuthuko S. Zungu[a], Thandeka Khoza[a], Adeyemi O. Aremu[a,b], Jiri Gruz[c], María Pérez-Fernández[d], Anathi Magadlela[a]*

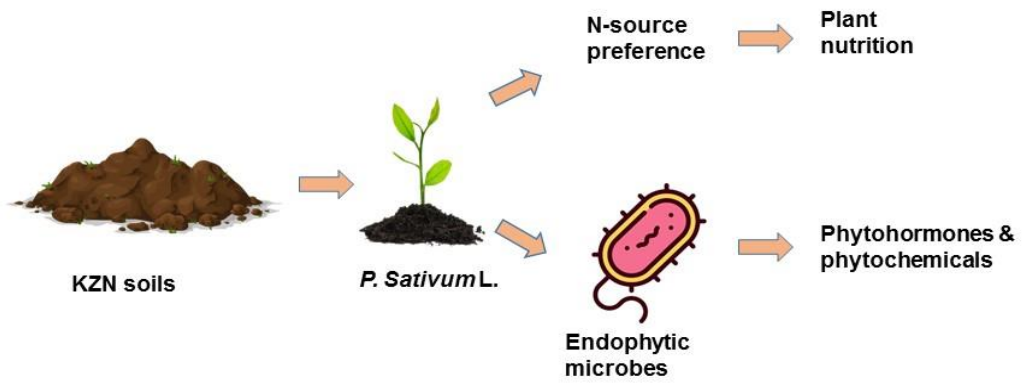
[a]*School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, KwaZulu-Natal, South Africa*

[b]*Food Security and Safety Niche Area, Faculty of Natural and Agricultural Sciences, North- West University, Private Bag X2046, Mmabatho, North West Province South Africa*

[c]*Laboratory of Growth Regulators, Faculty of Science, Palacký University & Institute of Experimental Botany AS CR, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic*

[d]*Ecology Area, University Pablo de Olavide, Carretera de Utrera Km 1, 41013 Sevilla, Spain*

***Correspondence:** anathimagadlela@icloud.com



Graphical abstract depicting the research conducted and presented in this section.

Abstract

The agricultural sector is a major contributor to many economies across the world, especially in developing countries. Unfortunately, soil quality degradation and low soil fertility presents a major threat to sustainable agriculture. Legumes have been used to improve soil fertility, however most legume research is usually focused on food and not forage legumes, especially in nutrient-deficient agri-systems. Forage legumes like *Pisum sativum* (L.) are important in these systems as they increase the nutritional value in pastures and provide relatively high amounts of protein and minerals when used as animal feed. Therefore, this study examined the effects of KwaZulu-Natal grasslands and savanna ecosystem nutrient deficient soils in plant-microbe symbiosis, plant nutrition and carbon growth costs in a forage legume, *P. sativum* (L.). Also, the phenolic acids and cytokinin concentrations in this forage legume were elucidated. Soils from four KZN geographical distinct regions covering grasslands and savanna ecosystems were used as growth substrates. These soils were nutrient deficient, acidic with varying microflora. *P. sativum* (L.) maintained its root dry weights and plant growth rates among all the soils. Low pH, total cations and high exchange acidity in Bergville soils resulted in decreased total plant dry weights. Also, Bergville soil grown plants showed decreased growth-promoting compounds (i.e. kinetin, active cytokinins, and salicylic acid) and increased cis-zeatin type and storage cytokinins. *P. sativum* (L.) grown in Izingolweni soils were more reliant on atmospheric N fixed by endophytic/associative bacteria from the genera *Cupriavidus*, *Paenibacillus*, *Cohnella* and *Bacillus*. While plants grown in Hluhluwe soils, relied on soil N. Furthermore, *P. sativum* (L.) grown in Izingolweni soil decreased shoot growth and overall plant nutrient concentration in comparison to Hluhluwe and Ashburton plants. Hluhluwe and Ashburton soil grown plants accumulated the most biomass and N nutrient concentration via N-source switching, increasing N nutrient absorption and/or decreasing N utilization rates. Therefore, plant associative microbes might modulate nutrient availability for plant uptake in grassland and savanna nutrient poor ecosystems. *P. sativum* (L.) may acclimatize to these low nutrient and acidic ecosystem soils by changing N source preferences, phenolic and cytokinin concentrations.

Keywords: *Pisum sativum* L., nutrient deficient, endophytic/associative bacteria, phenolic acids and cytokinin.

4.1. Introduction

Sustainable soil management is viewed as a fundamental aspect of sustainable agriculture for supporting the ever-increasing population and biodiversity (Nambiar *et al*., 2001; Lal, 2008; Ohyama, 2017). However, African countries are still faced with accelerating declines in agricultural yield due to unsustainable agricultural practices and environmental factors. The environmental factors include climate change, rainfall variability, soil quality degradation, and recurrent droughts (Lema and Majule, 2009; Doso Jnr, 2014). These aforementioned environmental factors do not only have detrimental effects on soil fertility but on ecosystem functioning and vegetation structure and composition (Lema and Majule, 2009; Doso Jnr, 2014). The agricultural sector plays a pivotal role in most economies and since the soil is a non-renewable resource (Doran and Zeiss, 2000), sustainable strategies need to be developed to help mitigate the negative impact on soil quality and yield potential. To achieve this, the importance of soil must be recapitulated and more research must be aimed at the identification of plants that can withstand such environmental stress conditions. This would enable the establishment of high nutrient pastures for animal feed and also improved soil fertility through the use of legumes.

Soil is a sink of essential minerals, nutrients, and microflora that contribute to the growth of plants (Rutigliano *et al*., 2004). Soil is also involved in the direct and/or indirect cycling of essential elements such as nitrogen (N), carbon (C) and oxygen (O) (Harper and Pendleton, 1993; Nannipieri *et al.*, 2003; Marschner, 2012). Soil fertility and functionality are based on the soil composition (i.e. clay content), relative acidity (pH), aeration (redox potential), microbial diversity, moisture content, temperature, nutrient availability and other chemical and physical characteristics (Voroney, 2007; Husson, 2013). All these soil characteristics are essential for plant growth and persistence during harsh environmental conditions (Voroney, 2007; Husson, 2013). However, microbial diversity tends to stand out due to the ability of some microorganisms to converts insoluble nutrients into plant-available form (Rengel and Marschner, 2005). These microorganisms can either be host-dependent or free-living such as legume *Rhizobia* and *Aspergillus niger*. *Rhizobia* are known to reduce atmospheric nitrogen (N_2) through nitrogenase activity, while *Aspergillus niger* can hydrolyze phytic acid through phytase activity (Rengel and Marschner, 2005). An example of a key plant nutrient that microorganisms recycle is phosphorus (P) and is usually present in low concentration ($\leq$10 μM) in acid soils (Kunwar *et al.*, 2018).

South Africa (SA) is one of the developing African countries that have experienced soil quality degradation due to drought and poor remediation strategies which include replenishing soil organic matter (Williams *et al*., 2004). Furthermore, SA soils are not classified as high fertility soils and those that are, tend to be easily degraded (Roberts *et al.*, 2003; Mandiringana *et al*., 2005). There are three distinct rain seasonality regions in SA i.e. the Mediterranean-like region (Western to southern Cape) that is characterized by winter rainfall, the summer rainfall region (KZN and Highveld ≥ 600 mm annual rainfall) and the bimodal region (Eastern Cape). All these regions constitute the average annual rainfall of ~450 mm, which makes SA a semi-arid country. It is also important to note that out of the 14 soil groups in SA, 12 are distributed across KZN (Fey, 2010). This makes KZN suitable for agricultural practices but nutrient availability is usually the limiting factor in areas like KZN and Highveld that receive ≥ 600 mm annual rainfall.

This can negatively affect agricultural productivity (Onwuka *et al*., 2016), especially if the soils are also acidic (Maisels *et al.*, 1994; Haumaier and Zech, 1995; Buresh *et al*., 1997). Luckily, legumes have developed adaptations to withstand these soil conditions through the establishment of tripartite symbiosis between the host plant and beneficial microorganisms (Ohyama, 2017; Sanz-Saez *et al.*, 2017). The limiting nutrients are made available in these symbiotic systems through biological N-fixation (BNF) in root nodules and P scavenging hyphae of arbuscular mycorrhizal (AM) fungi (Lambers *et al.*, 2008). However, legume research is usually focused on food and not forage legumes, especially in nutrient-deficient agri-systems. While forage legumes like *Pisum sativum* (L.) are also important in sustainable agricultural systems as they increase the nutritional value in pastures and provide relatively high amounts of N in soils required for plant growth (McCallum *et al*., 2000). Thus, the aim of this research was to investigate the effects of distinct soil types with varying nutrient concentrations and pH on plant-microbe symbiosis, biomass accumulation, N-source preference and carbon growth costs of *Pisum sativum* (L.), and to determine the effects of these distinct soil types on phenolic and cytokinin concentrations in *Pisum sativum* (L.).

4.2. Methods and materials

4.2.1. Soil collection sites

Four geographical distinct regions in KwaZulu Natal province, South Africa, covering grassland and savanna ecosystems were used for soil sampling: Hluhluwe, Northern KZN (28°0'58''S, 30°26'42''E); Izingolweni, Southern KZN (30°43'32''S, 30°6'10''E); Bergville, Mountainous KZN (28°34'14''S, 29°4'17''E); Ashburton, Midlands KZN (29°38'55''S, 30°26'42''E). Approximately 10 soil samples (0-30 cm depth and 2 m apart) were collected in the rhizosphere from each of the four sites and pooled for homogeneity.

4.2.2. Soil geochemistry and arbuscular mycorrhizal fungi analysis

Five 50 g soil samples from each respective site were sent to the KwaZulu-Natal Department of Agriculture and Rural Development's Analytical Services Unit at Cedara College of Agriculture, South Africa, for total soil nutrient and cation concentrations, cation exchange acidity and pH analysis. Arbuscular mycorrhizal spore count analysis was conducted according to Smith and Dickson (1997) at Mycoroot (Pty) Ltd, Rhodes University, South Africa in soil samples ranging from 250 to 300 g from each site. The method used for this analysis was the wet sieving and decanting technique. Thereafter, sucrose centrifugation and filtration followed and the spores were observed and enumerated using a microscope.

4.2.3. Seed germination, bacterial inoculation, and growth

P. sativum (L.) seeds obtained from AGT foods Africa, Marji Mizuri farm, KZN, were planted in soils collected from the four KZN sampling regions. The seeds were sown ~2 cm deep in 19 cm diameter pots containing soil used as natural inoculum and growth substrate. The seeds were watered daily with ~300 ml H_2O pre-germination and every second or third day post-germination. Each treatment was replicated 20 times and seedling emergence was assessed for up to 10 days from sowing. Initial and final harvests were conducted 25 and 55 days after sowing, respectively. The experiment was conducted under ambient conditions in glasshouse No. 12 at the University of KwaZulu Natal botanical gardens, Pietermaritzburg, South Africa. The daytime and night-time temperatures ranged from 30 to 35 °C and 12 to 14 °C, respectively. The daily average humidity was 75% and the irradiance was ca. 35% of full sunlight (415.6 μmol m^2 sec^{-1}).

4.2.4. Plant nutrient analysis

Five plants per treatment were separated into different organs (leaves, stem and roots) and oven-dried at 80 °C for 7 days and the dry weights (DW) recorded. The dry plant material was ground in a mortar and pestle using liquid nitrogen and analyzed for their C, P and $\delta^{15}N$ concentrations. $\delta^{15}N$ concentrations analysis was conducted using a LECO-nitrogen analyzer at the Archaeometry Department, University of Cape Town, South Africa, and C and P concentrations were analysed using the inductively coupled mass spectrometry (ICP-MS) at the Central Analytical Facilities, Stellenbosch University, South Africa.

4.2.5. Growth calculations

4.2.5.1.Relative growth rate (RGR)

Agren and Franklin (2003) method was used to calculate the relative growth rate.

$$RGR = [(\ln W_2 - \ln W_1) / t_2 - t_1]$$

Where *W* is the dry weights accumulated from initial to final harvest and *t* is the time for plant growth.

4.2.5.2.Specific N absorption rate (SNAR)

Plant total N content was used to calculate the specific N absorption rate according to Nielsen *et al*. (2001).

$$SNAR = (L_2 - L_1 / t_2 - t_1)*[(\log_e R_2 - \log_e R_1) / (R2 - R1)]$$

Where *L, t* and *R* represent total N content, duration of plant growth and root dry weight, respectively.

4.2.5.3.Specific N/P utilization rates (SNUR/SPUR)

Plant total N/P content was used to calculate the specific N/P utilization rate according to Nielsen *et al*. (2001).

$$SNUR = (W_2 - W_1 / t_2 - t_1)*[(\log_e L_2 - \log_e L_1) / (L_2 - L_1)]$$

$$SPUR = (W_2 - W_1 / t_2 - t_1)*[(\log_e M_2 - \log_e M_1) / (M_2 - M_1)]$$

Where *W, L* and *M* represent plant DW, total N and P content, respectively.

4.2.5.4.Percentage N derived from the atmosphere (%NDFA)

%NDFA was calculated according to Shearer and Kohl (1986) as $\delta = 1000 (R_{sample} / R_{standard})$, where *R* is the molar ratio of ^{15}N and ^{14}N of the samples and standards. Sample preparation and analysis was conducted according to Matiwane *et al*. (2019) using the same equipment, standards, and facilities.

$$\delta = 100 ((\delta^{15}N_{reference\ plant} - \delta^{15}N_{legume}) / (\delta^{15}N_{reference\ plant} - \beta))$$

Where β value represents the $\delta^{15}N$ natural abundance of the N derived from biological N_2-fixation of *P. sativum L*., grown in a N-free culture. The β value of *P. sativum* was -2.58%.

4.2.5.5.Carbon cost (Cw)

Carbon growth cost was calculated using the formula modified by Peng *et al.* (1993).

$$Cw = (C + kN / 14*180 / 24) (1 / 0.89) (6000 / 180)$$

Where *Cw* represents the tissues' total carbon construction cost, *C* is the total concentration of carbon, *k* is the reduced state of N substrate (i.e. NH_3 = -3) and *N* is the total organic nitrogen content of the tissue (Williams *et al.*, 1987).

Given: Atomic mass of N = 14; Conversion factor from mol to g glucose = 180; No. of e^- in glucose molecule = 24; Estimate of growth efficiency = 0.89; Constant conversion factor = 6000 / 180

4.2.6. Bacterial isolation, colony PCR and identification

Because Izingolweni soil grown *P. sativum* (L.) were the only plants that formed root nodules, root nodules from these plants were rinsed with ddH_2O to remove residual soil particles. The nodules were then surface sterilized using 70% (v/v) ethanol for 30 seconds before submerging them in 3.5% (v/v) sodium hypochlorite solution for 3 minutes. Thereafter, the nodules were thoroughly rinsed (~10 times) with dH_2O and stored in vials containing silica gel. The vials were subsequently placed inside a fridge at 4°C. Endophytic bacteria were isolated by crushing the stored root nodules in 15% (v/v) glycerol and culturing the suspension on yeast mannitol agar (YMA) and incubated at 28°C under micro-aerobic conditions. Single colonies were obtained by repeated streaking and culturing at 72 h incubation periods at 28°C under micro-aerobic conditions.

Colony PCR to amplify a portion of the 16S rDNA gene was carried out using the following primers set: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR reaction volumes (for a total of 25 µl) was 11 µl sterile distilled water, 12.5 µl TAKARA-EmeraldAmpGT PCR Master Mix (Separations, South Africa), 1 µl colony, 0.25 µl forward primer and 0.25 µl reverse primer. The PCR conditions were: Initial denaturation at 94°C for 5 minutes; 30 cycles of denaturation at 94°C for seconds; annealing at 55°C for 30 seconds; extension at 72°C for 2 minutes; final elongation step at 72°C for 10 minutes. The resulting PCR products were resolved on a 1% (w/v) agarose gel using 1x TAE buffer and run at 100V for 40 minutes. The PCR products were then sent for sequencing at the Central Analytical Facilities at Stellenbosch University, South Africa. The resulting sequences were subjected to BLASTN searches for identification (National Center for Biotechnology Information, NCBI (https//www.ncbi.nlm.nih.go).

4.2.7. Cytokinin and phenolic acids quantification

Nine plants per treatment were pulverized in liquid nitrogen using a mortar and pestle and freeze dried. The samples were then sent for cytokinin and phenolic acids quantification at the Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University & Institute of Experimental Botany AS CR, Šlechtitelů, Czech Republic. Plant cytokinin concentrations were determined according to Novák *et al.* (2008) with slight modification (Novák *et al.*, 2003), while phenolic acid concentrations were determined by extracting the compounds with 80% (v/v) methanol and metal bead with 20 µl internal standard [10^{-4} mol/L; solution of salicylic acid (3.4.5.6-$^{2}H_4$) and 4- hydroxybenzoic acid (2.3.5.6-$^{2}H_4$)]. Thereafter, the mixture was homogenized for a period of 3 minutes in the oscillatory ball mill homogenizer (frequency 27 sec^{-1}) then subjected to the ultrasonicator for 15 minutes and further centrifuged for 10 min at 17 000 RPM at temperature of 4 °C. Then the supernatants were transferred into Eppendorf tubes and the pellet was re-extracted. The extracts were then combined and evaporated to dryness in an oil evaporator for a period of 6-8 hours.

The ultra-high-performance liquid chromatography-tandem mass spectrometry quantification was determined using a UHPLC^{TM} system (Waters. Milford. MA. USA) connected simultaneously to both a PDA 2996 photodiode 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 ionization (ESI) source operating in negative mode (Gruz et al., 2008). The extracts were injected into a reversed-phase column (BEH C_8. 1.7 µm. 2.1 × 150 mm. Waters. Milford. MA) and incubated at a temperature of 30 °C. Linear gradients and isocratic flows of the mobile phase had a sequencing of 9.5 min with solvent B (acetonitrile) balanced with solvent A (aqueous 7.5 mM HCOOH) at a flow rate of 250 µl min^{-1}. After sequencing, the column was equilibrated for 2.5 min under initial conditions with pressure from 4000 to 8000 psi during the chromatographic run. The eluent was added into a PDA detector (scanning range 210-600 nm. resolution 1.2 nm). thereafter into an electrospray source (source block temperature 100 °C. desolation temperature 350 °C. capillary voltage 2.5 kV. cone voltage 25 V). Argon was applied as a collision gas (collision energy 16 eV) and N was the desolvation gas (500 L h^{-1}). Different retention windows were used for quantification

4.2.8. Statistical analysis

IBM SPSS Statistics 25 software was used for all data analysis. Analysis of variance (ANOVA) statistical model was used to compare means and values were considered significant when $P \leq 0.05$. Where the ANOVA showed significant differences between treatments, a Bonferroni's post hoc test was used to separate the means. The least significant difference test was used to compare cytokinin concentrations.

4.3. Results

4.3.1. Geochemistry and microflora

The geochemical analysis revealed that the KZN soils differed in macronutrient concentrations, relative acidity and microflora. Bergville soil had the highest P concentration whilst having the second and third highest N and K concentration respectively (Table 4.1). Hluhluwe soil had the highest N and K concentration but had the lowest P concentration (Table 4.1). Izingolweni and Ashburton soils fell in-between the two extremes for N and P concentration (Table 4.1). However, Izingolweni soil had the lowest K concentration (Table 4.1). All the sites had acidic soils, with Bergville having the lowest pH, total cations, and the highest exchange acidity (Table 4.1). All the sites had relatively low AM fungi spore count per 100 g of soil. The average spore count ranged between 5.20 - 15.80 spores per 100 g of soil (Table 4.1). Ashburton and Hluhluwe soils had the

most bacterial colony forming units, while Izingolweni and Bergville had the first and second lowest bacterial CFU, respectively (Table 4.1).

4.3.2. Seedling emergence and growth kinetics

A comparison of seedling emergence showed that Hluhluwe and Ashburton had the highest and similar percentage seedling emergence after four (91%) and seven days (100%) of planting, followed by Izingolweni (four days = 82%; seven days = 95%) and Bergville (four days = 86%; seven days = 91%) (Table 4.2). All plants grown in these different experimental soils maintained their growth rates (Table 4.3). Hluhluwe soil grown plants had the highest total plant biomass followed by Ashburton grown plants then Izingolweni and Bergville soil grown plants (Table 4.3). Hluhluwe and Ashburton soil grown plants had the highest aboveground biomass and decreased root-shoot (Table 4.3). Izingolweni and Bergville soil grown plants had root-shoot ratio of 1.39 and 5.52 respectively (Table 4.3). Bergville plants also had the lowest above-ground biomass whilst having the highest root biomass (Table 4.3).

Plants grown in soils from all four sites relied on both soil and atmospheric N for growth. There was no significant difference in total plant N concentration in plants grown in Ashburton (2.23 ± 0.087) and Hluhluwe soils (2.14 ± 0.093) (Table 4.3). Plants grown in Izingolweni soils (1.12 ± 0.15) and Bergville (0.70 ± 0.080) had the lowest total plant N concentration, respectively (Table 4.3). Izingolweni soil grown plants relied more on N_2-fixation (~55%) than Ashburton (~43%) then Bergville (~31%) and Hluhluwe soil grown plants (~5%) (Table 4.3). Ashburton and Izingolweni soil grown plants had the highest concentration of N derived from the atmosphere (Table 4.3). Hluhluwe soil grown plants had the most soil-derived N (~95%), followed by Bergville (~69%), Ashburton (~57%), and Izingolweni (~45%) (Table 4.3). Hluhluwe and Ashburton soil grown plants assimilated the most N, while Bergville soil grown plants had the least N absorption rate (Table 4.3). However, Ashburton and Hluhluwe soil grown plants utilized the least N per day in comparison to Izingolweni and Bergville plants which had the highest specific N utilization rate (Table 4.3).

Ashburton soil grown plants had the highest total plant P concentration, while Bergville soil grown plants accumulated the least total plant P concentration (Table 4.3). Izingolweni and Hluhluwe soil grown plants had the second and third least total plant P concentration, respectively (Table 4.3).

However, there was no significant difference between the treatments when it comes to the carbon construction costs (Table 4.3) and specific P utilization rates (Table 4.3).

4.3.3. Endophytic bacteria identification

Izingolweni soil grown plants were the only plants that developed nodules and were selected for endophytic bacteria identification. Amplification and comparison of the 16S ribosomal RNA gene partial sequence revealed that the root-nodule inhabiting bacteria were from the genera *Paenibacillus, Cupriavidus*, *Ralstonia, Saccharibacillus, Cohnella,* and *Bacillus* (Table 4.4). The species identity ranged between 90 – 100% (Table 4.4). Isolates from *Paenibacillus* were *P. polymyxa, P. jamilae*, *Paenibacillus. sp., P. favisporus,* and *P. endophyticus* (Table 4.4). Members of *Cupriavidus* were *C. necator, C. oxalaticus*, *Cupriavidus. sp.*, and *C. metallidurans* (Table 4.4). Isolates from *Bacillus* were *B. cereus, Bacillus sp.,* and *B. thuringiensis* (Table 4.4). Other nodule isolates were *Ralstonia sp., Saccharibacillus sp.,* and *Cohnella plantaginis* (Table 4.4).

4.3.4. Phenolic acids concentration

The concentration of phenolic acids was higher in plant shoots than in the roots, with the exception of salicyclic and vanillic acid (Table 4.5). The only phenolic acid concentrations that showed significant differences were the shoot concentrations of caffeic, vanillic, and sinapic acid (Table 4.5). Izingolweni soil grown plant shoots had the highest caffeic and vanillic acid concentration, while Hluhluwe soil grown plant shoots had the highest sinapic acid concentration (Table 4.5). Izingolweni and Bergville soil grown plants had an overall increase in the concentration of most phenolic acids, with the exception of salicyclic acid in which Bergville soil grown plants had the least concentration (Table 4.5). Furthermore, Bergville soil grown plants had the highest 4-coumaric acid concentration (Table 4.5).

4.3.5. Cytokinin concentration

The concentration of most of the cytokinins was higher in plant roots than in the shoots (Table 4.6). Bergville soil grown plants had high concentrations of *O*-glucoside and riboside *O*-glucoside containing cytokinins, as well as the cis isomer of zeatin (cZ) and DHZR (Table 4.6). However, Bergville plants lacked tZ7G, DHZ, iP, and kinetin (Table 4.6). Hluhluwe and Ashburton plants

had higher concentrations of the active cytokinins and their ribosides (i.e. Z, iP, DHZR, kinetin) but lacked DHZ (Ashburton) and tZROG (Table 4.6). Ashburton plants also had elevated tZ7G concentration. Izingolweni plants lacked iP and had moderate to high concentrations of all the other cytokinins (Table 4.6).

4.4. Discussion

Pisum sativum L. was able to grow in soils from KZN savanna and grassland ecosystems by modulating their growth kinetics, plant nutrient assimilation and utilization rates and regulating their phenolic acid and cytokinin concentrations. Additionally, Izingolweni soil grown plants established symbiosis with multiple bacterial genera.

Plants grown in Bergville soils had deceased total plant dry weight, however their root dry weights increased significantly. This may be due to the significant decrease in pH of Bergville soils. Low soil pH decreases the base saturation, nutrient availability, total cations and increase exchange acidity in soils (Stiles, 2004). This results in limited essential nutrients and co-factors required for growth and development. For instance, under slightly acidic conditions, phosphate available for plants is sequestered into insoluble cation bound phosphate making it unavailable for plant assimilation (Giesler *et al*., 2002). Additionally, Low pH alters the soil microflora composition, diversity, abundance and viability (Zhalnina *et al*., 2015). This results in decreased efficiency of plant growth-promoting microorganisms (Siciliano *et al*., 2014; Zhalnina *et al*., 2015). However, plants have been reported to have increased root systems under stress conditions to mitigate the effects and prevent senescence (Huang and Fry, 1998; Franco *et al*., 2011). This agrees with our observations in Bergville soil grown plants increased root dry weights, increasing root surface area increasing the probability that plant roots will contact more plant growth-promoting microorganisms and soil nutrients. This is evident in Bergville soil grown plants as they increased their reliance on soil-derived N rather than N derived from the atmosphere. The low plant nutrient concentrations and absorption rates could indicate resource management by Bergville soil grown plants. Resource management is one of many survival strategies that plants employ under unfavorable conditions (Chapin, 1991; Robert *et al*., 2014). Regulation of metabolites like phenolic acids has also been reported to play a role in plant stress response through antioxidant activity (Jaleel *et al.*, 2009). Bergville soil grown plants showed an increased vanillic, 4-coumaric, ferulic, and hydrobenzoic acid concentration but decreased salicylic acid concentration. Increased phenolic acid concentration are usually associated with unfavorable soil conditions, weather,

radiation and limited water availability (2000). Ghasemzadeh *et al*. (2010) reported increases in antioxidant activity and phenolic acid concentrations (gallic, vanillic, ferulic, cinnamic, and salicylic acid) in *Zingiber officinale* Roscoe under high CO_2 levels. This corresponds with the findings of the present study since plant phenolic acid concentrations increased as a result of nutrient deficient soils (Ghasemzadeh *et al*., 2010). Salicylic acid appears to be the key phenolic acid that directly affects the growth of Bergville soil grown plants since other phenolic acid are known for their antioxidant activity (Rivas-San Vicente and Plasencia, 2011; Saxena *et al*., 2012). In addition, salicylic acid also plays a role in ion uptake and photosynthesis (Horváth *et al.*, 2007; Rivas-San Vicente and Plasencia, 2011).

Cytokinin concentrations also support the notion of resource management in Bergville soil grown plants since these plants showed increased concentrations of *Cis*-Zeatin(s) (cZ), *O*-glucosides (~OG) and riboside *O*-glucosides (~ROG). *C*Z concentrations are known to increase in response to resource reallocation when plant growth is decreased or stopped to prevent senescence (Gajdošová *et al*., 2011). Furthermore, OG and ROG are viewed as storage molecules as they are shut down during unfavorable conditions and activated under favorable conditions (Mok *et al*., 1992). However, in Bergville soils grown plants DHZ, iP, tZ7G, and kinetin were not detected. Contrary to OGs and ROGs, DHZ and iP are some of the more active compounds during plant growth phase (McGaw, 1987). While kinetin promotes cell division, hence it was non-detection, this could be indicative of decreased plant growth in these plants (Mukherjee and Kumar, 2007).

Izingolweni soils had adequate P and N amounts but decreased K concentration. K plays an essential role in plant growth and development (Prajapati and Modi, 2012). Plants with decreased K concentrations, photosynthates, nutrients and H_2O movement are negatively affected (Prajapati and Modi, 2012). Also, K acts as a co-factor in enzyme activation, therefore, limited K affects the rate of photosynthesis and production of proteins and energy (Gajdanowicz *et al*., 2011). Koksal *et al*. (1988) reported that K deficiency resulted in increased abscisic acid (ABA) concentration, causing inhibition to shoot growth. This means Izingolweni soil grown plants would have decreased above-ground biomass or shoot length, nutrient concentrations and growth kinetics. However, the impact of K deficiency may have been reduced in these plants by establishment of symbiosis with endophytic microorganisms. Different species from the genera *Bacillus, Pseudomonas/Cupriavidus, Paenibacillus* and *Burkholderia* possess K solubilizing capabilities

which help by increasing exchangeable K in soils for plant uptake (Sheng *et al.*, 2008; Rajawat *et al*., 2012; Syed and Patel, 2014). Sequence analysis of the 16S rRNA gene revealed that *P. sativum* (L.) formed a symbiotic relationship with multiple genera of root nodule inhabiting bacteria, some of which are efficient N_2-fixers.

Members of *Paenibacillus* like P. *polymyxa* have been previously reported to be efficient N_2-fixers (free-living and/or symbiotic) and increase plant-available P through solubilization and mineralization (Wang *et al*., 2012; Puri *et al*., 2016; Villadas *et al*., 2019). Also, members of *Ralstonia* (*Ralstonia taiwanensis*) and *Cohnella* have been reported to fix N_2 in *Mimosa* roots (Chen *et al*., 2003; Wang *et al*., 2015). While members of *Bacillus* (including *B. cereus* and *B. thuringiensis*) promote root growth and nodulation and N_2 fixation in co-inoculation experiments (John Bullied *et al*., 2002; Mishra *et al*., 2009). These may have resulted in Izingolweni soil grown plants increased reliance in atmosphere derived N. Changes in phenolic acid and cytokinin concentrations could be a response to reactive oxygen species caused by the soil condition (endogenous production) or released exogenously by rhizobacteria (Ghasemzadeh *et al*., 2010).

Compared to Hluhluwe soil grown plants, Ashburton soil grown plants had decreased plant biomass, N absorption rate, salicylic acid and active CK concentration (iP, DHZ, Z, and their ribosides). The biomass could be attributed to decreased phytohormone and phytochemical concentrations (Thiruvengadam *et al*., 2016). The inverse might be true for Hluhluwe soil grown plants which had higher concentrations of molecules like kinetin, salicylic acid, and other active CKs. Ashburton plants were reliant on both soil N and atmosphere N during growth but the plants did not develop nodules. This suggests the possibility of Ashburton soils containing free-living N_2-fixing bacteria reducing atmosphere N for plant uptake. Both Hluhluwe and Ashburton soils showed relative soil acidity but Hluhluwe soils had lower P concentration, however Hluhluwe soil grown plants showed a significantly increased P concentration compared to Izingolweni and Bergville soil grown plants. In all the four treatments there is the possibility that P-solubilizing microorganisms, especially microbial fungi like *Aspergillus, Fusarium, Trichoderma*, AM fungi could have assisted in P acquisition (Sharma *et al*., 2013) as fungal spore enumeration conducted in this study does not reflect viability.

The varying concentration of cytokinins and phenolic acids in different plant parts might indicate that these compounds have different roles in process regulation and as signaling molecules that

elicit localized and/or systemic responses. The presence of the cytokinin tZ7G in three of the treatments maybe an example of this. Since tZ7G is regarded as an irreversible inactivation product, its presence could induce a homeostatic response (i.e. to initiate a different phase like flowering) by influencing the transcriptome (Schäfer *et al*., 2015; Raines *et al*., 2016).

4.5. Conclusion

Pisum sativum (L.) was able to grow under nutrient-poor and acidic grassland and savanna soils by maintaining their growth rates and relying on both soil and atmospheric N-source. Nutrient deficient soils induced changes in phenolic acids and cytokinin concentrations to enable the persistence of *P. sativum* (L.) to grassland and savanna ecosystem soils. Therefore, *Pisum sativum* (L.) may have a potential to be used as a forage and biofertilizer in sustainable agricultural practices in these nutrient poor ecosystems.

4.6. Acknowledgements

The authors would like to thank the National Research Foundation, South Africa for funding this work (NRF grant no. UID 113576). We also appreciate the financial support from the Czech Science Foundation (no. 17-06613S) and ERDF project "Development of pre-applied research in nanotechnology and biotechnology" (No. CZ.02.1.01/0.0/0.0/17_048/0007323). We acknowledge the Central Analytical Facilities at Stellenbosch University and the Archeometry Department at the University of Cape Town for their research facilities.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

4.7. References

1. Buresh, R.J., Smithson, P.C. and Hellums, D.T., 1997. Building soil phosphorus capital in Africa (No. replenishingsoi, pp. 111-149). *Soil Science Society of America and American Society of Agronomy*.
2. Chapin, F.S., 1991. Integrated responses of plants to stress. *BioScience*, 41(1), pp.29-36.

3. Chen, W.M., James, E.K., Prescott, A.R., Kierans, M. and Sprent, J.I., 2003. Nodulation of *Mimosa spp*. by the β-proteobacterium *Ralstonia taiwanensis*. *Molecular Plant-Microbe Interactions*, 16(12), pp.1051-1061.
4. Croteau, R., Kutchan, T.M. and Lewis, N.G., 2000. Natural products (secondary metabolites). *Biochemistry and Molecular Biology of Plants*, 24, pp.1250-1319.
5. Doran, J.W. and Zeiss, M.R., 2000. Soil health and sustainability: managing the biotic component of soil quality. *Applied Soil Ecology*, 15(1), pp.3-11.
6. Fey, M.V., 2010, August. A short guide to the soils of South Africa, their distribution and correlation with World Reference Base soil groups. *In Proceedings*, 19, pp. 32-35.
7. Franco, J.A., Bañón, S., Vicente, M.J., Miralles, J. and Martínez-Sánchez, J.J., 2011. Root development in horticultural plants grown under abiotic stress conditions–a review. *The Journal of Horticultural Science and Biotechnology*, 86(6), pp.543-556.
8. Gajdanowicz, P., Michard, E., Sandmann, M., Rocha, M., Corrêa, L.G.G., Ramírez-Aguilar, S.J., Gomez-Porras, J.L., González, W., Thibaud, J.B., Van Dongen, J.T. and Dreyer, I., 2011. Potassium (K+) gradients serve as a mobile energy source in plant vascular tissues. *Proceedings of the National Academy of Sciences*, 108(2), pp.864-869.
9. Gajdošová, S., Spíchal, L., Kamínek, M., Hoyerová, K., Novák, O., Dobrev, P.I., Galuszka, P., Klíma, P., Gaudinová, A., Žižková, E. and Hanuš, J., 2011. Distribution, biological activities, metabolism, and the conceivable function of cis-zeatin-type cytokinins in plants. *Journal of Experimental Botany*, 62(8), pp.2827-2840.
10. Ghasemzadeh, A., Jaafar, H.Z. and Rahmat, A., 2010. Elevated carbon dioxide increases contents of flavonoids and phenolic compounds, and antioxidant activities in Malaysian young ginger (Zingiber officinale Roscoe.) varieties. *Molecules*, 15(11), pp.7907-7922.
11. Ghasemzadeh, A., Jaafar, H.Z., Rahmat, A., Wahab, P.E.M. and Halim, M.R.A., 2010. Effect of different light intensities on total phenolics and flavonoids synthesis and anti-oxidant activities in young ginger varieties (Zingiber officinale Roscoe). *International Journal of Molecular Sciences*, 11(10), pp.3885-3897.
12. Giesler, R., Petersson, T. and Högberg, P., 2002. Phosphorus limitation in boreal forests: effects of aluminum and iron accumulation in the humus layer. *Ecosystems*, 5(3), pp.300-314.

13. Gruz, J., Novák, O. and Strnad, M., 2008. Rapid analysis of phenolic acids in beverages by UPLC–MS/MS. *Food Chemistry*, 111(3), pp.789-794.
14. Gryndler, M., Leština, J., Moravec, V., Přikryl, Z. and Lipavský, J., 1990. Colonization of maize roots by VAM-fungi under conditions of long-term fertilization of varying intensity. *Agriculture, Ecosystems & Environment*, 29(1-4), pp.183-186.
15. Harper, K.T. and Pendleton, R.L., 1993. Cyanobacteria and cyanolichens: can they enhance availability of essential minerals for higher plants?. *The Great Basin Naturalist*, pp.59-72.
16. Haumaier, L. and Zech, W., 1995. Black carbon—possible source of highly aromatic components of soil humic acids. *Organic Geochemistry*, 23(3), pp.191-196.
17. Horváth, E., Szalai, G. and Janda, T., 2007. Induction of abiotic stress tolerance by salicylic acid signaling. *Journal of Plant Growth Regulation*, 26(3), pp.290-300.
18. Huang, B. and Fry, J.D., 1998. Root anatomical, physiological, and morphological responses to drought stress for tall fescue cultivars. *Crop Science*, 38(4), pp.1017-1022.
19. Husson, O., 2013. Redox potential (Eh) and pH as drivers of soil/plant/microorganism systems: a transdisciplinary overview pointing to integrative opportunities for agronomy. *Plant and Soil*, 362(1-2), pp.389-417.
20. Jaleel, C.A., Riadh, K., Gopi, R., Manivannan, P., Ines, J., Al-Juburi, H.J., Chang-Xing, Z., Hong-Bo, S. and Panneerselvam, R., 2009. Antioxidant defense responses: physiological plasticity in higher plants under abiotic constraints. *Acta Physiologiae Plantarum*, 31(3), pp.427-436.
21. Doso Jnr, S., 2014. Land degradation and agriculture in the Sahel of Africa: causes, impacts and recommendations. *Journal of Agricultural Science and Applications*, 3(03), pp.67-73.
22. John Bullied, W., Buss, T.J. and Kevin Vessey, J., 2002. *Bacillus cereus* UW85 inoculation effects on growth, nodulation, and N accumulation in grain legumes: field studies. *Canadian Journal of Plant Science*, 82(2), pp.291-298.
23. Kiba, T., Kudo, T., Kojima, M. and Sakakibara, H., 2010. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. *Journal of Experimental Botany*, 62(4), pp.1399-1409.
24. Koksal, G., Gurkan, E. and Kucukcezzar, R., 1988. Relationship of potassium deficiency and abscisic acid metabolism in Soyabean plants. *Journal of Plant Nutrition*, 11(5), pp.517-523.

25. Kunwar, V.S., Lamichhane, J. and Gauchan, D.P., 2018. Strategies to Improve Phosphorus Availability in A Sustainable Agricultural System. *International Journal of Innovative Science and Research Technology*, 3(9), pp.323-331.
26. Lal, R., 2008. Soils and sustainable agriculture. A review. *Agronomy for Sustainable Development*, 28(1), pp.57-64.
27. Lambers, H., Raven, J.A., Shaver, G.R. and Smith, S.E., 2008. Plant nutrient-acquisition strategies change with soil age. *Trends in Ecology & Evolution*, 23(2), pp.95-103.
28. Lema, M.A. and Majule, A.E., 2009. Impacts of climate change, variability and adaptation strategies on agriculture in semi-arid areas of Tanzania: The case of Manyoni District in Singida Region, Tanzania. *African Journal of Environmental Science and Technology*, 3(8), pp.206-218.
29. Maisels, F., Gautier-Hion, A. and Gautier, J.P., 1994. Diets of two sympatric colobines in Zaire: more evidence on seed-eating in forests on poor soils. *International Journal of Primatology*, 15(5), pp.681.
30. Mandiringana, O.T., Mnkeni, P.N.S., Mkile, Z., Van Averbeke, W., Van Ranst, E. and Verplancke, H., 2005. Mineralogy and fertility status of selected soils of the Eastern Cape Province, South Africa. *Communications in Soil Science and Plant Analysis*, 36(17-18), pp.2431-2446.
31. Marschner, P., 2012. Rhizosphere biology. *In Marschner's Mineral Nutrition of Higher Plants* (Third Edition) (pp. 369-388).
32. McCallum, M.H., Peoples, M.B. and Connor, D.J., 2000. Contributions of nitrogen by field pea (Pisum sativum L.) in a continuous cropping sequence compared with a lucerne (Medicago sativa L.)-based pasture ley in the Victorian Wimmera. Australian *Journal of Agricultural Research*, 51(1), pp.13-22.
33. McGaw, B.A., 1987. Cytokinin biosynthesis and metabolism. *In Plant hormones and their role in plant growth and development* (pp. 76-93). *Springer*, Dordrecht.
34. Mishra, P.K., Mishra, S., Selvakumar, G., Kundu, S. and Shankar Gupta, H., 2009. Enhanced soybean (*Glycine max* L.) plant growth and nodulation by Bradyrhizobium japonicum-SB1 in presence of *Bacillus thuringiensis*-KR1. *Acta Agriculturae Scandinavica Section B–Soil and Plant Science*, 59(2), pp.189-196.

35. Mok, M.C., Martin, R.C., Mok, D.W.S. and Shaw, G., 1992. Cytokinin activity, metabolism and function in Phaseolus. *Physiology and biochemistry of cytokinins in plants. The Hague: SPB Academic Publishers*, pp.41-46.
36. Mukherjee, D. and Kumar, R., 2007. Kinetin regulates plant growth and biochemical changes during maturation and senescence of leaves, flowers, and pods of *Cajanus cajan* L. *Biologia plantarum*, 51(1), pp.80-85.
37. Nambiar, K.K.M., Gupta, A.P., Fu, Q. and Li, S., 2001. Biophysical, chemical and socio-economic indicators for assessing agricultural sustainability in the Chinese coastal zone. *Agriculture, Ecosystems & Environment*, 87(2), pp.209-214.
38. Nannipieri, P., Ascher, J., Ceccherini, M., Landi, L., Pietramellara, G. and Renella, G., 2003. Microbial diversity and soil functions. *European Journal of Soil Science*, 54(4), pp.655-670.
39. Novák, O., Hauserová, E., Amakorová, P., Doležal, K. and Strnad, M., 2008. Cytokinin profiling in plant tissues using ultra-performance liquid chromatography–electrospray tandem mass spectrometry. *Phytochemistry*, 69(11), pp.2214-2224.
40. Novák, O., Tarkowski, P., Tarkowská, D., Doležal, K., Lenobel, R. and Strnad, M., 2003. Quantitative analysis of cytokinins in plants by liquid chromatography–single-quadrupole mass spectrometry. *Analytica Chimica Acta*, 480(2), pp.207-218.
41. Ohyama, T., 2017. The Role of Legume-Rhizobium Symbiosis in Sustainable Agriculture. *In Legume Nitrogen Fixation in Soils with Low Phosphorus Availability* (pp. 1-20). *Springer*, Cham.
42. Onwuka, M.I., Ozurumba, U.V. and Nkwocha, O.S., 2016. Changes in Soil pH and Exchangeable Acidity of Selected Parent Materials as Influenced by Amendments in South East of Nigeria. *Journal of Geoscience and Environment Protection*, 4(05), pp.80.
43. Prajapati, K. and Modi, H.A., 2012. The importance of potassium in plant growth–a review. *Indian Journal of Plant Sciences*, 1(2), pp.177-186.
44. Puri, A., Padda, K.P. and Chanway, C.P., 2016. Evidence of nitrogen fixation and growth promotion in canola (*Brassica napus* L.) by an endophytic diazotroph *Paenibacillus polymyxa* P2b-2R. *Biology and Fertility of Soils*, 52(1), pp.119-125.

45. Raines, T., Blakley, I.C., Tsai, Y.C., Worthen, J.M., Franco-Zorrilla, J.M., Solano, R., Schaller, G.E., Loraine, A.E. and Kieber, J.J., 2016. Characterization of the cytokinin-responsive transcriptome in rice. *BMC Plant Biology*, 16(1), pp.260.
46. Rajawat, M.V.S., Singh, S., Singh, G. and Saxena, A.K., 2012. Isolation and characterization of K-solubilizing bacteria isolated from different rhizospheric soil. *In Proceeding of 53rd Annual Conference of Association of Microbiologists of India* (Vol. 124).
47. Rengel, Z. and Marschner, P., 2005. Nutrient availability and management in the rhizosphere: exploiting genotypic differences. *New Phytologist*, 168(2), pp.305-312.
48. Rivas-San Vicente, M. and Plasencia, J., 2011. Salicylic acid beyond defence: its role in plant growth and development. *Journal of Experimental Botany*, 62(10), pp.3321-3338.
49. Robert, C.A., Ferrieri, R.A., Schirmer, S., Babst, B.A., Schueller, M.J., Machado, R.A., Arce, C.C., Hibbard, B.E., Gershenzon, J., Turlings, T.C. and Erb, M., 2014. Induced carbon reallocation and compensatory growth as root herbivore tolerance mechanisms. *Plant, Cell & Environment*, 37(11), pp.2613-2622.
50. Roberts, V.G., Adey, S. and Manson, A.D., 2003. An investigation into soil fertility in two resource-poor farming communities in KwaZulu-Natal (South Africa). *South African Journal of Plant and Soil*, 20(3), pp.146-151.
51. Samuelson, M.E., Eliasson, L. and Larsson, C.M., 1992. Nitrate-regulated growth and cytokinin responses in seminal roots of barley. *Plant Physiology*, 98(1), pp.309-315.
52. Sanz-Saez, A., Morales, F., Arrese-Igor, C. and Aranjuelo, I., 2017. P Deficiency: A Major Limiting Factor for Rhizobial Symbiosis. *In Legume Nitrogen Fixation in Soils with Low Phosphorus Availability* (pp. 21-39). *Springer*, Cham.
53. Saxena, M., Saxena, J. and Pradhan, A., 2012. Flavonoids and phenolic acids as antioxidants in plants and human health. *International Journal of Pharmaceutical Sciences and Research*, 16(2), pp.130-134.
54. Schäfer, M., Meza-Canales, I.D., Navarro-Quezada, A., Brütting, C., Vanková, R., Baldwin, I.T. and Meldau, S., 2015. Cytokinin levels and signaling respond to wounding and the perception of herbivore elicitors in *Nicotiana attenuata*. *Journal of Integrative Plant Biology*, 57(2), pp.198-212.

55. Sharma, S.B., Sayyed, R.Z., Trivedi, M.H. and Gobi, T.A., 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*, 2(1), pp.587.
56. Sheng, X.F., Zhao, F., He, L.Y., Qiu, G. and Chen, L., 2008. Isolation and characterization of silicate mineral-solubilizing *Bacillus globisporus* Q12 from the surfaces of weathered feldspar. *Canadian Journal of Microbiology*, 54(12), pp.1064-1068.
57. Siciliano, S.D., Palmer, A.S., Winsley, T., Lamb, E., Bissett, A., Brown, M.V., van Dorst, J., Ji, M., Ferrari, B.C., Grogan, P. and Chu, H., 2014. Soil fertility is associated with fungal and bacterial richness, whereas pH is associated with community composition in polar soil microbial communities. *Soil Biology and Biochemistry*, 78, pp.10-20.
58. Stiles, W.C., 2004. Soil analysis and interpretation. *New York Fruit Quarterly*, 12(1), pp.28-30.
59. Syed, B.A. and Patel, B., 2014. Investigation and Correlation of Soil Biotic and Abiotic Factors Affecting Agricultural Productivity in Semi-Arid Regions of North Gujarat, India. *International Journal of Research Studies in Biosciences*, 2, pp.18-29.
60. Thiruvengadam, M., Baskar, V., Kim, S.H. and Chung, I.M., 2016. Effects of abscisic acid, jasmonic acid and salicylic acid on the content of phytochemicals and their gene expression profiles and biological activity in turnip (*Brassica rapa ssp. rapa*). *Plant Growth Regulation*, 80(3), pp.377-390.
61. Villadas, P.J., Díaz-Díaz, S., Rodríguez-Rodríguez, A., Arco-Aguilar, M.D., Fernández-González, A.J., Pérez-Yépez, J., Arbelo, C., González-Mancebo, J.M., Fernández-López, M. and León-Barrios, M., 2019. The Soil Microbiome of the Laurel Forest in Garajonay National Park (La Gomera, Canary Islands): Comparing Unburned and Burned Habitats after a Wildfire. *Forests*, 10(12), pp.1051.
62. Voroney, R.P., 2007. The soil habitat. *In Soil Microbiology, Ecology and Biochemistry* (pp. 25-49). *Academic Press*.
63. Wang, L.Y., Wang, T.S. and Chen, S.F., 2015. Cohnella capsici sp. nov., a novel nitrogen-fixing species isolated from *Capsicum annuum* rhizosphere soil, and emended description of *Cohnella plantaginis*. *Antonie van Leeuwenhoek*, 107(1), pp.133-139.
64. Wang, Y., Shi, Y., Li, B., Shan, C., Ibrahim, M., Jabeen, A., Xie, G. and Sun, G., 2012. Phosphate solubilization of Paenibacillus polymyxa and Paenibacillus macerans from

mycorrhizal and non-*mycorrhizal* cucumber plants. *African Journal of Microbiology Research*, 6, pp.4567-4573.

65. Williams, J., Bordas, V. and Gascoigne, H., 2004. Conserving land and water for society: global challenges and perspectives. *Conserving soil and water for society*: *Sharing solutions 13th ISCO*, pp.1-16.
66. Zhalnina, K., Dias, R., de Quadros, P.D., Davis-Richardson, A., Camargo, F.A., Clark, I.M., McGrath, S.P., Hirsch, P.R. and Triplett, E.W., 2015. Soil pH determines microbial diversity and composition in the park grass experiment. *Microbial Ecology*, 69(2), pp.395-406.
67. Rutigliano, F.A., D'Ascoli, R. and De Santo, A.V., 2004. Soil microbial metabolism and nutrient status in a Mediterranean area as affected by plant cover. *Soil Biology and Biochemistry*, 36(11), pp.1719-1729.

Table 4.1. KwaZulu-Natal soil geochemistry and microflora data.

Parameter	Site			
	Hluhluwe	**Izingolweni**	**Bergville**	**Ashburton**
Soil macronutrients (µmol)				
Phosphate (P)	0.032 ± 0.00^{a}	0.084 ± 0.02^{bc}	0.11 ± 0.01^{bd}	0.065 ± 0.00^{b}
Nitrogen (N)	0.18 ± 0.01^{c}	0.13 ± 0.01^{b}	0.14 ± 0.01^{b}	0.093 ± 0.00^{a}
Potassium (K)	10.07 ± 0.41^{d}	1.20 ± 0.00^{a}	4.63 ± 0.03^{b}	6.49 ± 0.05^{c}
Relative acidity (cmol/L)				
pH	4.96 ± 0.00^{c}	4.63 ± 0.00^{b}	4.06 ± 0.00^{a}	5.07 ± 0.02^{d}
Exchange acidity	0.074 ± 0.00^{a}	0.060 ± 0.00^{a}	1.50 ± 0.04^{b}	0.056 ± 0.00^{a}
Total cations	21.01 ± 0.18^{d}	11.37 ± 0.10^{c}	4.18 ± 0.08^{a}	6.74 ± 0.07^{b}
Microflora				
Fungal spore count (no./100 g soil)	5.20 ± 1.07^{a}	11.60 ± 2.68^{b}	15.80 ± 1.80^{b}	13.40 ± 1.40^{b}
Bacteria CFU/ml	2.04×10^{5}	1.57×10^{4}	5.88×10^{4}	3.31×10^{5}

Values are means ± SE, n = 3. In each row, values with the same letter do not differ significantly ($p \leq 0.05$)

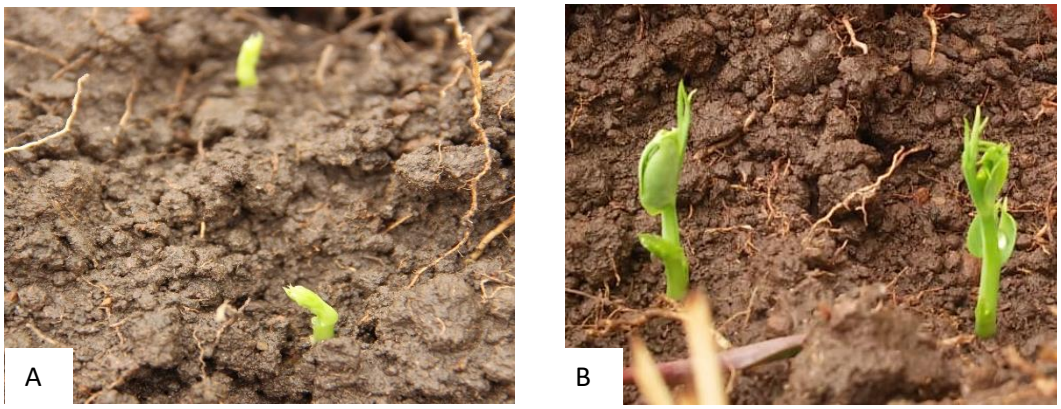


Figure 4.1. Forage pea (*Pisum sativum* L.) seedlings after (A) four and (B) seven days of growth in Ashburton soil.

Table 4.2. Relative seedling emergence comparison between soil sites.

Soil collection site	Seedling emergence (%)	
	Day 4	**Day 7**
Ashburton	91	100
Bergville	86	91
Hluhluwe	91	100
Izingolweni	82	95

Seedling emergence refers to the initial seedling visualization above soil surface. Where n = 32 per soil collection site.

Table 4.3. Growth kinetic of *Pisum sativum* L. grown in KwaZulu-Natal soils.

Parameter	Treatment			
	Hluhluwe	**Izingolweni**	**Bergville**	**Ashburton**
Biomass data				
Leaves (g)	0.35 ± 0.028^{a}	0.20 ± 0.047^{b}	0.06 ± 0.003^{c}	0.22 ± 0.041^{b}
Shoot (g)	0.63 ± 0.094^{a}	0.31 ± 0.066^{bc}	0.10 ± 0.023^{c}	0.52 ± 0.087^{ab}
Roots (g)	0.36 ± 0.048^{a}	0.40 ± 0.018^{a}	0.47 ± 0.088^{a}	0.43 ± 0.006^{a}
Total plant (g)	1.34 ± 0.064^{a}	0.92 ± 0.011^{b}	0.63 ± 0.080^{c}	1.16 ± 0.068^{ab}
Growth kinetics				
Growth rate (g/day)	0.024 ± 0.0012^{a}	0.017 ± 0.0020^{a}	0.011 ± 0.0015^{a}	0.021 ± 0.0012^{a}
Root: Shoot ratio	0.62 ± 0.15^{a}	1.39 ± 0.26^{a}	5.52 ± 2.24^{b}	0.88 ± 0.15^{a}
SNAR (mg N g^{-1} root DW day^{-1})	8.03 ± 0.36^{d}	3.80 ± 0.53^{b}	2.02 ± 0.24^{a}	7.05 ± 0.28^{c}
SNUR (mg N g^{-1} DW day^{-1})	3.75 ± 0.14^{b}	4.46 ± 0.42^{c}	4.38 ± 0.35^{c}	3.08 ± 0.10^{a}
SPUR (mg P g^{-1} plant DW day^{-1})	0.011 ± 0.00^{a}	0.011 ± 0.00^{a}	0.010 ± 0.00^{a}	0.009 ± 0.00^{a}
Carbon costs (mmol C g^{-1} DW)	0.0069 ± 0.00^{a}	0.0070 ± 0.00^{a}	0.0069 ± 0.00^{a}	0.0066 ± 0.00^{a}
N nutrition				
Total plant nitrogen (mmol N g^{-1})	2.14 ± 0.093^{c}	1.12 ± 0.15^{b}	0.70 ± 0.080^{a}	2.23 ± 0.087^{c}
Standard corrected $^{15}N/^{14}N$	4.00 ± 0.20^{c}	1.17 ± 0.31^{a}	2.17 ± 0.43^{b}	1.33 ± 0.13^{a}
%NDFA	4.43 ± 2.86^{a}	54.49 ± 4.43^{d}	31.00 ± 3.24^{b}	43.21 ± 1.94^{c}
Atmospheric derived N (mmol N g^{-1})	0.073 ± 0.046^{a}	0.70 ± 0.15^{c}	0.32 ± 0.015^{b}	0.83 ± 0.059^{c}
Soil derived N (mmol N g^{-1})	1.52 ± 0.073^{d}	0.52 ± 0.022^{a}	0.78 ± 0.13^{b}	1.09 ± 0.041^{c}
P nutrition				
Total plant phosphate (µmol P g^{-1})	56.08 ± 1.28^{c}	32.53 ± 2.15^{b}	23.52 ± 1.61^{a}	80.19 ± 8.47^{d}

Values are means ± SE, n = 3. In each row, values with the same letter do not differ significantly ($p \leq 0.05$)

Table 4.4. Identities of root nodule inhabiting microorganisms.

Site	Possible match	Identity (%)	Accession number
Izingolweni	*Paenibacillus polymyxa* BEb-40	95.78	AB863035.1
	Paenibacillus sp. Bs57	95.69	GU328694.1
	Paenibacillus jamilae IIF5SW-B4	95.68	KY218874.1
	Cupriavidus sp. Strain NGha2	91.46	MN197795.1
	Cupriavidus necator strain H16	91.46	CP039287.7
	Cupriavidus oxalaticus strain X32	91.46	CP038634.1
	Cupriavidus metallidurans I115	91.46	MK216985.1
	Ralstonia sp. AMF 1009	90.42	JQ316396.1
	Saccharibacillus sp. ATSA2	100	CP041217.1
	Paenibacillus polymyxa ZF197	100	MK961276.1
	Paenibacillus favisporus JF2-8	100	MK016488.1
	Paenibacillus endophyticus JBRI-MO-0041	100	MK302256.1
	Cohnella plantaginis JBRI-MO-0012	100	MK302227.1
	Bacillus cereus strain I7	93.01	MH892088.1
	Bacillus sp. KK-35	93.01	KU352795.1
	Bacillus thuringiensis strain BDzG	93.01	MN203618.1
	Bacillus sp. 69G6-C	93.01	JQ388720.1

Table 4.5. Concentration of phenolic acids (µg/g) in *Pisum sativum* L. grown in KwaZulu-Natal soils.

Compound	Part	Site			
		Hluhluwe	**Izingolweni**	**Bergville**	**Ashburton**
Protocatechuic acid:	Roots	0.97 ± 0.49^{a}	0.68 ± 0.22^{a}	1.20 ± 0.39^{a}	0.66 ± 0.12^{a}
	Shoot	1.85 ± 0.22^{a}	1.88 ± 0.029^{a}	1.79 ± 0.22^{a}	1.53 ± 0.10^{a}
4-hydroxybenzoic acid:	Roots	14.92 ± 6.78^{a}	18.28 ± 7.93^{a}	13.56 ± 1.44^{a}	10.11 ± 4.99^{a}
	Shoot	19.05 ± 3.20^{a}	27.93 ± 0.61^{a}	24.46 ± 10.14^{a}	10.23 ± 1.57^{a}
Caffeic acid:	Roots	0.73 ± 0.32^{a}	0.41 ± 0.24^{a}	0.19 ± 0.043^{a}	0.35 ± 0.19^{a}
	Shoot	1.14 ± 0.065^{a}	2.01 ± 0.23^{b}	1.01 ± 0.21^{a}	0.49 ± 0.089^{a}
Vanillic acid:	Roots	1.42 ± 0.40^{a}	1.18 ± 0.50^{a}	4.27 ± 1.38^{a}	1.89 ± 0.38^{a}
	Shoot	1.18 ± 0.17^{ab}	4.39 ± 0.24^{c}	2.61 ± 0.82^{bc}	1.33 ± 0.23^{ab}
Syringic acid:	Roots	0.61 ± 0.46^{a}	0.28 ± 0.11^{a}	0.27 ± 0.056^{a}	0.34 ± 0.041^{a}
	Shoot	0.77 ± 0.075^{a}	1.04 ± 0.10^{a}	1.31 ± 0.71^{a}	0.36 ± 0.040^{a}
4-coumaric acid:	Roots	13.57 ± 1.18^{a}	25.56 ± 11.82^{a}	14.23 ± 2.17^{a}	21.89 ± 3.54^{a}
	Shoot	87.79 ± 15.41^{a}	85.80 ± 2.64^{a}	105.47 ± 38.40^{a}	43.01 ± 4.73^{a}
Sinapic acid:	Roots	0.19 ± 0.042^{a}	0.25 ± 0.18^{a}	0.11 ± 0.00^{a}	0.26 ± 0.060^{a}
	Shoot	18.52 ± 1.37^{b}	10.13 ± 1.62^{a}	8.77 ± 1.53^{a}	5.42 ± 0.50^{a}
Ferulic acid:	Roots	6.03 ± 3.02^{a}	13.18 ± 11.42^{a}	3.05 ± 0.48^{a}	7.50 ± 1.60^{a}
	Shoot	26.69 ± 6.13^{a}	46.66 ± 1.45^{a}	35.15 ± 10.81^{a}	19.43 ± 2.70^{a}
Salicylic acid:	Roots	1.82 ± 0.71^{a}	3.90 ± 1.95^{a}	0.83 ± 0.12^{a}	0.92 ± 0.36^{a}
	Shoot	0.29 ± 0.060^{a}	0.34 ± 0.039^{a}	0.36 ± 0.13^{a}	0.31 ± 0.032^{a}

Values are means ± SE, n = 9. Rows with the same letter do not differ significantly ($p \leq 0.05$).

Table 4.6. Concentration of cytokinis (pmol/g) in *Pisum sativum* L. grown in KwaZulu-Natal soils.

Cytokinin metabolite	Part	Site			
		Hluhluwe	**Izingolweni**	**Bergville**	**Ashburton**
trans-Zeatin (tZ)	Roots	0.85 ± 0.85[a]	0.61 ± 0.61[a]	< LOD	1.69 ± 0.87[a]
	Shoot	0.47 ± 0.47[a]	< LOD	0.37 ± 0.37[a]	0.98 ± 0.02[a]
trans-Zeatin riboside (tZR)	Roots	1.38 ± 1.38[a]	2.90 ± 2.90[a]	< LOD	< LOD
	Shoot	2.00 ± 0.55[a]	2.05 ± 0.05[a]	4.92 ± 1.09[b]	1.21 ± 0.61[a]
trans-Zeatin riboside *O*-glucoside (tZROG)	Roots	< LOD	1.80 ± 1.80[a]	< LOD	< LOD
	Shoot	< LOD	< LOD	1.02 ± 1.02[a]	< LOD
trans-Zeatin 7-glucoside (tZ7G)	Roots	2.09 ± 1.09[ab]	0.96 ± 0.68[ab]	< LOD	5.76 ± 3.22[b]
	Shoot	0.78 ± 0.78[a]	< LOD	< LOD	1.59 ± 1.59[a]
cis-Zeatin (cZ)	Roots	4.48 ± 2.25[a]	8.67 ± 5.65[a]	3.88 ± 0.11[a]	4.72 ± 0.74[a]
	Shoot	9.34 ± 1.43[a]	3.50 ± 0.40[a]	13.08± 7.23[a]	4.52 ± 1.10[a]
cis-Zeatin *O*-glucoside (cZOG)	Roots	3.46 ± 1.18[a]	1.54 ± 0.80[a]	12.17± 2.24[b]	3.09 ± 0.63[a]
	Shoot	3.33 ± 0.40[b]	1.97 ± 0.99[ab]	5.91 ± 0.80[c]	< LOD
cis-Zeatin riboside (cZR)	Roots	79.21±52.56[a]	26.61±21.84[a]	27.63±8.04[a]	107.53±47.09[a]
	Shoot	210.01±22.69[ab]	200.03±2.50[ab]	177.15±15.76[a]	269.20±8.15[b]
cis-Zeatin riboside *O*-glucoside (cZROG)	Roots	14.24 ± 9.43[a]	5.29 ± 5.29[a]	9.89 ± 4.60[a]	17.48 ± 6.22[a]
	Shoot	38.51 ± 9.62[b]	7.76 ± 0.46[a]	15.23 ± 0.60[a]	15.62 ± 1.93[a]
Dihydrozeatin (DHZ)	Roots	17.96 ± 3.98[b]	< LOD	< LOD	< LOD
	Shoot	< LOD	6.82 ± 0.49[b]	< LOD	< LOD
Dihydrozeatin *O*-glucoside (DHZOG)	Roots	< LOD	< LOD	< LOD	< LOD
	Shoot	3.81 ± 0.20[a]	2.07 ± 0.04[a]	5.03 ± 1.93[a]	2.75 ± 0.29[a]
Dihydrozeatin riboside (DHZR)	Roots	2.91 ± 0.83[a]	3.02 ± 1.84[a]	1.88 ± 0.49[a]	1.93 ± 1.02[a]
	Shoot	5.04 ± 0.38[a]	9.05 ± 0.23[a]	10.99 ± 2.75[a]	5.14 ± 0.19[a]
Isopentenyladenine (iP)	Roots	0.79 ± 0.79[a]	< LOD	< LOD	1.91 ± 1.00[a]
	Shoot	7.22 ± 2.33[b]	< LOD	< LOD	3.60 ± 0.63[b]
Isopentenyladenine riboside (iPR)	Roots	11.75 ± 6.99[a]	10.3 ± 8.12[a]	4.44 ± 1.17[a]	19.06 ± 6.50[a]
	Shoot	271.88±15.59[c]	121.08±4.43[ab]	115.29±9.84[a]	173.98±4.23[b]
Kinetin (K)	Roots	7.88 ± 0.76[c]	7.26 ± 0.44[bc]	< LOD	5.71 ± 0.50[b]
	Shoot	8.51 ± 0.29[c]	5.64 ± 2.91[ab]	< LOD	6.20 ± 0.48[bc]

LOD: Below detection range or zero. Values are means ± SE, n = 9. Rows with the same letter do not differ significantly ($p \leq 0.05$).

Chapter 5

General discussion

5.1. General discussion

Nutrient availability and soil acidity are two of the major factors that influence plant establishment, growth, and functions in grassland and savanna ecosystems (Ledgard and Steele, 1992; Sarmiento *et al*., 2006). Potassium (K) is the only macronutrient that is infrequently reported as limited in these ecosystems (Han and Lee, 2005). However, nitrogen (N) and phosphorus (P) limitations are frequently reported as limited and this adversely affects ecosystem vegetation structures, crop production, quality and overall nutrition (Lima *et al.*, 2000; Augusto *et al*., 2013). The identification of leguminous plants and associated microbes as means to mitigate low soil N availability in soils has resulted in more research aimed at understanding the ecology and biochemistry used by these plants in nutrient-deficient ecosystems. However, nutrient deficiency (especially P) retards nodulation, biological N fixation (BNF), nutrient assimilation and metabolism and overall plant growth in legume plants (Plénet *et al*., 2000). Also, P deficiency negatively affects photosynthesis and ATP production leading to decreased plant growth (Uchida, 2000). Therefore, it is pertinent that more research is conducted on remediation procedures to improve and sustain nutrient fertility and plant establishment in these ecosystems by elucidating the role and importance of soil microflora and associated extracellular enzymes in soil nutrient cycling.

Chapter 3 investigated the potential of soil-borne microbes and extracellular enzymes in soil nutrient cycling in KZN grassland and savanna ecosystem soils to suit the establishment and growth of forage leguminous plants such as *Pisum sativum* (L.). Soil microflora plays a crucial role in terrestrial nutrient cycling through complex mechanisms like organic acid production and extracellular enzymes to solubilize sequestered soil nutrients (Schloter *et al*., 2003). In grassland and savanna nutrient-deficient ecosystem soils analyzed in the current study, we identified the presence of *Bacillus* and *Lysinibacillus* species. These bacterial species have been reported to solubilize cation-bound P, increasing soil available P for plant uptake in nutrient-poor soils (Lacava and Azevedo, 2013). Additionally, some *Bacillus* members are able to solubilize insoluble K and also fix atmospheric N (Raj, 2010; Ahmad *et al*., 2016). Potassium-solubilizing bacteria (KSB) are important when it comes to soil fertility because of their multifaceted mechanisms that promote plant growth. These mechanisms include (but not limited to) hormone production, root colonization, micronutrient solubilization, antibiotic production, chitinase and catalase activity

(Ahmad *et al*., 2016). Apart from *Bacillus*, other nutrient solubilizing bacteria include *Paenibacillus*, *Burkholderia*, and *Pseudomonas* (Sheng and Huang, 2002; Uroz *et al*., 2007; Sangeeth *et al*., 2017). However, the efficiency and method for nutrient solubilization vary between species (Ahmad *et al*., 2016).

In addition to beneficial growth-promoting and nutrient solubilizing bacteria, fungi may also facilitate plant growth more efficiently than bacteria since they transverse better and can form complex networks in soil (Sharma *et al.,* 2013). This is evident in the findings of this study as different fungal species were identified and quantified in experimental soils. Hluhluwe soils had low P concentration but showed the highest fungal species richness and this was associated with high phosphate solubilizing enzyme activities. Therefore, these nutrient solubilizing microbes and atmospheric N reducing bacteria may play an important role in KwaZulu-Natal (KZN) grassland and savanna nutrient-poor soil cycling. These soil-borne microbes (rhizobacteria and fungi) may also regulate the release of enzymes into the soils to cycling and mineralize soil nutrients. There are numerous soil microorganisms that contribute to the turnover of organic matter into plant-available nutrients (Richardson, 2001). Organic matter is the major source of soil organic P which usually exists as high molecular weight (MW) compounds that are typically resistant to chemical hydrolysis (Rodríguez and Fraga, 1999). This means the organic P compounds must be bio-converted into ionic phosphate or low MW organic P compounds by microbial phosphatases and/or phytases (Peix *et al*., 2001). This leads to increased P availability since the immobilized P undergoes a mineralization process, hence becoming readily available for plant absorption (Richardson and Simpson, 2011). Low soil P concentrations in KZN grassland and savanna ecosystems resulted in increased β-Phosphatase enzyme activities. Also increased activities of carbon (C) cycling, organic matter and lignin-degrading enzymes in these ecosystem soils were observed. Therefore, grassland and savanna ecosystem soil may support the establishment and growth of plants that define these ecosystems and forage legumes in sustainable agriculture practices. Given that these nutrient-poor and acidic ecosystem soils have varying diversity of microbes and soil enzyme activities that can solubilize and recycle nutrients.

In chapter 4 we investigated this assumption and the effects of KZN soils on the growth of *P. sativum* L. The results showed that *P. sativum* L. was adaptable to KZN grassland and savanna ecosystem soils by associating with beneficial soil microorganisms. *P. sativum* L. was able to

utilize both soil and atmospheric N, even in the absence of visible nodulation. The absence of nodulation can be attributed to the lack of a suitable *Rhizobial* strain or competition between the soil microbes since some can produce antimicrobial compounds (Wolpert and Albersheim, 1976; Vlassak *et al*., 1997). *Paenibacillus polymyxa* is one of the microbes that protect the plant host from pathogenic fungi and bacteria through antibiotic production (Storm *et al*., 1977; Jeong *et al*., 2019). However, some microbes could be complementary to one another and enhance their action (Jeong *et al*., 2019). For instance, a mycotoxin released by *Penicillium citrinum* induces swarming motility in *P. polymyxa* (Park *et al*., 2008). We assume that *P. sativum* L. was nodulated by *Cupriavidus* while the other bacteria like *Paenibacillus* and *Bacillus* acted as plant growth-promoting bacteria (Martínez-Hidalgo and Hirsch, 2017). The basis of this notion is that *Cupriavidus* is from the family *Burkholderiaceae* which has multiple members that induce nodulation in leguminous plants (Coenye, 2014). While *Paenibacillus* and *Bacillus* are unable to induce nodulation in species that they were previously identified as inhabitants of their root nodules (Velázquez *et al*., 2013; Lai *et al*., 2015). Furthermore, the plants were able to upregulate the production of antioxidants and storage molecules while downregulating growth-promoting compounds during stress conditions. In essence, this study has highlighted the beneficial role of soil microflora on plant growth and the synergy involved in plant physiology.

5.2. Future work

As more insights are gained on plant-microbe interactions, there is reason to believe that the knowledge, if applied appropriately, could contribute to sustainable agricultural and soil management systems. The present study delineates the role of soil microflora and extracellular enzymes in terrestrial nutrient cycling, mineralization, and their possible applications in nutrient-deficient grassland and savanna ecosystems. The persistence of forage pea employing the above-mentioned strategies in these nutrient stressed ecosystems suggests its potential use in improving soil fertility in nutrient stressed ecosystems and in sustainable agricultural practices. The simplicity, execution, and results obtained from this study can be translated into environmentally friendly low-input farming. However, more research is required to minimize the ambiguity, and the findings relayed to farmers in a more accessible format. The first step would be the inoculation of *P. sativum* L. with single bacterial strains of the isolated strains using selective media and exposing them to soil nutrient stress under glasshouse and/or field conditions. The plants could

then be subjected to transcriptomic and proteomic profile studies. This knowledge would provide insight into the efficiency of single bacterial strains in solubilizing limited soil nutrients and N_2 fixation. Additionally, the transcripts and proteins regulated enabling forage pea to cope in soil nutrient-poor ecosystems. These results will draw the full picture of how *P. sativum* L. is able to cope under nutrient-poor ecosystem soils.

5.3. References

1. Ahmad, M., Nadeem, S.M., Naveed, M. and Zahir, Z.A., 2016. Potassium-solubilizing bacteria and their application in agriculture. In *Potassium solubilizing microorganisms for sustainable agriculture* (pp. 293-313). *Springer*, New Delhi.
2. Augusto, L., Delerue, F., Gallet-Budynek, A. and Achat, D.L., 2013. Global assessment of limitation to symbiotic nitrogen fixation by phosphorus availability in terrestrial ecosystems using a meta-analysis approach. *Global Biogeochemical Cycles*, 27(3), pp.804-815.
3. Coenye, T., 2014. The family Burkholderiaceae. *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*, pp.759-776.
4. Han, H.S. and Lee, K.D., 2005. Phosphate and potassium solubilizing bacteria effect on mineral uptake, soil availability and growth of eggplant. *Research Journal of Agriculture and Biological Sciences*, 1(2), pp.176-180.
5. Jeong, H., Choi, S.K., Ryu, C.M. and Park, S.H., 2019. Chronicle of a Soil Bacterium: Paenibacillus polymyxa E681 as a Tiny Guardian of Plant and Human Health. *Frontiers in Microbiology*, 10.
6. Lacava, P.T. and Azevedo, J.L., 2013. Endophytic bacteria: a biotechnological potential in agrobiology system. In *Bacteria in Agrobiology*: Crop Productivity (pp. 1-44). *Springer*, Berlin, Heidelberg.
7. Lai, W.A., Hameed, A., Lin, S.Y., Hung, M.H., Hsu, Y.H., Liu, Y.C., Shahina, M., Shen, F.T. and Young, C.C., 2015. *Paenibacillus medicaginis* sp. nov. a chitinolytic endophyte isolated from a root nodule of alfalfa (*Medicago sativa* L.). *International Journal of Systematic and Evolutionary Microbiology*, 65(11), pp.3853-3860.

8. Ledgard, S.F. and Steele, K.W., 1992. Biological nitrogen fixation in mixed legume/grass pastures. *Plant and Soil*, 141(1-2), pp.137-153.
9. Lima, J.D., Da Matta, F.M. and Mosquim, P.R., 2000. Growth attributes, xylem sap composition, and photosynthesis in common bean as affected by nitrogen and phosphorus deficiency. *Journal of Plant Nutrition*, 23(7), pp.937-947.
10. Martínez-Hidalgo, P. and Hirsch, A.M., 2017. The nodule microbiome: N2-fixing rhizobia do not live alone. *Phytobiomes*, 1(2), pp.70-82.
11. Park, S.Y., Kim, R., Ryu, C.M., Choi, S.K., Lee, C.H., Kim, J.G. and Park, S.H., 2008. Citrinin, a mycotoxin from *Penicillium citrinum*, plays a role in inducing motility of *Paenibacillus polymyxa*. *FEMS Microbiology Ecology*, 65(2), pp.229-237.
12. Peix, A., Mateos, P.F., Rodriguez-Barrueco, C., Martinez-Molina, E. and Velazquez, E., 2001. Growth promotion of common bean (Phaseolus vulgaris L.) by a strain of Burkholderia cepacia under growth chamber conditions. *Soil Biology and Biochemistry*, 33(14), pp.1927-1935.
13. Plénet, D., Etchebest, S., Mollier, A. and Pellerin, S., 2000. Growth analysis of maize field crops under phosphorus deficiency. *Plant and Soil*, 223(1-2), pp.119-132.
14. Raj, S.A., 2010. Solubilization of silicate and concurrent release of phosphorus and potassium in rice ecosystem. *Biofertilizers Technology*, pp.372.
15. Richardson, A.E. and Simpson, R.J., 2011. Soil microorganisms mediating phosphorus availability update on microbial phosphorus. *Plant Physiology*, 156(3), pp.989-996.
16. Richardson, A.E., 2001. Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Functional Plant Biology*, 28(9), pp.897-906.
17. Rodríguez, H. and Fraga, R., 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, 17(4-5), pp.319-339.
18. Sangeeth, K.P., Bhai, R.S. and Srinivasan, V., 2012. Paenibacillus glucanolyticus, a promising potassium solubilizing bacterium isolated from black pepper (Piper nigrum L.) rhizosphere. *Journal of Spices and Aromatic Crops*, 21(2), pp.118-124.
19. Sarmiento, G., Da Silva, M.P., Naranjo, M.E. and Pinillos, M., 2006. Nitrogen and phosphorus as limiting factors for growth and primary production in a flooded savanna in the Venezuelan Llanos. *Journal of Tropical Ecology*, 22(2), pp.203-212.

20. Schloter, M., Dilly, O. and Munch, J.C., 2003. Indicators for evaluating soil quality. *Agriculture, Ecosystems & Environment*, 98(1-3), pp.255-262.
21. Sharma, S.B., Sayyed, R.Z., Trivedi, M.H. and Gobi, T.A., 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*, 2(1), pp.587.
22. Sheng, X. and Huang, W., 2002. Mechanism of potassium release from feldspar affected by the sprain Nbt of silicate bacterium. *Acta Pedologica Sinica*, 39(6), pp.863-871.
23. Storm, D.R., Rosenthal, K.S. and Swanson, P.E., 1977. Polymyxin and related peptide antibiotics. *Annual Review of Biochemistry*, 46(1), pp.723-763.
24. Uchida, R., 2000. Essential nutrients for plant growth: nutrient functions and deficiency symptoms. *Plant Nutrient Management in Hawaii's soils*, pp.31-55.
25. Uroz, S., Calvaruso, C., Turpault, M.P., Pierrat, J.C., Mustin, C. and Frey-Klett, P., 2007. Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved in mineral weathering in a forest soil. *Applied Environmental Microbiology*, 73(9), pp.3019-3027.
26. Velázquez, E., Martínez-Hidalgo, P., Carro, L., Alonso, P., Peix, A., Trujillo, M.E. and Martínez-Molina, E., 2013. Nodular endophytes: an untapped diversity. Beneficial Plant–Microbial Interactions: *Ecology and Applications*, pp.215-235.
27. Vlassak, K.M., Vanderleyden, J. and Graham, P.H., 1997. Factors influencing nodule occupancy by inoculant rhizobia. *Critical Reviews in Plant Sciences*, 16(2), pp.163-229.
28. Wolpert, J.S. and Albersheim, P., 1976. Host-symbiont interactions. I. The lectins of legumes interact with the o-antigen-containing lipopolysaccharides of their symbiont *Rhizobia*. *Biochemical and Biophysical Research Communications*, 70(3), pp.729-737.

APPENDIX A

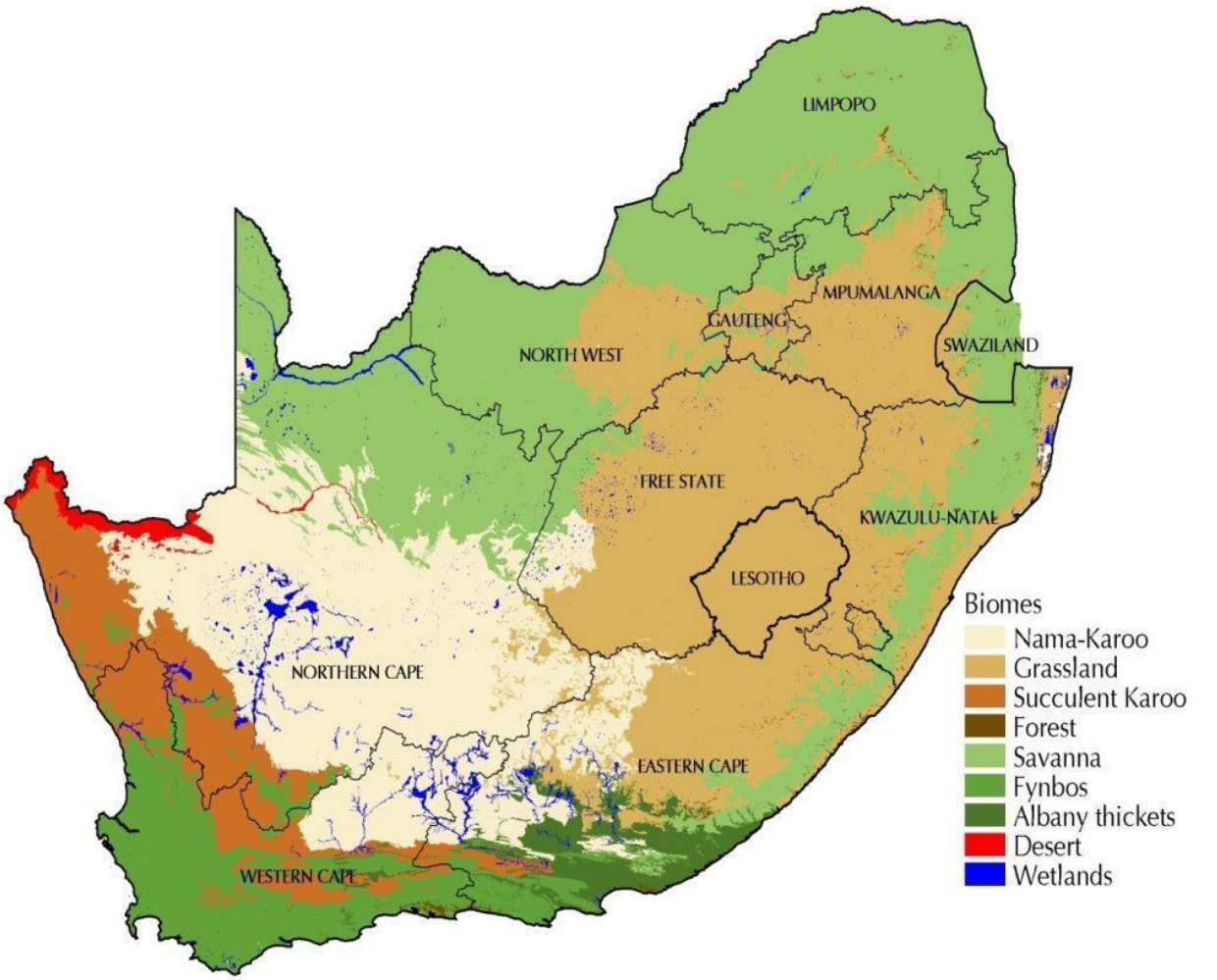


Figure S2: Biomes of South Africa. Retrieved from https://rangerdiaries.com/diary/south-africas-biodiversity-guides-perspective/