

Soil phosphorus deficiency affects the microbial symbiosis, nitrogen nutrition, and growth of *Vachellia nilotica* and *Themeda triandra* growing in competition in grassland ecosystems in KwaZulu-Natal, South Africa.

By

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SUMMARY

Soils of the Savanna and grassland ecosystems of southern Africa are acidic and nutrient deficient. Both these nutrients are essential for plant productivity and ecosystem composition with P being the energy driver during nodulation and biological N fixation (BNF) in legume plants. Legume establishment in nutrient-poor ecosystems has been reported to increase soil N due to their N fixing ability. Atmospheric N fixation by legumes can facilitate soil N inputs for co-occurring plant species, promoting plant growth and productivity and are considered for sustainable nutrient management practices in nutrient-poor ecosystems. Legumes such as *V. nilotica* (Benth.) Kyal. & Boatwr, are recommended in agroforestry because of their multipurpose usages including contributing soil N in nutrient-deficient grassland and savanna ecosystems. However, this could be affected by nutrient competition with co-occurring plants such as grass. Legume-grass interaction can result in reduced tree saplings and nodulation due to competition for available nutrients. Reduction in tree saplings is due to indirect competition for soil N and other vital nutrients that are required for plant growth and proliferation. A better understanding of the extracellular enzyme activities in soil, soil microbial composition, and established symbiosis, N nutrition, and growth carbon (C) costs during legume-grass competition in P-deficient grassland is required. It is therefore important to understand the mechanisms used by legume plants in low-nutrient soils in competition with grasses and identify N-fixing symbionts and bacterial strains that are tolerant of these environments. Information that will be obtained from this study is needed to understand the mechanism of legume plants' adaptations to acquire nutrients and metabolize N from different sources in nutrient-deficient environments.

The first experiment in this study investigated the effects of soil nutrient variability on microbial communities and associated enzymatic activities in soils collected in high and low P fertilized plots (366 kg of superphosphate per hectare per season (P) and control (-P)) from long-term fertilization trial plots at Ukulinga Research Farm, University of KwaZulu- Natal, Pietermaritzburg, South Africa. These soils were analyzed for nutrients, microbial composition, and extracellular enzymes activities. Overall, soil samples were acidic and soil nutrition, total cations and exchange acidity showed significant differences. The 16s RNA gene sequence analysis showed the presence of *Burkholderia contaminans sp*, an N fixing bacteria across all treatments. The most abundant strains identified with P solubilizing, N fixing, and N cycling functions belonged to the *Pseudomonas* genera. These *Pseudomonas* strains included *P. nitroreductase*, *P. putida* isolates, and *P. chlororaphis*. P solubilizing bacteria (*Rhodococcus erythropolis* and *P. nitroductens*) screened and assessed for P-solubilizing efficiency showed an efficiency of 3.5 and 3%, respectively. β -D-Glucosaminide, acid, and alkaline phosphatase enzyme activities increased under -P soils. Overall, these results outline various biogeochemical cycling bacteria, P solubilizing efficiency, soil enzyme activities, and the role these may collectively play in soil nutrient dynamics in KZN grasslands soils.

The second experiment investigated the effects of nutrient deficiency, grass competition, and physiological adaptations of *V. nilotica* in these acidic and nutrient-deficient KZN soils. Soils used as natural inoculum and growth substrate were collected from UGNE experimental trials located at the Ukulinga research. The soils were collected from superphosphate (P) and non-superphosphate (-P) fertilized trials. Seedlings of *V. nilotica* were subjected to competition with cuttings of *T. triandra* and *V. nilotica* cultivated independently. Plant biomass increased in plants growing in P soils. *V. nilotica* grown independently and in competition with *T. triandra* in P soils developed nodules while -P soils grown *V. nilotica* did not nodulate. *V. nilotica* grown independently and in competition with *T. triandra* in -P soils increased their reliance on soil N. P amended soils showed an increase in atmospheric derived percentage N (%Ndfa). This study shows that *V. nilotica* growing independently and in competition in -P soils rely more on soil available N than atmospheric derived N. *V. nilotica* grown in -P soils did not nodulate, but acquired atmospheric N suggesting association with endophytic bacteria.

PREFACE

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

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



Signed by: Zinhle Lembede (Student)

Date: August 2021

As the supervisor I have approved this thesis/dissertation for submission



Dr. A. Magadlela (Supervisor)

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE DECLARATION

PLAGIARISM

I, Zinhle Lembede (213558550) declare that:

1. The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work.
2. This dissertation has not been submitted in full or in part of any degree or examination to any other university.
3. This dissertation does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.
 - a) Their words have been re-written, but the general information attributed to them has been referenced.
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Date: August 2021



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GENERAL OVERVIEW OF CHAPTERS IN THIS DISSERTATION

This study consists of five chapters arranged as follows;

Chapter 1 introduces the background of nutrient deficiency and acid soils in southern African ecosystems and agricultural production and the use of legume plants to enrich soil nutrients. The competitive effect of nearby plant growth on these legumes is also explored. This chapter further discusses the significance of the study, hypotheses, aims, research questions, and objectives.

Chapter 2 is a literature review that outlines in details *Vachellia nilotica* and *Themeda triandra*, effects of P deficiency on legume growth, and nitrogen fixation from the atmosphere. The interaction of legume plants with associated grasses, competitive effect on legumes saplings, and incorporating legumes in sustainable agriculture practices are also explored. Also, the physiological and morphological survival strategies of legumes during phosphorus deficiency and the presence of grasses are investigated.

Chapter 3 investigates the effects of soil nutrient variability, microbial communities and associated enzymatic activities in KwaZulu-Natal grassland ecosystem soils. The study also assesses bacterial strains for potential P-solubilization efficiency and their role in this ecosystem soil.

Chapter 4 focuses on physiological adaptations of the legume, *V. nilotica*, growing in P deficient soils and in competition with *T. triandra*.

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LIST OF ABBREVIATION

Al-Aluminium

AM-Arbuscular mycorrhiza

ATP-Adenosine triphosphate

BNF-Biological Nitrogen Fixation

C-Carbon

Ca-Calcium

CFU-Colony Forming Units

Cl-Chloride

DAP-Days after planting

Fe-Iron

GPB-Growth-promoting bacteria

H-Hydrogen

K-Potassium

KZN-KwaZulu-Natal

Mg-Magnesium

N-Nitrogen

NDFA-Nitrogen derived from the atmosphere

NDFS-Nitrogen derived from the soil

NEDD-ethylenediamine dihydrochloride

NH₃-Ammonia

NR-Nitrate reductase

P-Phosphorus

PCR-Polymerase chain reaction

RGR-Relative growth rate

T-*Themeda*

UGNE-Ukulinga Grassland Nutrient Experiment

V-*Vachellia*

VFT-veld fertilized trials

Xg-Times gravity

CHAPTER ONE
GENERAL INTRODUCTION

1.1 Background

Acidic and nutrient-poor soils have been reported to occur in South African ecosystems that contain endemic species of which legumes and grasses are included (Goldbalt and Manning, 2010). Soil acidity and nutrient-poor soils stress agricultural systems (Suleiman and Tran, 2015) and have an impact on soil microbes and their enzyme activities (Utobo and Tewari, 2015). The demands on agriculture are increasing every day due to the continuously increasing human population with needs to be fulfilled currently and in the future (Velten *et al.*, 2015). This emphasizes the role of soil fertility in agricultural production. Phosphorus (P) and nitrogen (N) are the most limiting soil nutrients in savannas and grasslands ecosystems (Crane and Jackson, 2010). Atmospheric N fixation via symbiotic association with bacteria strongly depends on P availability (Vardien *et al.*, 2014). Insufficient P may have direct and indirect effects on legumes and their ability to fix atmospheric nitrogen, which ultimately affects plant growth productivity (Valentine *et al.*, 2011; Divito and Sadras, 2014). The process of biological nitrogen fixation (BNF) by legumes results in distribution of N back to the soil ecosystem (Chalk *et al.*, 2006). These plants associate with rhizobia and arbuscular mycorrhizal fungi (AM) in nutrient-deficient environments (Mortimer *et al.*, 2008). During symbiotic association, AM fungi improve P and N nutrients acquisition (Vance *et al.*, 2003). This is referred to as the tripartite relationship and results in atmospheric N₂ fixation, nodulation, and high yields in legumes plants (Harnett and Wilson, 2002). The tripartite mutualistic relationship among AM fungi, rhizobia, and legume plants results in increased legume growth (Chalk *et al.*, 2006) and P uptake by plants (De Varennes and Gross, 2006). An increased and abundancy in plant community biomass of semiarid grasslands resulted from an influence of AM fungi (Yang *et al.*, 2018).

The processes of nodulation and BNF by legumes are disturbed by the presence of other plant species, for example, grasses that also acquire nutrient P from the same soil (Thomas and Sumberg, 1995). Competition for nutrients between two species further results in legumes switching their limited N sources in poor P soils where resources must be shared (Mortimer *et al.*, 2008; Ndzwanana *et al.*, 2019). Switching N sources by legumes reduces their participation in atmospheric N fixation and favors the uptake of soil N via roots which are linked to the carbon economy of the plant (Ledgard, 2001; Magadlela *et al.*, 2016). The host plants depend on energy from carbon to maintain these symbiotic relationships as well as maintain their growth and productivity (Mortimer *et al.*, 2008, Magadlela *et al.*, 2016). Here the effect of the competition from *Themeda triandra* growing alongside legumes (*Vachellia nilotica*), N source preference and N assimilation rates is investigated. Legumes such as *V. nilotica* (Skowno *et al.*, 1999) and grass-like *T. triandra* are present (Snyman *et al.*, 2013). The presence of grasses is a known competitor with leguminous woody species at the seedling stage and may affect their establishment (Bond *et al.*, 2001). Factors such as grass fires, and excessive grazing can affect the coexistence of trees and grasses in savanna herbivory (Higgins *et al.*, 2003). The interaction of

these two species may be important in structuring savannas (Muvengwi *et al.*, 2017) because they dominate certain ecosystems such as at Ukulinga.

Legumes have been reported to evolve adaptation mechanisms to grow and cope with P stress. They include the development of cluster roots and root exudation of organic acids and acid phosphatase (Suleiman and Tran, 2015). For example, *Lupinus albus* responds to low P environments through AM fungi symbiosis, formation of cluster roots, and roots exudation (Schulze *et al.*, 2006). Enhancement of soil nutrient status results from the activities of soil microorganisms (Singh *et al.*, 2011; Gupta, 2012; Martínez-Hidalgo and Hirsch, 2017) and grass-legume mixture (Dhakal and Islam, 2018). Many studies have demonstrated that microbial communities improve soil nutrient status and tolerance to unfavorable environmental conditions (Di Stasio, 2016). Microbial organisms associate with extracellular enzymes whose activities enhance nutrients cycling and uptake by plants in savanna and grassland ecosystems (Burns *et al.*, 2013).

There are very limited studies on how competition on nearby grass influences N nutrition and biomass of *V. nilotica* in P poor soils of grasslands in southern Africa. *V. nilotica* (previously known as *Acacia nilotica*), is a multipurpose medicinal legume (Boon, 2010; Donaliso *et al.*, 2018) and has a distinguishing feature of fixing N₂ from the atmosphere (Woldemeskel and Sinclair, 1998). Both *V. nilotica* and *T. triandra* are dominant in savanna and grassland ecosystems (Kgope *et al.*, 2010). The presence of this native legume plant in these ecosystems is important for woody thickening (Kgope *et al.*, 2010). *T. triandra* is a keystone grass species that is reputed for its economic and ecological value in grazing systems within this ecosystem (Synman *et al.*, 2013). *T. triandra* also has the potential of indicating the health condition of the veld and is adapted to fire (Oudtshoorn *et al.*, 1999). Therefore the present study aimed at (1) evaluating the effect of variable P inputs on soil microbial communities and associated soil enzymes activities, (2) plant nutrition and biomass accumulation of *V. nilotica* growing independently and alongside *T. triandra*.

1.2 Justification of the Study

Legumes such as *V. nilotica* tolerates growth in harsh environmental conditions like P deficient soils (Suleiman and Tran, 2015) because of their ability to fix N from the atmosphere (Woldemeskel and Sinclair, 1998). However, competition for nutrients by grasses reduce BNF and legume production (Ledgard and Steede, 1992). During the interaction between legumes and grasses, there is a transfer of atmospherically fixed nitrogen from the legume to non-legume species. This transfer of N₂ stimulates non-legumes, however, it may alter the growth of the two species and N₂ fixation efficiency. (Hogh-Jensen and Schoering, 1997). Research studies focusing on grass-legume competition are few such that there is limited understanding of how the presence of *T. triandra* grasses on the same soil as the legume affects growth and N nutrition of the indigenous legumes such as *V. nilotica* in nutrient-deficient soils.

A better understanding of the extracellular enzyme activities in soil, soil microbial composition, and established symbiosis, nitrogen nutrition, and growth carbon (C) costs during legume-grass competition in P-deficient grassland is required. It is therefore important to understand the mechanisms used by legume plants in low-nutrient soils in competition with grasses and identify N-fixing symbionts and bacterial strains that are tolerant of these environments. Information that will be obtained from this study is needed to understand the mechanism of legume plants' adaptations to acquire nutrients and metabolize N from different sources in nutrient-deficient environments.

1.3 Hypotheses

- a) The growth of *V. nilotica* is negatively influenced by P-deficient soils and the presence of *T. triandra*.
- b) P-deficient soils and competition for nutrients from *T. triandra* negatively affect biological nitrogen fixation in *V. nilotica*.

1.4 Research questions

- a) How do P-deficient soils in KwaZulu-Natal affect enzyme activities, microbial species composition and N nutrition?
- b) Is *V. nilotica* establishment and growth affected by *T. triandra* growth on the same soil?
- c) How do KwaZulu-Natal nutrient-poor soils and competition for nutrients affect nodule development and N source preference in *V. nilotica*?

1.5 Aim and objectives

The study aimed at assessing the soil enzyme activities, soil microbial composition, and plant growth and N nutrition of legume during legume-grass competition in P-deficient grassland.

The objectives of this study will be addressed by:

- a) Studying the effect of P availability on soil enzyme activities and soil microbe composition in high and low P soils.
- b) Studying the effect of P deficient soils on plant nutrition, growth rate, N preferences (available soil N or atmospheric N₂), and associated carbon costs for *V. nilotica*.

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CHAPTER TWO

LITERATURE REVIEW

2.1. Plants in savannas and grasslands

Legumes (Leguminosae, or Fabaceae) are ecologically essential due to their ability to increase soil fertility by fixing atmospheric nitrogen (N) in association with rhizobacteria (Hussain, 2017). This contributes to soil fertility in ecosystems where plant growth is negatively affected by limited soil N (Cramer *et al.*, 2010). Legumes can be used to reclaim degraded lands in reforestation efforts (Chaer *et al.*, 2011), and social forestry programs (Skolmen, 1986). Legumes also contribute to animals feed as fodder in grass-legume mixtures and humans as a food source (Ahmed and Hasan, 2014). Legumes are commonly defined as podded fruits and contain important flowering plants with herbs, trees, and climbing species (Ahmed and Hasan, 2014). Leguminosae is the third most important family flowering plants, after the Poaceae grass family (Smýkal *et al.*, 2014).

Savanna ecosystems of southern Africa are dominated by and woody plants (Steven *et al.*, 2016) which include legumes (Cramer *et al.* 2010) and grasses (Bond, 2016). One grass species, *Themeda triandra*, occurs in all provinces of South Africa (Van Oudtshoorn, 2012) and attains a high abundance in grasslands and savannas (Mucina *et al.*, 2006). *Themeda triandra* is a keystone grass species that has considerable economic and ecological value (Synman *et al.*, 2013). Grasslands promote frequent fires and sustain large biomass of many types of grazing animals (Dunning *et al.*, 2017) and cover about 20% of the earth's land surface (Kellog, 2001). The co-occurrence of grasses and trees can lead to competition between these two species (Høgh-Jensen and Schjoerring, 1997; Cramer *et al.*, 2007). For example, grass species may compete for available resources (nutrients and water) in soil (Aerts *et al.*, 1991) and competition from grasses may suppress tree seedling growth (Mitchell *et al.*, 1999).

2.2 P deficiency, acidic soils, and N₂ fixation in legumes

Atmospheric N₂ fixation via symbiotic association with bacteria depends on soil phosphorus (P) availability (Vardien *et al.*, 2014). P is required by legumes during biological nitrogen fixation (BNF) in the form of adenosine triphosphate (ATP) which is the source of energy (Schulze *et al.*, 2006). Additionally, P is required for plants in a variety of functional, structural, and regulatory processes (Vance *et al.*, 2003). These metabolic processes include nucleic acid synthesis, respiration, photosynthesis, glycolysis, energy generation, carbohydrates metabolism, signaling, and enzymatic activation or inactivation (Hussain, 2017). Moreover, P is essential for nodule activity, growth, and functioning on legumes and in signal transduction, membrane biosynthesis, and (Divito and Sadras, 2014). A shortage of P negatively influences N accumulation and allocation of photosynthates in plants especially during BNF in legumes (Isidra-Arellano *et al.*, 2018). The effects of P deficiency on N fixation are evidenced through evaluation of biochemical and physiological parameters which include CO₂ fixation, nodule O₂ diffusion, and nitrogenase activity, and carbon (C) and N metabolizing enzyme activities (Hernandez *et al.*, 2009). These studies have been performed in different legume species, for example, on *Lupinus albus*, *Glycine max*, *Medicago sativa*, *Phaseolus vulgaris*, and

Virgilia divaricata (Hernandez *et al.*, 2009). Also, studies have been performed in indigenous legumes growing in nutrient-deficient soils of the Cape fynbos ecosystems (Power *et al.*, 2010; Magadlela *et al.*, 2017). Total plant P concentrations in *V. divaricata* during growth were reduced due to limited soil P availability (Magadlela *et al.*, 2016). Also, during P deficiency, symbiotic process and atmospheric N fixation were greatly reduced (Almeida *et al.*, 2000; Valentine *et al.*, 2011). The effect is direct as root nodules require P for their growth and metabolism or indirect as their high demand for P may be associated with the role in nodule carbon and energy metabolism (Valentine *et al.*, 2011; Divito and Sadras, 2014). This could result in poor carbon supply during nodulation with bacteria having greater respiratory demand on the host plant during nitrogen fixation thereby reducing plant growth (Valentine *et al.*, 2011).

Soil P deficiency is associated with soil acidity (Gurmessu, 2021). Soils with pH < 7 are considered acidic and associated with high concentrations of cations such as Al, Fe, and Mn (Tyler and Olsson, 2002). High cation concentrations in soils limit P assimilation by plants as P is bound to cations (Maathuis, 2009). Soil acidity is considered a major constraint on the growth and production of many cultivation systems across the world (Valentine *et al.*, 2011). Acidic soils have been widely reported in species-rich South African ecosystems (Dludlu *et al.*, 2018) that contain a variety of endemics, including legumes, which are continuously subjected to acidic and poor nutrient soils (Goldbalt and Manning, 2010). Acidic soils in southern African ecosystems such as grasslands, savanna, and Cape fynbos have varying nutrient concentrations; however, it has been reported that their soils are limited in N and P (Grigg *et al.*, 2008). Acidic soils negatively affect leguminous plants' symbiosis with the bacterium, and the formation and functioning of nodules (Sankar *et al.*, 2021). Fixation of N from the atmosphere is reduced by acidic soil conditions (Torabian *et al.*, 2019) and this is evidenced by reduced plant biomass in these ecosystems (Graham *et al.*, 1994). Soil nutrient deficiency harms N fixation and should be considered as a major limitation on legume plants (Valentine *et al.*, 2011).

However, microbial organisms in association with plants influence nutrient uptake in conditions of limited soil nutrient availability (Jeffries *et al.*, 2003). Microbes are beneficial in nutrient cycling and increasing nutrient concentrations in soils. These microbes include rhizobia, *Bacillus*, and arbuscular mycorrhizal (AM) fungi (Miransari and Smith, 2007). The solubilization of fixed P is driven by a soil-borne microorganism such as rhizobacteria and AM fungi (Sharma *et al.*, 2013). Overall fixing bacteria positively influence soils and can withstand acidic and nutrient-stressed environments (Mehta *et al.*, 2015). Microbes acquire limited nutrients by allocating their resources to extracellular enzymes. These extracellular enzymes recycle and mineralize major nutrients such as N, P, and C in soils (Das and Varma, 2010; Nanda *et al.*, 2010).

2.3 Legume symbiosis with arbuscular mycorrhizae (AM) fungi

AM fungi are distributed globally and considered as one of the most abundant microbes occurring below-ground (Munkvold *et al.*, 2004). The established symbiotic relationship between plants and AM fungi is important and contributes to nutrient cycling (Chalk *et al.*, 2006). AM fungi association with plants promotes

soil nutrient assimilation resulting in improved plant growth and development (Harrison, 1997; Chalk *et al.*, 2006; Veresoglou *et al.*, 2012). It is then not surprising that AM plants are more tolerant of nutrient stress compared to non-AM plants (Maruland *et al.*, 2003; Aroca *et al.*, 2007). Rhizobia and AM fungi in legume plant symbiosis is a promising interaction for further research in savannas but has been shown to improve plant performance, productivity, nutrition, and inhibition of infection by fungal plant pathogens (Abd-Alla *et al.*, 2014). The interaction between rhizobia bacteria, the fungi, and roots of legume plants improves host plant growth and increases nodule biomass in symbiotic plants (Kaschuk *et al.*, 2009). Regulation of rhizobial and AM fungal symbiosis is associated with photosynthetic carbon to maintain growth and symbiotic activities (Kaschuk *et al.*, 2009). Legume plants use up to 4-16% of photosynthates for each rhizobia bacteria and AM fungal symbiosis and this may affect the yield of the host plant (Kaschuk *et al.*, 2009, 2010).

2.4 Cost-effectiveness of N₂ fixation

The process of N₂ fixation requires a high amount of energy (Thuynsma *et al.*, 2014) in the form of ATP to sustain the bacterial physiology (Burén and Rubio). The supply of 16 adenosine triphosphate (ATPs) is required by nitrogenase enzymes to maintain its activities during N₂ reduction (Frankow-lindberg and Dahlin, 2013). N₂ reduction requires a large amount of energy, partly because hydrogen is produced during the formation of ammonia (NH₃). There is a relationship between N fixation and hydrogen (H) metabolism and H production represents energy loss and reducing equivalent (Stam *et al.*, 1987). H₂ is produced independently as a product which can occur in N₂ fixing organisms (Bothe *et al.*, 2010). At least one mol H₂ is produced for each mol N₂ reduced so that the BNF follows the overall equation (Biswas and Gresshof, 2014) indicated as $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP$. The respiration associated with nitrogenase activity for legumes requires 3.01 g C g⁻¹ N whereas the respiration of the entire N fixing nodules requires about 2.78 and 4.81 g C g⁻¹ N. Measurements of these values are based on roots respiration during the active N fixation period at the range of 5 - 10 g C g⁻¹ N with an average value of 6.5 g C g⁻¹ N (Minch and Witty, 2005). However, these costs differ according to species and specific genotype as some plants rely on both N fixation and soil N sources, specifically when under stress (Valentine *et al.*, 2011).

2.5 N use preferences of legumes

As legume plants are known to be dependent on atmospheric N₂ fixation for growth and development (Kaschuk *et al.*, 2009), during P deficiency they reduce their participation in the reduction and uptake of atmospheric N and switch to soil N to save energy (Magadlela *et al.*, 2016). Commonly, it is cost-effective for plants to assimilate soil inorganic N sources than atmospheric fixed N (Minch and Witty, 2005). Legume plants switch their N preferences due to nutrient stress conditions favoring soil N uptake to reduce energy costs (Mortimer *et al.*, 2008). Magadlela *et al.* (2016) concluded that *V. divaricata* plants growing in nutrient-poor Mediterranean-type ecosystems switch to soil N absorption and this was evidenced by reduced BNF. In nutrient-poor ecosystems, plants can establish an association with AM fungi. The plant-AM fungus symbiosis

contributes to increased assimilation efficiency of P and N resulting in improved N₂ fixation (Birhane *et al.*, 2012). In nutrient-poor ecosystems with variable plant mixtures, legumes may rely on limited N in soils resulting in competition with grasses (Nyfeler *et al.*, 2011). However, the unique ability of legumes to fix atmospheric N may be beneficial when there is competition for soil N (Ledgard, 2001).

2.6 Interaction of legume plants with associated grasses

Nitrogen is among essential nutrients within ecosystems, therefore plant mixtures and microbes often compete for available N pools (Veresoglou *et al.*, 2012). The coexistence of grasses and trees in savannas has been reported by (Høgh-Jensen and Schjoerring 1997, Cramer *et al.* (2007) and Ndzwanana *et al.* (2019) and the proximity between legume plants and C₄ grasses in savannas results in competition for nutrients (Cramer *et al.*, 2010). During this interaction, there is a transfer of atmospherically fixed N from legumes to non-legume plants which then stimulates the growth of the non-legumes. During this interaction, roots of grasses and trees share the same soil layer, and this interaction can result in suppression of tree growth, especially at the seedling stages of the trees (Muir *et al.*, 2011).

Currently, only a few studies (Cramer *et al.*, 2007, 2010) have been conducted to understand the interactions of native legume trees and grasses in savannas of southern Africa. Ndzwanana *et al.* (2019) studied the competitive performance of *Vachellia sieberiana* tree seedlings grown in competition with an invasive shrub *Chromolaena odorata* on nutrient-poor soils and found that *V. sieberiana* maintained its growth and was able to adapt during competition with the invasive shrub. This was observed by increased below-ground biomass and utilization of both atmospheric and soil-derived nitrogen by *V. sieberiana* (Ndzwanana *et al.*, 2019).

2.7 Legumes and grasses adaptations to P deficiency and competition

Legumes have evolved adaptive strategies for growth under P-deficient soil conditions (Sulieman and Tran, 2015; Valentines *et al.*, 2017). These plants have coordinated different gene expressions that allow them to survive under such conditions. They include root exudation of organic acids and development of cluster roots (Sulieman and Tran, 2015; Gaiand, 2017). Metabolic analyses have revealed that during P deprivation there is accumulation and exudation of organic and amino acids into the rhizosphere to solubilize P then increase uptake (Castro-Guerrero *et al.*, 2018). Under P starvation, legumes such as *Lupinus albus* may exude about 23% of plant organic acids and acid phosphatase and this solubilizes soil cation bound P making it available for plant uptake (Sprenst, 1999). Legume plants form cluster roots thereby increasing the root surface area and increasing the number of nodules for efficient P uptake (Lazali and Drevon, 2021).

This commonly occurs in non-mycorrhizal plants such as *L. albus* growing in low P soils (Uhde-Stone *et al.*, 2003a).

Additionally, legumes can change their respiration pathways to non-P requiring pathways and remobilizing their internal inorganic P (Pi) to maintain the P homeostasis (Uhde-Stone *et al.*, 2003a). For example, during P starvation, *Phaseolus vulgaris* can recycle and remobilize its internal P sources such as nucleic acids and membrane phospholipids (Castro-Guerrero *et al.*, 2018). Furthermore, preservation of P concentration in nodules and increasing N₂ fixation per unit of nodule mass allow plants to cope under insufficient P soils (Suliman and Tran, 2015). Nodules have the highest concentration of P than other plant parts during P stress. This suggests that nodules can regulate their C/N flux between the bacteria and the plant (Hernandez *et al.*, 2009; Castro-Guerrero *et al.*, 2018). Legumes further cope with unfavorable environmental conditions through stress-tolerant microbes made up of the fungi-bacteria interaction. This is because they have special features or traits that allow them to survive in nutrient-stressed environments (Zahran, 2017). Caradus (1980) compared several grass and legumes growing independently to observe differences in response to low P. Grasses had a more intensive roots system, thinner roots, longer total roots length, and longer roots hairs. Therefore, responsiveness to low P between these species was determined by roots morphology. These findings are similar to those of Fitter (1977), who found that grass maintains adequate P adaptively by roots morphology changes. Furthermore, P uptake is also influenced by a mycorrhizal infection which modulates the growth of grasses under nutrient stress (Fitter, 1977). To date, no study has focused on how grass such as *T. triandra* and indigenous legumes such as *V. nilotica* grown together may affect their microbial symbiosis, N nutrition, and biomass in nutrient-poor soils.

2.8 Native legume species: *Vachellia nilotica*

Vachellia nilotica (Fabaceae; Mimosoideae) is widely known as the taxonomic synonym *Acacia nilotica* (L.) (Donalisio *et al.*, 2018) and is commonly known as Indian gum or the Arabic tree (Ali *et al.*, 2012; Bargali and Bargali, 2009). This species is widely distributed in parts of Africa (Senegal, Egypt, and South Africa), Asia and is very common in India (Rather *et al.*, 2015). *V. nilotica* has a key feature which is the fixation of N from the atmosphere (Rajendra and Mohan, 2014), and their seed pods and barks contain secondary metabolites such as tannins (Boon, 2010). It is then not surprising that it increases soil fertility from a symbiotic relationship with rhizobium and mycorrhizal fungi and can thrive and tolerate moisture stress together with extreme temperatures (> 50 °C) (Rajendra and Mohan, 2014). Since it is a multipurpose tree (Rather *et al.*, 2015), tree parts like leaves, barks, and roots can be used for medicinal purposes (Boon, 2010). *V. nilotica* is also a good source of forage for animals (Rather *et al.*, 2015). The structural form of *V. nilotica* includes a round shape and flattened crown and has thorns as indicated in Figure 2.1a. Figure 2.1b illustrates pods that contain seeds. This plant has outgrowth-like structures called nodules (Figure 2.1 c) which are located on the roots. Nodules are the site of N fixation by N₂ fixing rhizobacteria (Broughton *et al.*, 2003; Hernandez *et al.*, 2009). The leaves are clustered and pinna pairs and arranged in 7-36 pairs with green-grey and bright green colors. The bark from this tree is black and grey and rough (Boon, 2012).



Figure 2.1: Images of *V. nilotica* (a) mature tree, (b) seeds and pods (<https://www.randomharvest.co.za>), and nodules (190 days after planting (DAP) -research study experiment).

2.9 Native grass species: *Themeda triandra*

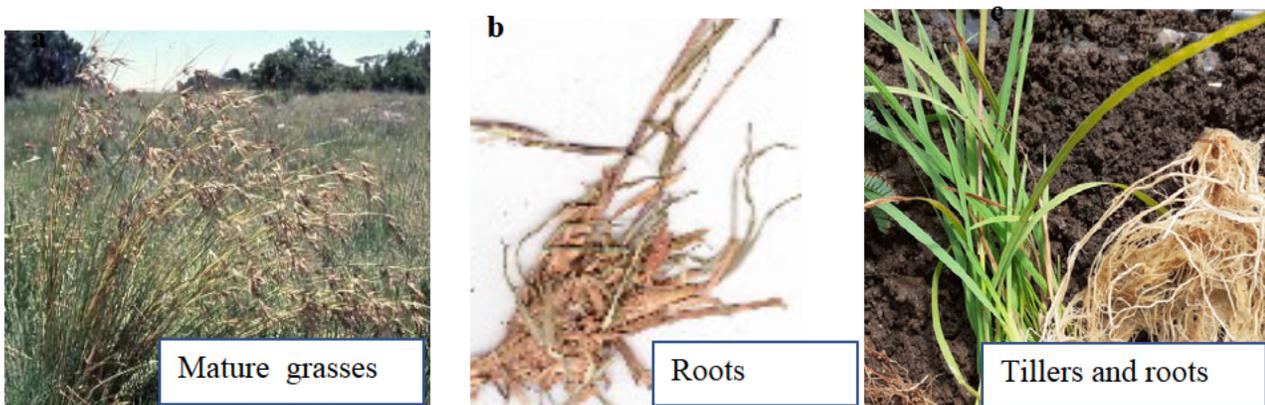


Figure 2.2: Images of *Themeda triandra* (a) mature grasses (<https://www.pza.sanbi.org>), (b) roots (<https://www.v3.boldsystems.org>) and tillers and roots (c) (190 days after planting (DAP)-research study experiment).

The description of *Themeda triandra* grass (Figure 2.2 a) is presented by Van Oudtshoorn *et al.* (1999). *T. triandra* only occurs in southern Africa and is commonly known as red grass. This grass grows with average rainfall of 1 300-3 000mm above sea level, palatable for grazing animals, and is dominant in grasslands of southern and East Africa.

2.10 Research objectives

The main objectives of the study are to:

- a) Study the effect of P availability on soil enzyme activities and soil microbe composition on soils supplemented with P.
- b) Study the effect of P deficient soils on plant nutrition, growth rate, nitrogen preferences (available soil N or atmospheric N₂), and associated carbon costs for *V. nilotica* and *T. triandra*.

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CHAPTER THREE
SOIL GEOCHEMISTRY AND MICROFLORA

Soil microbes and extracellular enzymes contribute to the nutrient dynamics in phosphorus-deficient KwaZulu-Natal grassland ecosystem soils.

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ABSTRACT

Soil nutrient deficiency, specifically nitrogen (N) and phosphorus (P), has been reported in grassland and savanna ecosystems. Soil microbial communities with associated soil enzymes influence soil nutrition within these ecosystems. The present study examined soil nutrient concentrations, microbial diversity and composition, P solubilizing efficiency, and extracellular enzymes activities in KwaZulu-Natal grassland ecosystem soils. The study's experimental soil samples were randomly collected from Ukulinga Grassland Nutrient Experiment (UGNE) trials at the Ukulinga research farm of the University of KwaZulu - Natal, Pietermaritzburg, South Africa. The experimental soil trials were fertilized with 366 kg of superphosphate per hectare per season (P) and soils not fertilized with superphosphate fertilizer (-P). The P soil treatments showed some significant differences in soil parameters examined. Overall, soil samples were acidic. Soils collected from P trials showed high P and Ca concentrations compared to -P soils. N concentrations across soil treatments revealed no significant differences. The 16s RNA gene sequence analysis showed *Burkholderia contaminans* sp an N fixing bacteria across all treatments. However, the most abundant strains identified with P solubilizing, N fixing, and N cycling functions belonged to the *Pseudomonas* genera. These *Pseudomonas* strains included *P. nitroreductase*, *P. putida* isolates, and *P. chlororaphis*, respectively. P solubilizing bacteria (*Rhodococcus erythropolis* and *P. nitroductens*) screened and assessed for P-solubilizing efficiency showed an efficiency of 3.5 and 3%, respectively. β -D-Glucosaminide, acid, and alkaline phosphatase enzyme activities increased under -P soils. Overall, these results outline various biogeochemical cycling bacteria, P solubilizing efficiency, soil enzyme activities, and the role these may collectively play in soil nutrient dynamics in KZN grasslands soils.

Keywords: Grassland ecosystems, soil acidity, microbial species richness and diversity, β -D-Glucosaminide, acid phosphatase, and alkaline phosphatase

3.1 Introduction

Soil acidity and nutrient deficiencies are global problems (Schjoerring *et al.*, 2018), as documented reports have shown that 30% of the world's agricultural suitable land is acidic (Gurmessa, 2021). This has been attributed to climate change (Mellor, 2014) and increased unsustainable agricultural practices for food production to support the world's growing population (Livi Bacci, 2017). Nitrogen (N) and Phosphorus (P) are key plant-growth-limiting nutrients (Richardson *et al.*, 2009) despite being abundantly present in soils in both inorganic and organic forms (Widding *et al.*, 2019; Pueyo *et al.*, 2021). Usually, P forms precipitates and complexes with other soil nutrients which makes them non-bioavailable to plants (Kalayu, 2019). In such ecosystem soils, growth-promoting bacteria (GPB) have helped in the solubilization of P and fixing of N for plants uptakes (Gupta *et al.*, 2013). Amongst the GPB, P solubilizing and N fixing bacteria (Saeid *et al.*, 2018) belonging to the family *Bacillaceae* including *Bacillus subtilis* (Moreno-Lora *et al.*, 2019) and *Bacillus firmus* (El-Esawi *et al.*, 2018) have been reported in stressed soils, including acidic and nutrient deficient KwaZulu-Natal ecosystem soils (Zungu *et al.*, 2020). Similarly, bacteria belonging to the family *Pseudomonadaceae* and *Actinomycetaceae* have also been implicated in nutrient-stressed ecosystem (Martínez-Hidalgo and Hirsch, 2017). Soil extracellular enzymes play a major role in conserving (Qin *et al.*, 2020) and recycling key nutrients in nutrient-poor ecosystem soils (Guo *et al.*, 2019).

These extracellular enzymes recycle and mineralize major nutrients such as N, P, and carbon (C) in soil, thus increasing nutrient availability for plant uptake (Rao *et al.*, 2014). Enzymes involved in N cycling include *acetylglucosaminase* (Ma *et al.*, 2019), urease (Burns *et al.*, 2013), and β -D- *Glucosaminidase* (Fujita *et al.*, 2018). Phosphatase plays a vital role in P cycling, mineralization (Adetunji *et al.*, 2017), and production through the hydrolysis of phosphoric acid monoester to produce a phosphate anion (Kalsi *et al.*, 2016). Common phosphatase enzymes that have been extensively studied including acid and alkaline phosphatase enzymes, and their activities have been influenced by soil pH and P availability (Touhami *et al.*, 2020).

Mineral fertilizer applications to soil, including Southern African grassland ecosystems, have always been considered as the most suitable and efficient method to increase world food production and income generation (Nandwa, 2001). The continuous applications of these mineral fertilizers in modern day agriculture is expensive and environmentally unfriendly (Herridge *et al.*, 2005). For instance, the use of chemical fertilizers contributes to algal bloom in aquatic systems through leaching (Herridge *et al.*, 2005). Also to a large extent, it often influences the soil microbial community structures and its enzyme activities. However, the available information on the responses of soil microbial communities and enzyme activities to nutrient stress in the southern African grassland ecosystem is limited.

Thus, this study seeks to 1.) assess the physiochemical parameters of phosphorus-deficient soil, 2.) evaluate the response of soil microbial community structures and specific soil microbial enzyme activities in the

phosphorus-deficient soil, 3.) isolate, characterize, identify and evaluate the GPB strains which can supplement the P deficiency of the grassland ecosystem soil cost-effectively without compromising crop yields, and 4.) ascertain the response of the acidic and alkaline phosphate enzymes to phosphate solubilization.

3.2 Materials and Methods

3.2.1 The soil sampling site description and vegetation overview

The soil samples for this study were collected at the Ukulinga Grassland Nutrient Experiment (UGNE) farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa (29°37'S; 30°16'E). The altitude gradient in which the UGNE trials were set up ranged from 838 to 847 m above sea level (Ward *et al.*, 2017). The climatic conditions at Ukulinga experimental farm are as follows; the annual mean precipitation and temperature of the area are approximately 838 mm and 18°C, respectively (Ward *et al.*, 2020). The soils were derived from shale under the Westleigh classification and are relatively infertile and acidic. The surrounding area is classified as a southern tall grass veld (Fynn and O'Connor, 2005). The UGNE area is dominated by dense and tall grassland and legume species such as *Vachellia sieberiana* and *Vachellia nilotica*. Grass patches include species of *Hyparrhenia hirta*, *Themeda triandra*, and various herbaceous species (Fynn and O'Connor, 2005). The trials of UGNE were initiated in 1951 through the implementation of N, P, and lime (L) and are considered the longest-running nutrient experiments across Africa (Morris and Fynn, 2001). Initially, it consisted of 96 plots from the years 1951-2019 and each plot was 9.0 x 2.7 meters (m) in size with a 1 m spacing between plots. The experiment is a 4 x 2³ factorial design, with three replicated blocks and each block contains 32 plots (Le Roux and Mentis, 1986). The objectives of the long-term veld fertilized trials (VFT) were agricultural and to increase the productivity of fodder which was of interest. The pH value of Ukulinga topsoil is acidic (5.9) (Widdig *et al.*, 2019).

3.2.2 Experimental soils and soil nutrient analysis

Soils samples were randomly collected within the long-term VFT, and these sites were fertilized with 366 kg of superphosphate per hectare per season (P) and non-phosphate fertilizer soils (-P) at the depth of 30 cm to minimize damage during the trials. The 20 soil samples collected from each P treatment were pooled for nutrient and microbial homogeneity. Five soil subsamples of 50 g from each treatment were sent for P, N, K, pH, acidity exchange, and total cation analysis at the KwaZulu- Natal Department of Agriculture and Rural Development's Analytical Services Unit, Cedara, South Africa. An additional 5 soil samples (250-300 g) from each treatment were used for microbial identification and enzymatic analysis.

3.2.3 Microbial population analysis

Soil samples were subjected to serial dilution and spread on selective media agar plates per standard solid plating techniques. Phosphate solubilizing bacteria were isolated and enumerated using Pikovskaya's agar

plates containing tricalcium phosphate (TCP) as the phosphate source. The nitrogen cycling bacteria were enumerated on Simmons Citrate agar plates which contain citrate as its carbon source and inorganic ammonium salts as its only source of nitrogen, while that of the nitrogen cycling bacteria were carried out on Jensen's media agar (nitrogen-free media). Each selective media plate was replicated in triplicate and incubated at 30 °C for 5 days. The microbial population was expressed as colony forming units (CFU/mL).

3.2.4 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 minutes, and placed on ice for 5 minutes. The suspension was centrifuged at 13817 x g in a micro-centrifuge (Spectrafuge 16M, Labnet) for 5 minutes and the supernatant ($\sim 50 \mu\text{L}$) was transferred to sterile Eppendorf tubes.

3.2.5 DNA amplification, sequencing, and identification

The extracted bacterial DNA was amplified using the 63F (5'-CAG GCCTAACACATGCAAGTC -3') and 1387R (5'-GGGCGGTGTGTACAA GGC -3') primers in the PCR. The PCR mixtures consisted of 10 μL DNA, 5 μL of 10 X reaction buffer, 2 μL 25 mM MgCl_2 , 2.5 μL of each primer, 0.25 μL of Taq DNA polymerase, 1 μL of 10 mM 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 94°C for 2 minutes followed by 30 cycles of denaturation at 92°C for 30 s, then annealing at 56°C for 45 seconds and elongation at 75°C for 45 seconds with a final elongation at 75°C for 10 minutes. The PCR products were resolved on 1.0% (w/v) agarose gels (Seakem) and visualized after staining with ethidium bromide (0.5 $\mu\text{g}/\text{mL}$) using the Chemigenius Bio-imaging System (Syngiene, England). Positive amplicons (~ 1324 bp) were excised and sequenced at Inqaba Biotechnical Industries and the sequences were compared against the GenBank database. Homologs were identified using the BLASTn program at the National Center for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3.2.6 Extracellular soil enzyme activities

The activities of N and P cycling enzymes; (β -glucosaminidase), acid phosphatase, and alkaline phosphatase were assayed using the fluorescence-based method described by Goyal *et al.* (2019) and expressed in units of $\text{nmol h}^{-1}\text{g}^{-1}$. In brief, 5 g soil samples were homogenized at low speed in 50 mL Milli-Q H_2O for 2 hours at 4 °C. The supernatants were transferred into black 96-well microplates before adding the respective substrates. Each 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 + 50 μL buffer), negative controls (200 μL buffer + 50 μL substrate) and blanks (250 μL buffer). The reaction was stopped with 0.5 M NaOH after 2 hours incubation period at 30 °C. Thereafter, fluorescent absorbance was measured at 450 nm on a Glomax Multi Plus microplate reader

(BioTek, USA). Noteworthy, before the determination of acid phosphatase activity both the buffer and standards, was adjusted to pH 5.

Nitrate reductase activity (NR) was determined according to a slightly modified method of Kandeler (1995). Briefly, 5 g of soil was added to a solution consisting of 1 mL of 25 mM KNO₃, 4 mL of 0.9 mM 2,4-dinitrophenol, and 5 mL of Milli-Q H₂O in a sealed 50 mL centrifuge tube. The mixture was then briefly mixed before incubation at 30 °C in the dark for 24 hours. After the incubation period, 10 ml of 4 M KCl solution was added to each sample, mixed briefly, and passed through a Whatman number 1 filter.

The enzymatic reaction was initiated by adding 500 µl of filtrates with 300 µl of 0.19 M ammonium chloride buffer (pH 8.5) and 200 µl of a color reagent (1% sulfanilamide in 1 N HCl and 0.02% N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) before incubating in dark at 30 °C for 30 mins. The absorbance was measured at 520 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, USA). The amount of nitrite (NO₂⁻) liberated into the medium was extrapolated from a prepared standard curve with KNO₃. Nitrate reductase activity was expressed as 0.1 µmol h⁻¹ g⁻¹.

3.2.7 Ecological parameters (structural indices)

The bacterial community richness and diversity was analyzed based on total CFU/mL, Shannon-Wiener index of diversity (H), Simpson index of dominance (D), and Evenness

Species diversity measurement

Shannon-Wiener's (1949) diversity index was used to determine the microbial diversity of each fertilization site.

$$H = - \sum P_i \ln P_i$$

Where $P_i = S/N$ S= number of individuals of one species

N= total number of all individuals in the sample

Ln= natural logarithm to base e

Species richness measurement

The Margalef's (1985) index was used to calculate the species richness of microbes of each fertilization site.

$$\text{Margalef's index} = (S - 1) / \ln N$$

S = total number of species

N = total number of individuals in the sample

Ln = natural logarithm to base e

Species evenness measurement

The Pielou's evenness (1966) index was used to measure the species evenness of each site

$$e = H / \ln S$$

H = Shannon–Wiener diversity index

S = total number of species in the soil sample.

3.2.8 Assessment of P-solubilization efficiency

Media composition: Pikovskaya's broth containing 1 g glucose, 0.5 g MgCl₂ · 6H₂O, 0.025 g MgSO₄ · 7H₂O, 0.02 g KCl, 0.01 g (NH₄)₂SO₄, 2.0 g NaCl while 0.5 g tricalcium phosphate was used as the source of insoluble P. The media pH was adjusted to 7.

Preparation of bacterial suspensions

The bacterial suspension was conducted as described by Pikovskaya (1948) with a slight modification. All the potential phosphate solubilizing bacteria strains were individually cultured in nutrient broth overnight under aseptic conditions at 30 °C and 150 Xg. The suspension was centrifuged for 10 minutes at 8,000 Xg to obtain the bacterial cells pellet. The pellet was later resuspended in 0.85% saline solution and standardized to MacFarland standard of 0.5 at λ_{max} 600 nm.

Screening for potential inorganic phosphates solubilization

Preliminary screening for potential high inorganic phosphate solubilizing bacteria was carried out using a solid plate media following the modified method of Liu *et al.* (2015). Briefly, 10 µL of the standardized culture was spot inoculated on Pikovskaya agar plates in triplicates before incubation at 30 °C for 48 hours. The colony and halo zones (clear zones) diameters were measured with a meter ruler calibrated in mm

The following formula measured solubilization Index (SI):

$$SI = \frac{\text{Colony diameter} + \text{halo zone diameter}}{\text{Colony diameter}}$$

Quantification of inorganic phosphate solubilization in liquid medium

The strain with the highest solubilization index was further evaluated for its inorganic phosphate solubilization activity in Pikovskaya's broth. The assay was carried out following a modified method of Wan *et al.* (2020). Briefly, a sterile 100 ml Pikovskaya broth inoculated with 10 % (v/v) of standardized bacterial culture $0.5 (\lambda_{\max} 600 \text{ nm})$ in a 250 ml Erlenmeyer flask and incubated at 30 °C under continuous agitation on a rotary shaker at 180 Xg for ten days.

Extraction and quantification of P -solubilized

The extraction and analytical quantification of phosphate solubilization of $\text{Ca}_3(\text{PO}_4)_2$ was carried out using the standard colorimetric method described by Waterlot (2018) with a slight modification. For brevity, 10 mL aliquots of the culture broth, periodically collected at two-day intervals, were centrifuged at 6000 Xg for 15 minutes. The biomass-free supernatants were used for pH determination, P- solubilization, and solubilization enzyme production analysis. The pH of the supernatant was measured using a digital pH meter (Hanna Instruments, USA). Simultaneously, the P- solubilization was quantified using the standard colorimetric assay via the molybdenum blue method using KH_2PO_4 as standard. The soluble P was expressed as $\mu\text{g/mL}$.

The solubilized phosphate concentration in the bacterial biomass at each sampling period was accounted for by following the method of Perez *et al.* (2007) with slight modification. The bacterial biomass containing $\text{Ca}_3(\text{PO}_4)_2$ was resuspended in an equal volume of MilliQ water and vortexed thoroughly. Thereafter, the mixture was rested for 15 minutes at room temperature before centrifuging at 500 Xg for 2 minutes to recover the P-solubilized biomass. Also, residual insoluble phosphate of $\text{Ca}_3(\text{PO}_4)_2$ in bacterial biomass was retrieved by resuspension of the biomass in 1 mL of 1 N HCL and centrifuging at 500 Xg for 2 minutes before subjecting the supernatant to colorimetric analysis. The insoluble P was expressed as g/L.

Determination of acid and alkaline phosphatase activities of the isolates in liquid broth

This was carried out using the standard colorimetric method with *p*-nitrophenol as standard as described by Dick *et al.* (2000) with a slight modification.

Acidic Phosphatase activity

The reaction mixture contained 50 mM sodium acetate (pH 5.0), 1mM Dithiothreitol, 20 mM MgCl_2 , 20 mM *p*-nitrophenyl phosphate in a total volume of 0.5 mL and 100 μL of enzyme extract (culture supernatant). The enzymatic reaction was catalyzed with 0.4 mL of 1M Na_2CO_3 before incubating at 30 °C for 15 minutes. The color change was measured using a spectrophotometer (Agilent Cary 60 UV-Vis) at 410 nm. The enzyme activities were expressed as units per mL, where 1U was defined as 1 μM of substrate oxidized per minute with an extinction coefficient of $18000 \text{ cm}^{-1} \text{ M}^{-1}$).

Alkaline Phosphatase activity

The alkaline phosphatase activity was carried out according to the acidic phosphate procedure. The reaction mixture contained supplementation with 50mM Tris-HCL (pH 8.6) in lieu of 50 mM sodium acetate (pH 5.0) (Dick *et al.*, 2000).

Data analysis

SPSS 24 (IBM Corp, Armonk, New York, USA) was used to test for differences in soil nutrients, pH, exchange activity, and total cations, soil enzyme activities between the two soil treatments of KwaZulu-Natal, using One-way analysis of variance (ANOVA). Where the ANOVA showed significant differences between treatments, a Welch test was used to separate the means (≤ 0.05).

3.3 Results

3.3.1 Soil nutrient analysis

The physicochemical parameters of the P and -P soil fertilizations are shown in Table 1. Both the soil samples from P and -P fertilizations were acidic with a pH value of 4.59 and 4.29 respectively. In comparison to the P, P-fertilized soil sample was very rich in Ca, P, total cation, and exchangeable acidity contents with 38.24 mmol/g, 0.308 mmol/g, 14.99 cmol⁻¹, and 0.133 cmol⁻¹ respectively. No significant differences ($P < 0.05$) were observed in the pH, K, N, Mg and C contents of both P and -P fertilization soil samples while a significant difference was only observed in the Ca, total cations, and exchangeable acidity of both assessed soil samples.

3.3.2 Functional microbial diversity

The functional microbial diversity structure under the P and -P fertilization soil samples (P solubilizing, N cycling, and N fixing bacteria) is shown in Table 2. The P-fertilized soil sample is very rich in microbial community structures with a population of 9.11 X10⁴ CFU/mL for N cycling bacteria and P-solubilizing bacteria with 1.23 X 10⁶ CFU/mL while 4.44 X 10⁴ CFU/mL was enumerated for N cycling bacteria and 0.84 X 10⁶ CFU/mL for P-solubilizing bacteria in a -P fertilized soil sample. Conversely, -P fertilized soil sample was very rich in N-fixing bacteria with a population of 5.11 X10⁶ CFU/mL when compared with the P fertilized soil samples with 2.77X10⁶. Both P and -P fertilization soil samples have the same species richness with 2,3 and 2 species for N-cycling bacteria, N-fixing bacteria, and P-solubilizing bacteria respectively. A similar trend was also observed in their diversity index and R_{margalef}.

3.3.3 Relative abundance of microbial communities

The relative abundance of the microbial community structures is presented in figure 1 (a-c). For the P-fertilization soil sample, *Burkholderia contaminans* account for the 50 % relative abundance of N-fixing bacteria followed by *Pseudomonas sp.* with 26%, *Caulobacter rhizosphaerae* with 14.5 %, and *Sphingomonas sp* with 9.5 %. A similar trend was also observed in -P fertilization soil samples. N fixing bacteria showed

high species relative abundance in *Burkholderia contaminans* J8A6SARS of 50% and 40% in P supplemented and -P soil treatments, respectively. This was followed by *Pseudomonas sp.* with a relative abundance of 26% and 25% and *Caulobacter rhizosphaera* IMCC34905 at 14.5% and 15% in P supplemented and -P soil treatments, respectively. *Kitasataspora grisela* bacterial strain showed a 10% relative abundance and was unique in -P soils. *Sphigomonas sp.* N-9 had a relative abundance of 9.6% and 10% in P supplemented and -P soil treatments, respectively. Moreover, N cycling bacteria showed high species relative abundance in *Pseudomonas chlororaphis* of 30.5% in -P soils. This was followed by *Pseudomonas sp.* BRJH1 with 20% and 14.5% in P supplemented and -P soils, respectively. *Pandora oxalativorans* KSI 1495 had 12.5% and 7.5% relative abundance and *Pseudomonas koreensis* showed a 10% and 5% relative abundance in P and -P soil treatments, respectively.

3.3.1 Extracellular enzyme activities

The assessment of nutrient cycling enzyme activities was conducted to determine factors influencing soil nutrient availability. There was a significant increase ($p \leq 0.05$) in β -Glucosaminidase activity between -P soils ($\sim 180 \text{ nmol h}^{-1} \text{ g}^{-1}$) and P supplemented soils ($\sim 155 \text{ nmol h}^{-1} \text{ g}^{-1}$) (figure 3.7b). However, there was no significant difference ($p \geq 0.05$) in N reductase activity between P supplemented and -P soils (figure 3.7b). Acid and alkaline phosphatase activity were higher in -P soils, compared to P supplemented soils (figure 3.7c and d).

3.3.5 Qualitative screening for potential P-solubilizing bacteria

The potential phosphate solubilizing bacteria performance on solid Pikovskaya's agar plate is presented in Figure 2 (a-b) and Figure 3. From the four P solubilizing bacterial strains that were screened on Pikovskaya's agar plate, *R. erythropolis* showed the halo zone diameter of 42 mm followed by *P. nitroreducens* with 40 mm while *Pseudomonas sp.* had the least halo zone (30 mm). Conversely, *Pseudomonas sp.* had the superior colony diameter with 23 mm followed by *P. kribbensis* while *R. erythropolis* had the least colony diameter of 15 mm (Figure. 2a and Figure 3). In addition, the highest solubilization index was observed in *R. erythropolis* (3.8) followed by *P. nitroreducens* with 3 while *Pseudomonas sp.* has the least solubilization index (2.3) (Figure 2b).

An evaluation of the phosphate solubilizing activity by *P. nitroreducens* is presented in Figure 3 (a-d). The water-soluble P-concentration was observed to increase with an increase in the incubation period. The optimum P-solubilization period was recorded on day 8 after the incubation period with a P-solubilization concentration of 332 $\mu\text{g/mL}$ while no significant changes were observed in the abiotic control (Figure 1a). A similar trend was observed during the bacterial growth determination with a significant increase in growth which was optimal at day 8 after the incubation period (Figure 3b). Conversely, the *P. nitroreducens* was active at a slight acid pH (4.9-7) during the P-solubilization activity. The pH decreases concurrently with the increase

in the incubation period. However, a slight decrease in the insoluble P was recorded at day 10 after the incubation period with a residual value of 4.68 (g/L).

Acid and alkaline phosphatase enzymes assays reactions were carried out and acid phosphatase activity of *R. erythropolis* rapidly increased from zero to 20 U/ml after day two of incubation and peaked at ~23 U/ml on day four (Figure 3.6a). This was followed by a gradual decrease in activity to ~14 U/ml by day 10 of incubation (Figure 3.6a). Contrary to acid phosphatase activity, alkaline phosphatase activity gradually increased from zero to ~20 U/ml after six days of incubation, followed by a rapid increase to 75 U/ml by day 10 of incubation (Figure 3.6b).

3.4 Discussion

The relationship between soil nutrition, soil functional microbes, and extracellular soil enzymes activities in this study are important as reported by Kotzé *et al.* (2017) where soil geochemical properties, texture, and exchange acidity are the main determinants for soil nutrient composition and its bioavailability to plants and soil microbial biota. Grassland and savanna ecosystems of southern Africa are reported to occur in different geographical ranges (Mukwanda, 2018) that are acidic and N and P deficient (Pellegrini, 2016). The soil nutrient analysis from our study showed that soils were acidic and nutrient-poor. Acidic soils tend to be high in cations such as Al, Mn, and Fe (Afonso *et al.*, 2020), cations sequester P making it unavailable for plant uptake even when available in soils (Mitra *et al.*, 2020). However, the presence of identified nutrient cycling and fixing microbes in our experimental soils may have regulated nutrient concentrations, including P. These findings concur with Zhu *et al.* (2018) who that reported high P concentrations in acidic soils were related to increasing hydroxide ions due to the presence of phosphorus solubilizing microbes. The necessity of P in soils comes with a positive influence on agricultural productivity and ecosystem composition (Guignard *et al.*, 2017). Phosphorus further assists during plant growth development, atmospheric N fixation by legumes, and enzyme activities (Pueyo *et al.*, 2021). Soil microbes and extracellular enzymes have been reported to be a useful soil indicator (Raiesi *et al.*, 2018) and contribute to increased P concentrations in acidic soils by solubilizing cation-bound P (Adeleke *et al.*, 2017).

Our study soils presented a high diversity of P solubilizing, N cycling, and N fixing bacteria. The highly dominant identified bacterial species were *Burkholderia contaminans* and *Pseudomonas* species such as *P. nitroreducens*, *P. denitrificans*, and *P. chlororaphis*. Species of *Burkholderia* genus isolated from the soils are classified as plant growth-promoting (PGP) bacteria and contribute greatly to N₂ fixation (Zuleta *et al.*, 2014). Also, *B. contaminans* species are said to contain occidiofungin antifungal activities (Yurnaliza *et al.*, 2020) that inhibit pathogen growth (Lu *et al.*, 2009). Microbes belonging to the *Burkholderia* genus are also tolerant of acidic soils (Maxton *et al.*, 2018) and this was confirmed by their high abundance in our collected soils which were found to be acidic. Free-living N₂ fixing *Caulobacter rhizosphaera* was identified in our study soils and was reported to improve soil health status and produce activities of alkaline and acid phosphatase (Sun *et al.*,

2017). The diversity of microbes identified in our study soils further revealed the presence of *Kitasataspora grisela* bacteria. This bacterium is classified as actinomycetes (Takahashi *et al.*, 2017) and contains secondary metabolized gene clusters such as hopanoids and spore-associated proteins (Arens *et al.*, 2015) which are involved in the synthesis and degradation of bacteria (Hermanas *et al.*, 2021) and contains antimicrobial activities that are beneficial in nutrient stressed soils (Yun *et al.*, 2020). The common activities of *Pseudomonas* species include plant growth (Goud *et al.*, 2018), tolerance to stressed soil conditions, and control of pathogens (Nandi *et al.*, 2017). For example, *P. nitroreducens* contains plant growth-promoting activity, however, its potential use in agriculture has not been explored yet (Trinh *et al.*, 2018). Furthermore, Nandi *et al.* (2017) reported *P. chlororaphis* for improving crop productivity and plant growth under stressed soil conditions (Yuan *et al.*, 2020). The presence of *P. chlororaphis* in the study soils may have enhanced legume plant growth under low P soil treatments due to its high tolerance to stressed soil conditions.

The presence of these identified soil microbes may adjust P concentrations and fix N₂ for plants and this has been observed and studied extensively in most P solubilizing and N fixing bacteria (Bargaz *et al.*, 2018). Insoluble P is considered a challenge for plants' direct absorption therefore P solubilizing microbes are vital in converting insoluble and fixed P to plant usable forms (Eida *et al.*, 2018). Examining the efficiency of these bacterial strains before application in agricultural or ecosystem soils is important (Soumare *et al.*, 2020). The current study evaluated the P solubilizing efficiency of bacteria isolated in long-term veld fertilized grasslands soil trials. *P. nitroreducens*, *P. kribbenis*, *Pseudomonas sp.* and *R. erythropolis* strains were screened and isolated, and among them, *R. erythropolis* and *P. nitroreducens* showed the highest P solubilizing efficiency in the Ca₃(PO₄)₂ culture media. *Pseudomonas sp.* (Babalola and Glick, 2012b) and *Rhodococcus* (Raj *et al.*, 2014) have been reported to mobilize unavailable P through solubilization and mineralization (Istina *et al.*, 2015). *Triticum aestivum* inoculated with *Pseudomonas sp.* showed increased growth and productivity which was an indication of the effectiveness of P solubilizing microbes in soil for plant assimilation (Tahir *et al.*, 2020). P solubilizing microbes further increase the efficiency of N₂ fixation by legumes, thus promoting legume N concentrations and plant growth (Alemneh *et al.*, 2020). Growth of the *P. nitroreducens* under a wide range of pH was observed. This showed that this bacterial strain was tolerant to acidity and efficient P solubilization. In addition, *R. erythropolis* displayed high P solubilization capacity than other microbes due to increased production of acid and alkaline phosphate enzyme activities.

As much as southern African ecosystem soils are characterized as nutrient stressed (Ezeokoli *et al.*, 2020), well-managed agricultural practices contribute to soil microbes and extracellular enzymes efficient cycling and absorption of nutrients (Schröder *et al.*, 2016). Soil extracellular enzymes pose a great necessity in soils as they contribute to the cycling and solubilization of nutrients promoting the role of plants and microbe interactions (Schofield *et al.*, 2019). Moreover, these enzyme activities are soil health indicators (Ghosh *et al.*, 2020) and acid phosphatase is P hydrolyzing extracellular enzymes that contribute to catalyzation of hydrolytic organic P making it accessible to plants (Baleni and Negisho, 2012). Plant available H₂PO₄ has

been reported to be derived by soil microbial communities in response to deficient inorganic P (Antonious *et al.*, 2020). Acid phosphatase has been reported in many studies to increase during P deficiency and allows plants to access P from organic sources. Alkaline phosphatase is another essential extracellular enzyme (Tang *et al.*, 2019) which in our study increased phosphatase activity during nutrient-deficient soils. These phosphatase enzymes may be essential for P mineralization in grassland ecosystem soils which is vital for plant growth and productivity. The current soil analyses further showed that β -D-Glucosaminidase activity was increased under low P soils. This enzyme plays a role in N and C nutrient cycling and contributes to N and C concentration in soils (Parham and Deng, 2000). Similar results were obtained by Loeppmann *et al.* (2016) where β -D- *Glucosaminidase* activities increased soil N concentration during P deficiency.

3.5 Conclusion

Key findings of the conducted study provided an overview of the functions and vital role of soil microbes and extracellular enzyme activities may play in nutrient cycling in P deficient grassland soils. These nutrient cycling dynamics may be important in maintaining grasslands ecosystems functions in South African KwaZulu-Natal province. Also, some of these microbes showed increased P solubilizing efficiency which can be useful when applied in impoverished soil recovery practices. From this study, it can be concluded that these KwaZulu-Natal grassland soils may support plants growth and isolated bacteria can contribute to sustainable agricultural practices as bio-fertilizers and thereby promote less usage of expensive and environmentally harmful chemical fertilizers.

Conflicts of Interest

We declare no conflict of interest concerning this study. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The opinions, findings, and conclusions/recommendations expressed in this work are that of the authors, and that NRF accepts no liability whatsoever in this regard.

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Table and figure legends

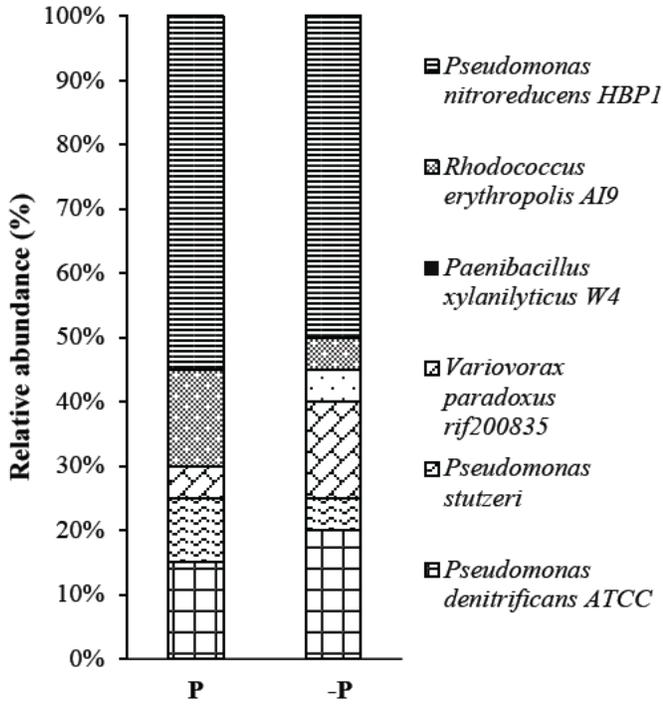
Parameters	P soils	- P soils
pH	4.587±0.044 ^a	4.289±0.222 ^a
Ca concentration (mmol/g)	38.240±1.548 ^a	26.541±1.921 ^b
P concentration (mmol/g)	0.308±0.026 ^a	0.072±0.016 ^b
K concentration (mmol/g)	4.848±0.849 ^a	2.953±0.364 ^a
N concentration (mmol/g)	0.205±0.003 ^a	0.200±0.007 ^a
Mg concentration (mmol/g)	15.740±1.273 ^a	14.361±2.678 ^a
C concentration (mmol/g)	3.810±0.102 ^a	3.616±0.0345 ^a
Total cations (mmol L ⁻¹)	14.990±8.352 ^a	11.240±5.882 ^b
Exchangeable acidity (mmol L ⁻¹)	0.133±0.009 ^a	1.967±1.279 ^b

Table 3.1: Macronutrient concentrations (P, N, K, C, Ca, and Mg), exchange acidity, total cations, and pH in soils fertilized with P and -P at Ukulinga Grassland Nutrient Experiment (UGNE) trials. Values are expressed as means ± SE, n=5. Different letters denote significant difference across the rows for each parameter.

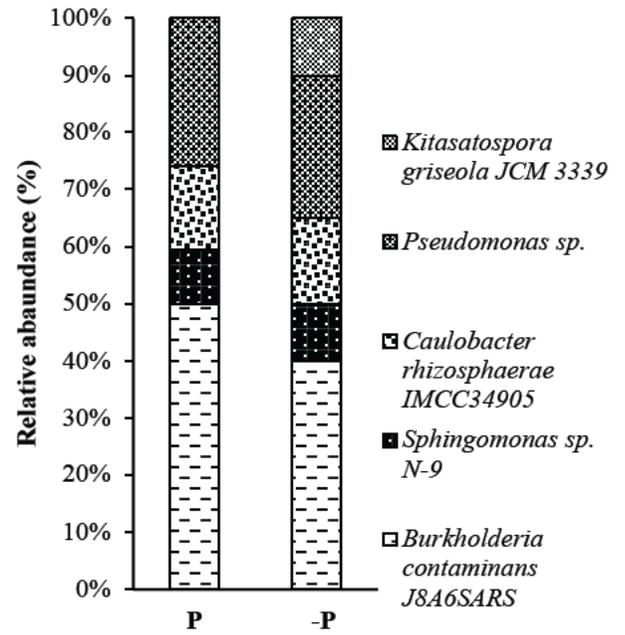
Parameters	Treatments	
	P	-P
N cycling bacteria		
Total CFU (mL)	9.11X10 ⁴	4.44X10 ⁴
Species Richness	2	2
Simpson diversity index	0.5	0.416
Shannon diversity index	0.693	0.607
R _{margalef}	0.088	0.093
N fixing bacteria		
Total CFU (mL)	2.77X10 ⁶	5.11X10 ⁶
Species Richness	3	3
Simpson diversity index	0.552	0.48
Shannon diversity index	0.889	0.828
R _{margalef}	0.135	0.13
P solubilizing bacteria		
Total CFU (mL)	1.23X10 ⁶	0.84X10 ⁶
Species Richness	2	2
Simpson diversity index	0.477	0.103
Shannon diversity index	0.67	0.211
R _{margalef}	0.071	0.069

Table 3.1: Bacterial Colony Forming Units (CFU), Species Richness, Shannon Diversity Index, Simpson Diversity Index, R_{margalef}, and E_{pielou} values in soils fertilized with P and -P at Ukulinga Grassland Nutrient Experiment (UGNE) trials.

A.



B.



C.

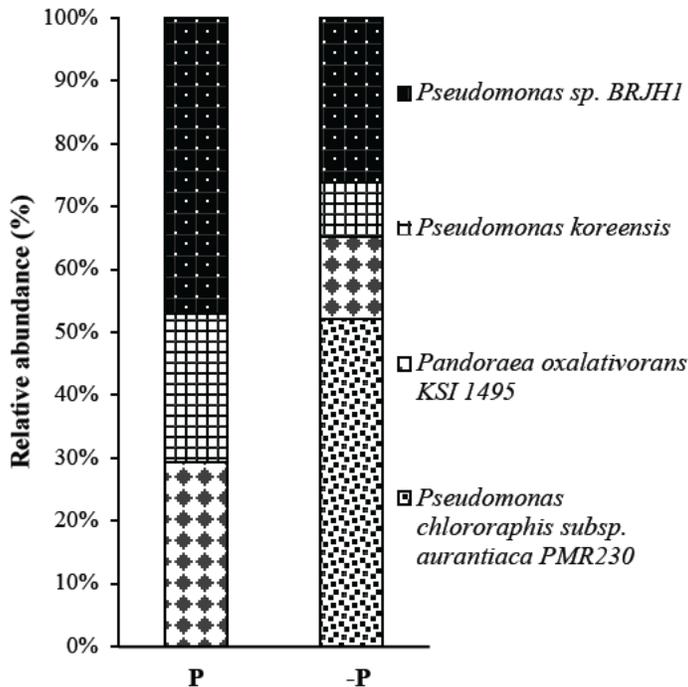


Figure 3.1: Relative abundance of microbial communities (A) P solubilizing, (B) N fixing, and (C) N cycling bacteria in soils fertilized with P and -P at Ukulinga Grassland Nutrient Experiment (UGNE) trials.

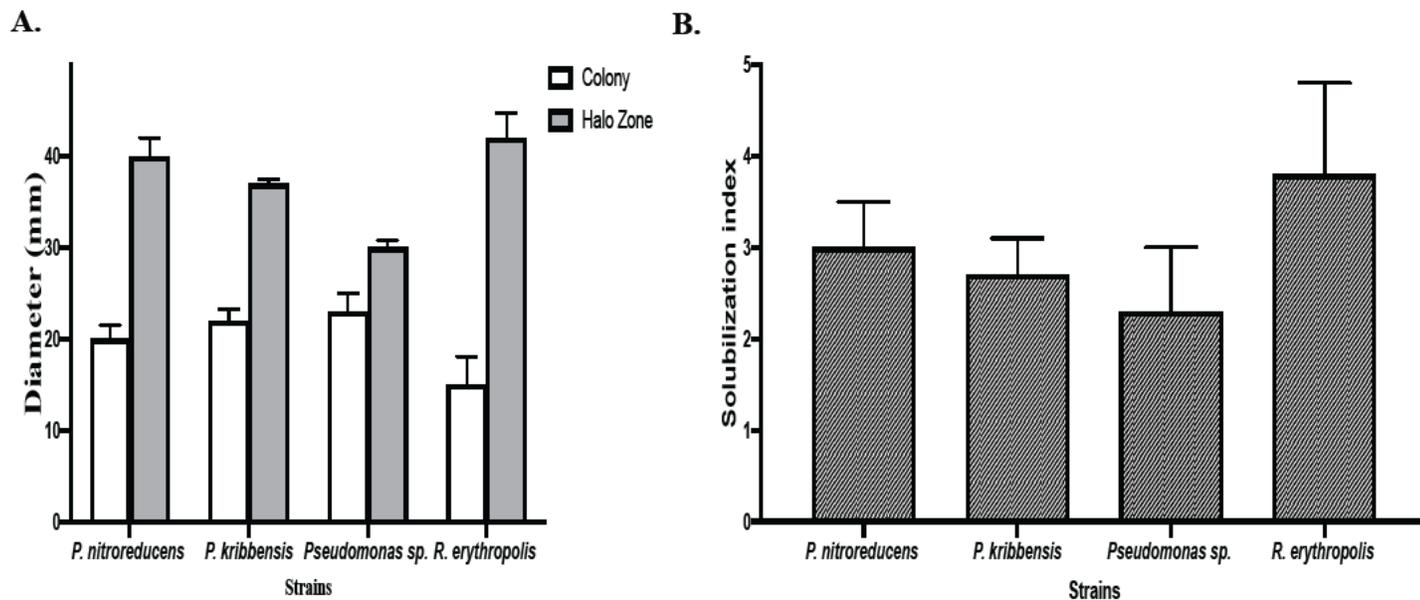


Figure 3.2: Potential P solubilizing bacteria performance on solid Pikovskaya's agar plate. (A) average colony and phosphate solubilization zone (halo zones) diameters and (B) solubilization index.

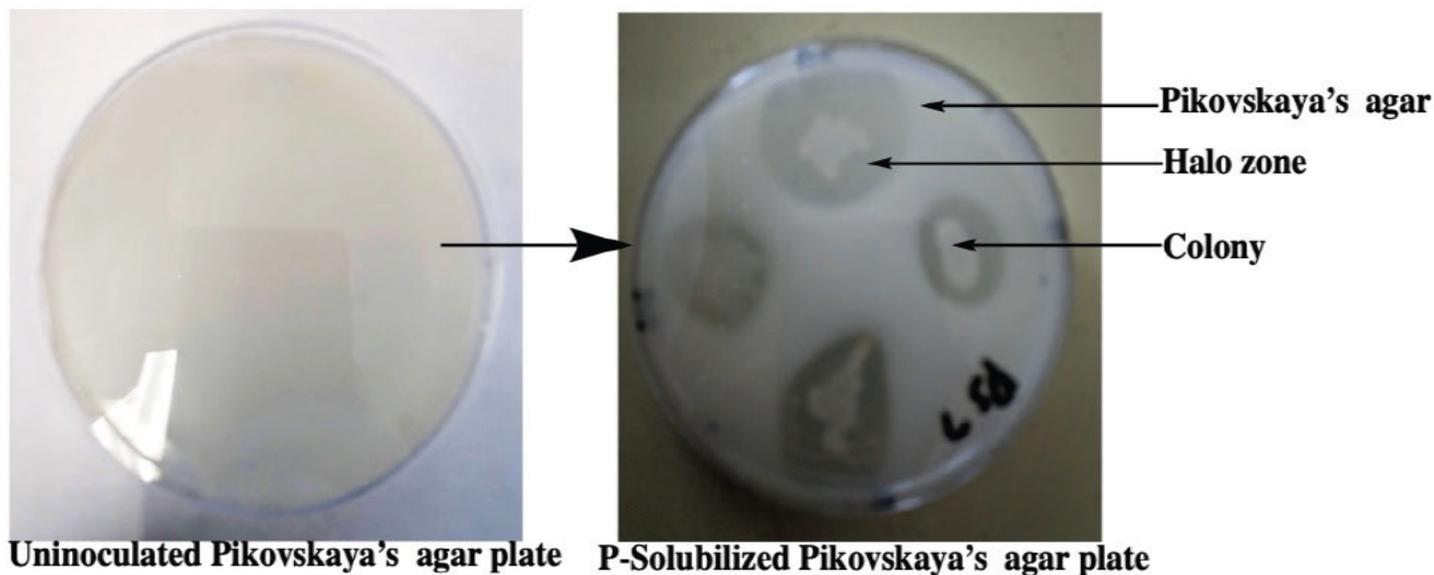


Figure 3.3: Potential P solubilizing bacteria performance on solid Pikovskaya's agar plate and uninoculated Pikovskaya's agar plate.

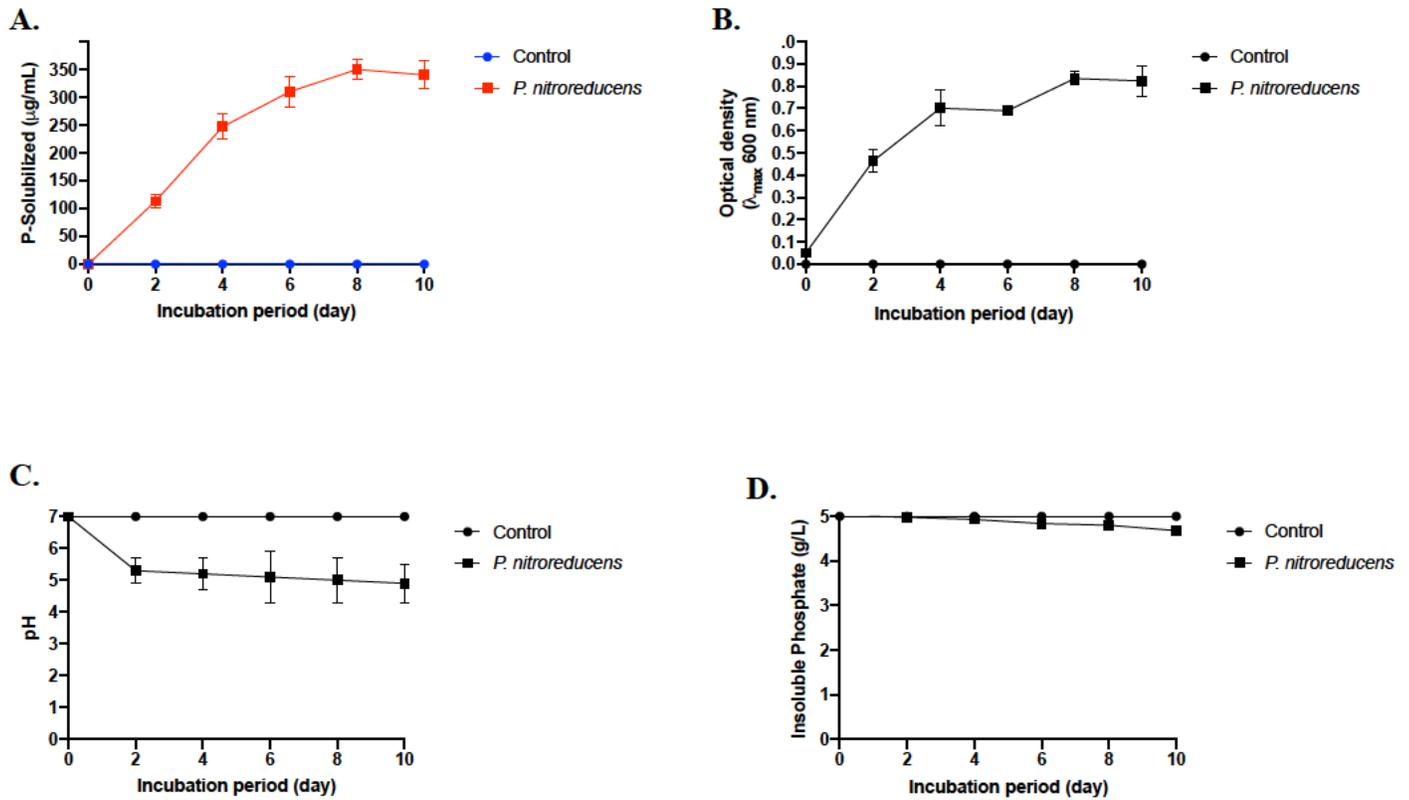


Figure 3.4: Phosphate solubilization of $\text{Ca}_3(\text{PO}_4)_2$ by *P. nitroreducens*. (A) Amount of P-solubilized in $\text{Ca}_3(\text{PO}_4)_2$, (B) *P. nitroreducens* growth curve, (C) Change in pH condition of the media, (D) The residual P-solubilized in $\text{Ca}_3(\text{PO}_4)_2$.

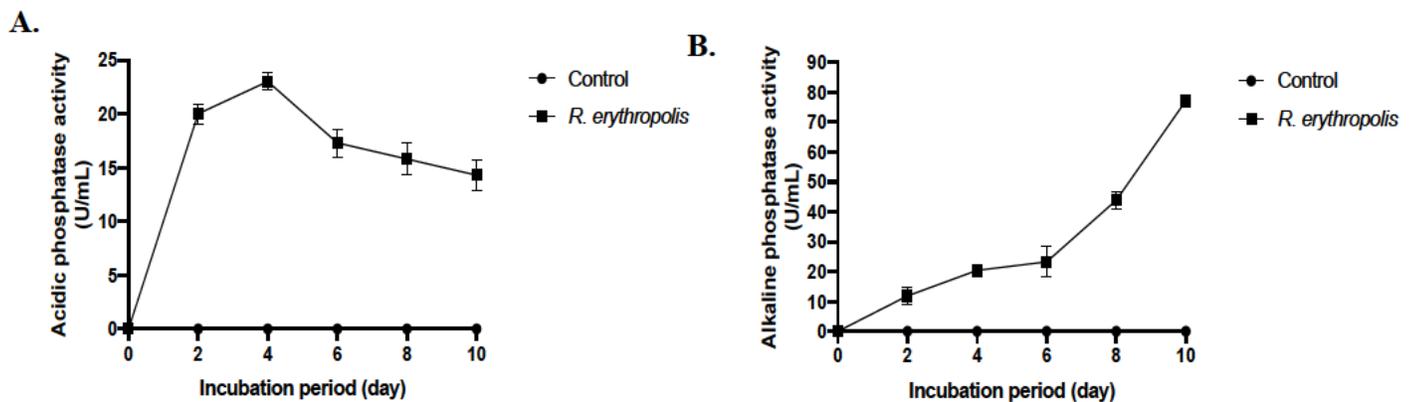


Figure 3.5: Phosphate solubilization enzymes production in $\text{Ca}_3(\text{PO}_4)_2$ (A) acidic phosphatase activity and (B) alkaline phosphatase activity

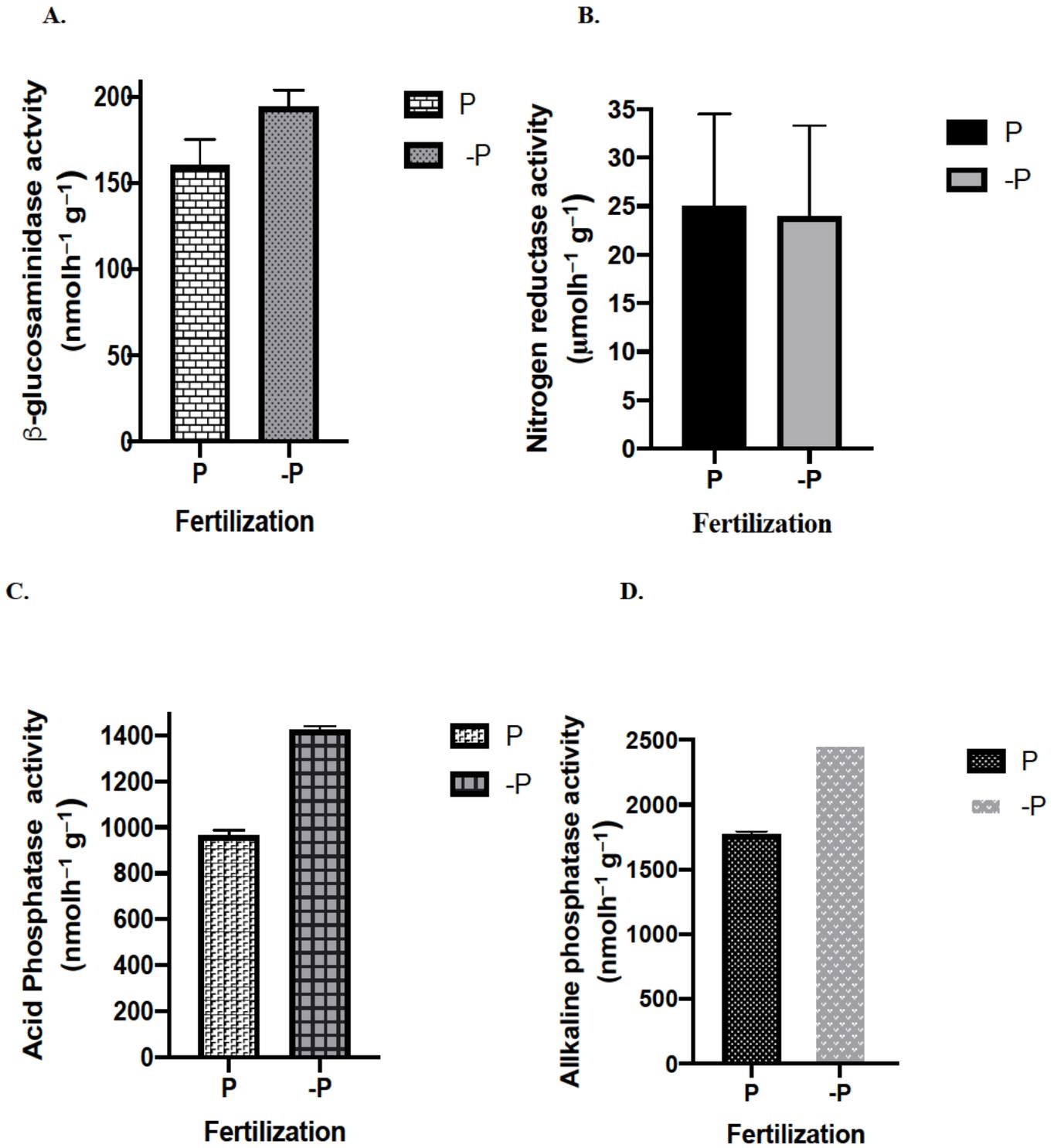


Figure 3.6: N and P cycling and hydrolyzing enzyme activity determined in phosphorus and non-phosphorus fertilized soils fertilized with P and -P at Ukulinga Grassland Nutrient Experiment (UGNE) trials.

CHAPTER 4

PLANT GROWTH AND NUTRITION

***Vachellia nilotica* growing in competition with *Themeda triandra* in phosphorus (P) deficient grassland soils switched nitrogen (N) preference to soil available N to reserve energy for growth.**

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ABSTRACT

Vachellia nilotica plants are important in agroforestry due to their nitrogen (N) inputs in grassland and savanna ecosystems including those in KwaZulu- Natal (KZN). However, these ecosystem soils are acidic and nutrient deficient specifically with regards to nitrogen (N) and phosphorus (P). Within grassland and savanna ecosystems, native legumes co-occur with grasses which may result in competition for nutrients including N and P. Knowledge on *V. nilotica* nodulation and N nutrition in P deficient grassland soils co-occurring with grasses is limited. Therefore, this study seeks to understand the effects of nutrient deficiency, grass competition, and physiological adaptations of *V. nilotica* in these acidic and nutrient-deficient KZN soils. Soils used as natural inoculum and growth substrate were collected from UGNE experimental trials located at the Ukulinga research farm of the University of KwaZulu- Natal, Pietermaritzburg, South Africa. The soils were collected from superphosphate (P) and non-superphosphate (-P) fertilized trials and seedlings of *V. nilotica* were subjected to competition with cuttings of *T. triandra* and cultivated independently. Plant biomass increased in plants growing in P soils. *V. nilotica* grown independently and in competition with *T. triandra* in P soils developed nodules while -P soils grown *V. nilotica* did not nodulate. *V. nilotica* grown independently and in competition with *T. triandra* in -P soils increased their reliance on soil N. *V. nilotica* growing with *T. triandra* in P soils showed increase percentage N derived from atmospheric (%NDFa). This study shows that *V. nilotica* growing in independently and in competition in P deficient soils rely more on soil available N than atmospheric derived N. *V. nilotica* grown in -P soils did not nodulate, however, was able to acquire atmospheric N suggesting association with endophytic bacteria.

Keywords: *V. nilotica*, P deficiency, competition, N₂ fixation, *pseudomonas*

4.1 Introduction

Savanna and grassland ecosystems soils of southern Africa are reported to be nutrient deficient and acidic (Goldblatt and Manning, 2010). Nitrogen (N) and phosphorus (P) are the main nutrients deficient in these ecosystems (Sanchez *et al.*, 1997). Both these nutrients are essential for plant productivity and ecosystem composition (Ferguson *et al.*, 2010) with P being the energy driver during nodulation and biological N fixation (BNF) in legume plants (Vardien *et al.*, 2014). Legume plants require P as adenosine triphosphate (ATP) during BNF (Sanginga *et al.*, 2000) and 16 ATP molecules are required to reduce atmospheric dinitrogen (N₂) to plant usable ammonium (NH₄) (Mus *et al.*, 2016). Therefore, nodulation and BNF in legumes are reduced under P deficient soil conditions (Yahiya and Fatma, 1995). Also, soil acidity negatively affects legume symbiosis with growth-promoting bacteria, nodule formation and functioning and consequently BNF (Rice *et al.*, 1977). Acidic soils are reported to have a high concentration of cations (Eswaran *et al.*, 1997) that form insoluble complexes with P, resulting in soil P being unavailable for plant uptake (Vitousek *et al.*, 2010). Therefore, acidic soil pH exacerbates the effects of P deficiency on nodulation and BNF, resulting in reduced legume plant biomass in these ecosystems (Graham *et al.*, 1994).

Legume establishment in nutrient-poor ecosystems has been reported to increase soil N due to their N fixing ability (Jhariya *et al.*, 2018). Atmospheric N fixation by legumes can facilitate soil N inputs for co-occurring plant species, promoting plant growth and productivity and are considered for sustainable nutrient management practices in nutrient-poor ecosystems (Pirhofer-Walzl *et al.*, 2012). Legumes such as *V. nilotica* (Benth.) Kyal. & Boatwr, are recommended in agroforestry because of their multipurpose usages including contributing soil N in nutrient-deficient grassland and savanna ecosystems (Rajendren and Mohan, 2014). However, this could be affected by nutrient competition with co-occurring plants (Hogh-Jensen and Schoering, 1997) such as grass (Cramer *et al.*, 2010). Legume-grass interaction can result in reduced tree saplings and nodulation due to competition for available nutrients (Cramer *et al.*, 2010). Reduction in tree saplings is due to indirect competition for soil N and other vital nutrients that are required for plant growth and proliferation (Bhadouria *et al.*, 2017). Legume-grass interaction study by Donzelli *et al.* (2013) revealed that due to competition for soil nutrients, legumes utilize N derived from the atmosphere, while grass species utilize soil available N.

Grasses such as *T. triandra* Forssk. maximise nutrient uptake by increasing root surface area through alteration of root morphology as a response to P deficiency. P uptake by this species is further influenced by a mycorrhizal infection which modulates the growth and development of grasses under soil nutrient stress (Fitter, 1977). During legume-grass interactions, N transfer occurs through mycorrhizal fungi where roots of both species are interconnected, nutrient absorption by grasses is via rhizodeposition into the soil (Hogh-Jensen and Schjoering, 2001). Despite all the above-mentioned, legume species survive in N deficient

environments and nutrient competition (Thomas and Sumberg, 1995). Cramer *et al.* (2007) highlighted that legume's capability to fix N from the atmosphere contributes to their survival and growth during competition with grasses. Legumes have been reported to switch N sources in nutrient-poor soils and when nutrient resources are shared among plants (Mortimer *et al.*, 2008; Ndzwanana *et al.*, 2018). N-source switching by legumes reduces the carbon economy in the plants since N₂-reduction requires large amounts of respired carbohydrates, especially during nutrient deficiency (Ledgard, 2001, Magadlela *et al.*, 2016). It is yet to be determined if the same is true during nutrient deficiency and legume-grass competition. Other adaptations that enable legumes to optimally access nutrients include the development of cluster roots and secretion of organic acids and acid phosphatase to solubilize cation bound P to overcome nutrient deficiency (Schulze *et al.*, 2006; Suleiman and Tran, 2015). Additionally, legume plants have been reported to establish a symbiotic association with arbuscular mycorrhizae (AM) fungi, resulting in improved soil nutrients acquisition (Vance *et al.*, 2003; Schulze *et al.*, 2006). AM fungi and rhizobia are common microbes (Gupta, 2012) in legume-plant symbiosis with a very promising interaction because they improve host plant growth and increase nodulation (Kaschuk *et al.*, 2009). However, the N source preference and N assimilation rates in legume plants have not been studied during grass competition in nutrient-deficient grassland ecosystem soils. The interaction between legumes and grasses and sharing of resources in nutrient poor ecosystems is poorly understood. Therefore, the present study examined the N source preference and N assimilation rates in *V. nilotica* growing independently and in competition with *Themeda triandra* in grassland ecosystem soils.

4.2 Materials and methods

4.2.1 The soil sample collection site description

The soil samples for this study were collected at the Ukulinga Grassland Nutrient Experiment (UGNE) farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa (29°37'S; 30°16'E). The altitudinal gradient in which the UGNE trials are set up ranges from 838 to 847 m above sea level (Ward *et al.*, 2017). The climatic conditions at Ukulinga experimental farm are as follows; mean precipitation and temperature of the area are approximately 838 mm and 18°C respectively (Ward *et al.*, 2020). The Ukulinga experimental farm soils were derived from shales under the Westleigh classification and is reported to be relatively infertile and acidic (Ward *et al.*, 2020). The vegetation of the UGNE and surrounding area are classified as southern tall grassveld (Fynn & O'Connor, 2005). The area is a dense tall grassland consisting of leguminous trees such as *Vachellia sieberiana* DC and *V. nilotica* with grass patches such as *Hyparrhenia hirta* L, *Themeda triandra* (Forssk.) and various herbaceous species (Fynn & O'Connor, 2005). The UGNE was initiated in 1951 through the manipulation of nitrogen (N), phosphorus (P), and lime (L) without any disturbances and is the longest-running field experiment in Africa (Morris and Fynn, 2001). There were initially 96 plots from the years 1951-2019 and each plot was 9.0 x 2.7 meters (m) in size with a 1 m spacing between plots. The experiment was replicated in three blocks, each block containing 32 plots, resulting in a 4 x 2³ factorial design

(Le Roux and Mentis, 1986). The objectives of the long-term veld fertilized trials (VFT) were to increase the productivity of fodder which was of interest. The VFT sites are fertilized with different elements such as lime, N, and P applied at different concentrations (Morris and Fynn, 2001). The pH value of Ukulinga topsoil is acidic by (5.9) (Widdig *et al.*, 2019).

4.2.2 Experimental soils and soil nutrient analysis

The soils samples were randomly collected within the long-term VFT plots and for this study, soils were collected from plots fertilized with superphosphate (336 kg/ha/season) (P) and plots not fertilized with superphosphate (-P). Soils were collected at the depth of 30 cm to minimize the damage to the trials. These two plots were treated as two different treatments and 20 soil samples were collected from each treatment were pooled for nutrient and microbial homogeneity. Five 50 g soil subsamples from each treatment were sent for P, N, K, pH, acidity exchange, and total cation analysis at the KwaZulu- Natal Department of Agriculture and Rural Development's Analytical Services Unit, Cedara, South Africa. Soil nutrient analysis was performed to determine the grassland soil's geochemistry, inclusive of exchangeable acidity and pH (Table S4.1). The soils were acidic across all treatments (P and -P) with pH values of 4.587 and 4.289, respectively. Ca concentration was significantly higher in the P supplemented soils compared to -P soils. P concentration was more than three-fold higher in the P supplemented soils (0.308 ± 0.026 mmol/g) compared to -P soils (0.072 ± 0.016 mmol/g). K, N, Mg, and organic C concentrations showed no significant differences statistically between P supplemented and -P soils. Total cations concentrations were significantly higher in the P supplemented soils than -P soils. Contrary to total cation concentrations, exchangeable acidity was significantly higher in -P soil treatment compared to P supplemented soil treatments.

4.2.3 Seed germination and growth conditions

The experiment was conducted under ambient conditions in greenhouse no. 12 at the University of KwaZulu-Natal Botanical gardens, Pietermaritzburg, South Africa. The greenhouse conditions were 12 to 14°C and 30 to 35°C at night and day temperature, respectively. With humidity that ranged from 70% to 80% and irradiance was ~35% of full sunlight (i.e., $415.6 \mu\text{mol m}^{-2} \text{s}^{-1}$). Fertilized soils obtained from UGNE were used for seed germination and as growth substrates in 19 cm pots. *V. nilotica* seeds were randomly collected from Mposhini Nature Reserve, Pietermaritzburg, South Africa. Before germination, the seeds were scarified by soaking in 5% sodium hypochlorite for 20 minutes for sanitation. Thereafter, seeds were rinsed 5 times with distilled water. Seeds were scarified by clipping with a nail cutter away from the embryo, this is to allow the sourcing of the nutrients and air to contact with epicotyl (embryo shoot). Ten seeds were laid in petri dishes with filter papers for germination. Filter paper was watered when necessary to avoid drying as that can inhibit or delay the germination process. This was done until seedling emergence. Thereafter, seedlings were planted at a depth of 1-2 cm in 19 cm diameter pots. The experiment was a randomized block design.

T. triandra grass species were collected from the University of KwaZulu-Natal grassland located at the Agricultural campus, Pietermaritzburg, South Africa. A total of 10 genotypes was collected from different locations within the same grassland. The whole grass tussock was removed from the ground with its root system where the tiller segment (leaves) was cut off. The remaining root stem was separated into three smaller root stems and planted in pots. The competition experimental setup was a factorial randomized block design and replicated 10 times. *T. triandra* grass was planted 7 days after *V. nilotica* seedling transfer to pots. Each experimental setup was as follows; legume-grass growing in competition and legumes growing independently in P and -P soil treatments, making up four treatments. Plants were irrigated with tap water after every 2nd day, depending on the environmental temperature and soil moisture.

4.2.4 Plant harvesting and nutrients analysis

The first set of harvest (n=5) of initial plants was conducted after 30 days of seed germination. During harvest, plants were rinsed with distilled water separated into leaves, stems, roots, and nodules thereafter oven-dried at 65°C for 4 days. Their dry weight was recorded. Oven-dried plant material was pulverized to powder with a mortar and pestle using liquid N and sent for P, N, and C analysis and Nitrogen isotope analysis ($\delta^{15}\text{N}$) were inductively coupled mass spectrometry (ICP-MS) and LECO-nitrogen analyzer with suitable standards were used at Central Analytical Facilities, Stellenbosch University, and the Archeometry Department, University of Cape Town, South Africa, respectively.

4.2.5 Plant growth and nutrition calculations

4.2.5.1 Carbon construction cost

Carbon construction cost (CW) ($\text{mmol C g}^{-1} \text{ DW}$) was calculated according to (Mortimer *et al.*, 2005) equation modified from (Peng *et al.*, 1993) as follows;

$$\text{CW} = [(C + (kN/14) \times (180/24) \times (1/0.89) \times (6000/180)]$$

Where CW is the construction costs of the plant tissue ($\text{mmol C g}^{-1} \text{ DW}$), C represents the concentration of carbon (mmol C g^{-1}), *k* denotes the reduction state of N substrate (*k*= -3, NH_3), N represents nitrogen content of the tissue ($\text{g}^{-1} \text{ DW}$) (Williams *et al.*, 1987). The numerical value of 14 is the atomic mass of N, 180 is a conversion factor from moles to grams of glucose, and 24 is the electrons in a glucose molecule. The 1/0.86 fractions denote the estimates of growth efficiency (Peng *et al.*, 1993) and the 6000/180 is the conversion factor of units from g glucose DW^{-2} to $\text{mmol C g}^{-1} \text{ DW}$.

4.2.5.2 Specific N absorption rate (SNAR)

Total plant N was used to calculate the SNAR ($\text{mg N g}^{-1} \text{ root DW day}^{-1}$) which refers to the net nitrogen absorption rate per unit root DW and will be calculated as follows by (Nielsen *et al.*, 2001):

$$\text{SNAR} = [(N_2 - N_1/t_1 - t_2)] * [(\log_e R_2 - \log_e R_1) / (R_2 - R_1)]$$

Where N is the nitrogen content per plant and t is the time between germination and harvesting and R is the root dry weight.

4.2.5.3 Specific N utilization rate (SNUR)

Total plant N was used to calculate the SNUR ($\text{g DW mg}^{-1} \text{ N day}^{-1}$) to measure of the DW gained for the N and P taken up by the plant using the equation of Nielsen *et al.* (2001).

$$\text{SNUR} = (W_2 - W_1/t_2 - t_1) * [(\log_2 N_2 - (\log_2 N_1) / (N_2 - N_1)]$$

Where N is the nitrogen content per plant and t is the difference in time between germination and harvesting and R is the root dry weight.

4.2.5.4 Roots: shoot ratio (R: S)

Root: shoot ratio value of the plants was calculated as reported by (Agren and Frankin, 2003) equation:

$$\text{Root: shoot} = W_r / W_s$$

Where W represents the dry weight of the plants

4.2.5.5 Relative growth rate (RGR)

The relative growth (RGR) for both plants was calculated according (Agren and Frankin (2003):

$$\text{RGE} = ((\ln W_2 - \ln W_1) / (t_2 - t_1)),$$

Where W_1 AND W_2 represent the dry weight and t_1 and t_2 is the time taken for the plant to grow at initial and final harvest, respectively

4.2.6 Calculations of percentage N derived from the atmosphere

The nitrogen isotope analysis ($\delta^{15}\text{N}$) was conducted by a commercial laboratory, using inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyzer with suitable standards (Central Analytical Facilities, Stellenbosch University, and the Archeometry Department, University of Cape Town, South Africa). Thereafter percentage N derived from the atmosphere was calculated to determine the N source preference. The estimation of N_2 derived from the atmosphere to the plant budget will be calculated according to Cramer *et al.* (2010)

$$\% \text{Ndfa} = (\delta^{15}\text{N}_{\text{reference}} - \delta^{15}\text{N}_{\text{sample}}) / \delta^{15}\text{N} * 100,$$

Where %Ndfa is the percentage of nitrogen derived from the atmosphere. $\delta^{15}\text{N}_{\text{reference}}$ is the average $\delta^{15}\text{N}$ of the non-nodulating species and $\delta^{15}\text{N}_{\text{sample}}$ is the $\delta^{15}\text{N}$ of the *A. nilotica* species.

4.2.7 Data analysis

IBM Statistics 24 was used to analyze the effects of nutrient deficiency and nutrient competition on legume nodulation and N fixation in *V. nilotica* growing independently and with *Themeda triandra* in grassland ecosystem soils using one-way analysis of variance (ANOVA) (IBM SPSS Statistics 25). In cases where the data was not normally distributed, a Kruskal-Wallis test was performed and where the variances were not homogenous, a Welch test was performed along with a Temhane T2 Post-Hoc to determine differences within treatments. Different letters were used to show significant differences.

4.3 Results

4.3.1 Biomass and Growth kinetics

The biomass and growth kinetics, N and P mineral nutrition, and N preferences are presented in table 4.1. The total plant biomass was the highest from *V. nilotica* growing independently compared to *V. nilotica* growing with *T. triandra* across P and -P soils and significant difference were observed from -P treatment. The shoots biomass was significantly greater ($P \leq 0.05$) and the highest for *V. nilotica* growing independently in high P soils, no significant difference were observed from -P soils. Roots biomass of *V. nilotica* growing together with grass was significantly higher ($P \leq 0.05$) compared to *V. nilotica* growing independently in -P soils. Whereas roots biomass of *V. nilotica* growing independently was high. Only P soil grown plants nodulated and nodule biomass showed no significant differences ($P \geq 0.05$) between competition and independently grown experimental plants. *V. nilotica* grown in P soils had a significantly higher relative growth rate (RGR) compared to the *V. nilotica* grown in -P soils. RGR were observed to be higher in *V. nilotica* growing independently than *V. nilotica* growing with *Triandra triandra* in both P treatments. The C construction costs were higher in -P soils compared to P.

4.3.2 P and N mineral nutrition

P concentration of *V. nilotica* growing independently was significantly higher ($P \leq 0.05$) than *V. nilotica* growing with *T. triandra* across treatments. N concentration of *V. nilotica* growing together with *T. triandra* in P soils was higher than *V. nilotica* growing independently however, no significant difference was observed.

4.3.3 N source preferences

V. nilotica growing with *T. triandra* attained the highest ($78.760 \pm 5.316\%$) percentage of nitrogen derived from the atmosphere (%Ndfa) compared to legume *V. nilotica* growing independently ($71.120 \pm 6.192\%$) in P soils. This was followed by *V. nilotica* growing independently with $31.540 \pm 3.569\%$ and *V. nilotica* growing with competition with $27.43 \pm 0.168\%$ in -P soils. Plant N concentration derived from the soil (Ndfs) was high for *V. nilotica* growing independently and in competition in -P soils. Specific nitrogen absorption rates

(SNAR) were significantly higher for *V. nilotica* grown with *T. triandra* in P soils and *V. nilotica* growing independently in -P soils. Inversely, the specific nitrogen utilization rate (SNUR) was high for *V. nilotica* growing independently in P soils and *V. nilotica* growing with *T. triandra* in -P soils.

4.4 Discussion

Experimental soils were acidic and nutrient-poor across all treatments however, plant growth was observed and *V. nilotica* growing independently obtained the highest biomass in P soils. Miguez-Montero *et al.* (2020) obtained similar results where *Cytisus balangae* and *Cytisus scorparius* species showed 100% growth in 500 μM (high P) and decreased growth in 5 μM (low P) treatments. Also, under optimal P supply *Lupinus albus* and *Glycine max* increased shoots and roots growth whereas under low P there was a decrease in root and shoot growth (Wu *et al.*, 2021). Nutrient deficiency and induced competition by *T. triandra* might have contributed to *V. nilotica* plants increasing root biomass. Increasing root surface area for nutrient assimilation by increasing below-ground biomass is a survival strategy for legumes growing nutrient-deficient soils (Horst *et al.*, 2001). Minchin and Witty (2005) reported that increase in C-growth costs was associated with increased plant biomass. In our study, increased C-cost may be linked to increased roots biomass. Legume plants utilize both atmospheric and soil-derived N during soil nutrient deficiency and competition (Thomas and Sumberg, 1995). This concurs with our study results showing an increase soil N preference (NDFS) during nutrient stress and competition. During P deficiency, legumes tend to rely on N derived from the soils (Magadlela *et al.*, 2014) than atmospheric N due to high energetic requirement (Ledgard, 2001). Legumes growing in nutrient deficient soils and competition requires photosynthates for bacterial physiological processes, N fixation and production of plant tissues (Nielsen *et al.*, 1998). Total N concentration increased with an increase in SNAR in *V. nilotica* growing independently under -P soil treatment. These results concur with those of Ndzwana *et al.* (2018) where legumes growing independently assimilated soil N resulting in high absorption rate.

Interestingly, despite the lack of nodulation in P deficient soils, *V. nilotica* was still able to fix atmospheric N. This suggests that *V. nilotica* established symbiosis with free-living N fixing bacteria present in the soils. Legumes can rely on actinomycetes, gram-positive bacteria, for N fixation without nodulation (Bhatti *et al.*, 2017). Similarly, Matiwane *et al.* (2019) observed non-nodulation in *Vigna radiata* plants grown in acidic and nutrient poor soil conditions. Further explanations could be that the soil's high exchange acidity and low pH are the most likely reasons for non-nodulation (Li *et al.*, 2012). The possible reason for legume plant NDFA in soil grown plants may be the association of *V. nilotica* with bacteria in the rhizosphere or other endophytic spp. able to fix atmospheric N without inducing nodulation to reduces costs required by symbionts from plant hosts (Lu *et al.*, 2017; Martínez-Hidalgo and Hirsch, 2017). Hence, all soil bacterial identification showed the presence of various *Pseudomonas* spp. known as one of the most abundant found in the rhizosphere. Some *Pseudomonas* spp. have been reported to possess ability to fix atmospheric N by associated symbiotic combinations with non-legume plants (France *et al.*, 2009).

4.5 Conclusion

The results of this show that *V. nilotica* subjected to competition with grasses during nutrient stress increase below ground biomass. Also, the legume plant subjected to competition and grown independently in P deficient soils rely more on soil N. Also, *V. nilotica* established in -P soils assimilating atmospheric derived N suggests legume plant association with endophytic bacteria. Therefore, the results of this study show that grassland ecosystems in KwaZulu-Natal can support the co-occurrence of legume plants and grasses in these ecosystems.

Conflicts of Interest

We declare no conflict of interest concerning this study. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The opinions, findings, and conclusions/recommendations expressed in this work are that of the authors, and that NRF accepts no liability whatsoever in this regard.

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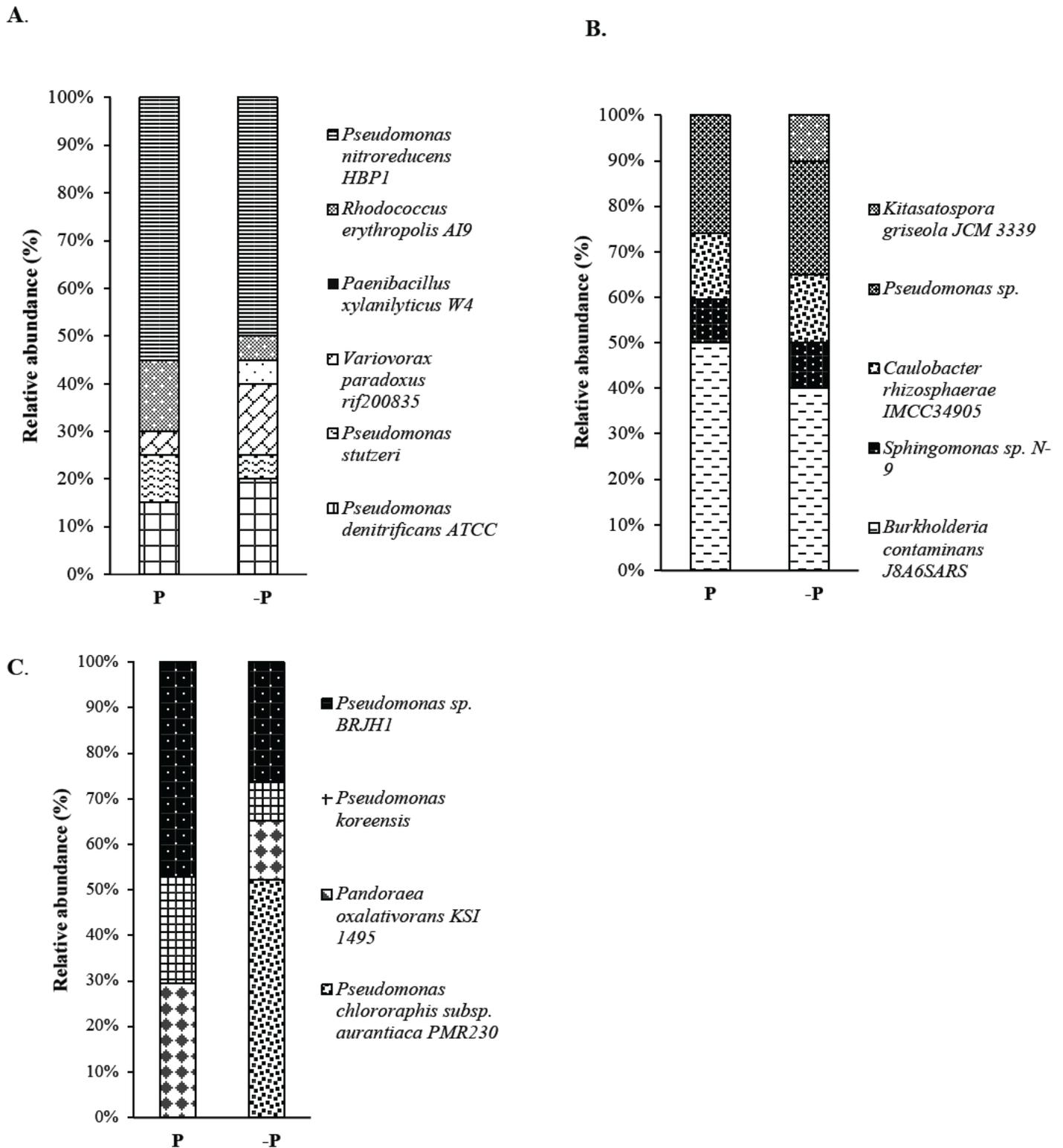
Table and figure legends

Table 4.1: Biomass accumulation, plant mineral nutrients, and growth kinetics of *V. nilotica* growing independently and in competing with *T. triandra* in phosphorus (P) and non-phosphorus fertilized soils (-P) at Ukulinga Grassland Nutrient Experiment (UGNE) trials. Values are expressed as means \pm SE, n=5. Significant differences ($p < 0.05$) among treatments are denoted by different superscript letters across the rows.

Parameters	+P soils		-P soils	
	<i>V.n</i> and <i>T.t</i>	<i>V.n</i>	<i>V.n</i> and <i>T.t</i>	<i>V.n</i>
Biomass				
Total plant biomass (g)	4.095 \pm 0.778 ^a	4.837 \pm 0.707 ^a	2.354 \pm 0.144 ^c	2.565 \pm 0.060 ^c
Shoots (g)	2.996 \pm 0.086 ^a	3.347 \pm 0.092 ^b	2.057 \pm 0.195 ^c	2.358 \pm 0.061 ^c
Nodules DW (g)	0.044 \pm 0.014 ^a	0.045 \pm 0.151 ^a	NN	NN
Roots DW (g)	1.085 \pm 0.698 ^a	1.448 \pm 0.701 ^a	0.297 \pm 0.066 ^c	0.208 \pm 0.062 ^d
RGR (g mg ⁻¹ day ⁻¹)	0.249 \pm 0.028 ^a	0.301 \pm 0.044 ^a	0.011 \pm 0.001 ^c	0.0120 \pm 0.0003 ^d
Plant mineral nutrition				
%NDFAs	78.760 \pm 5.316 ^a	71.120 \pm 6.192 ^b	27.43 \pm 0.168 ^c	31.540 \pm 3.569 ^d
NDFS (mmol N g ⁻¹)	0.286 \pm 0.050 ^a	0.347 \pm 0.066 ^b	0.657 \pm 0.044 ^c	0.6911 \pm 0.046 ^c
Total plant N (mmol N g ⁻¹)	1.347 \pm 0.246 ^a	1.203 \pm 0.159 ^a	0.9200 \pm 0.109 ^c	1.240 \pm 0.185 ^d
Total plant P (μ mol P g ⁻¹)	39.980 \pm 8.580 ^a	53.460 \pm 5.00 ^b	12.75 \pm 0.930 ^c	16.080 \pm 1.100 ^d
Std corrected (¹⁵ N/ ¹⁴ N)	-1.117 \pm 0.366 ^a	-0.590 \pm 0.419 ^a	2.4200 \pm 0.012 ^c	2.137 \pm 0.246 ^c
Carbon Construction Costs				
(mmol. C. g ⁻¹)	0.010 \pm 0.001 ^a	0.0101 \pm 0.0004 ^a	0.008 \pm 0.0007 ^c	0.007 \pm 0.0002 ^d
Shoot: roots ratio	0.350 \pm 0.2170 ^a	0.431 \pm 0.2076 ^a	0.153 \pm 0.066 ^c	0.089 \pm 0.062 ^d
SNAR (mg N g ⁻¹ root DW day ⁻¹)	11.001 \pm 2.014 ^a	8.503 \pm 1.146 ^b	5.352 \pm 1.187 ^c	7.078 \pm 1.490 ^d
SNUR (mg N g ⁻¹ root DW day ⁻¹)	9.830 \pm 0.701 ^a	11.238 \pm 1.257 ^b	4.4675 \pm 0.583 ^c	3.3243 \pm 0.083 ^d

Supplementary Table 4.1: Macronutrient concentrations (P, N, K, C, Ca, and Mg), exchange acidity, total cations, and pH in soils fertilized with P and -P at Ukulinga Grassland Nutrient Experiment (UGNE) trials. Values are expressed as means \pm SE, n=5. Significant differences ($p < 0.05$) among treatments are denoted by different superscript letters across the rows.

Parameters	P soils	- P soils
pH	4.587 \pm 0.044 ^a	4.289 \pm 0.222 ^a
Ca concentration (mmol/g)	38.240 \pm 1.548 ^a	26.541 \pm 1.921 ^b
P concentration (mmol/g)	0.308 \pm 0.026 ^a	0.072 \pm 0.016 ^b
K concentration (mmol/g)	4.848 \pm 0.849 ^a	2.953 \pm 0.364 ^a
N concentration (mmol/g)	0.205 \pm 0.003 ^a	0.200 \pm 0.007 ^a
Mg concentration (mmol/g)	15.740 \pm 1.273 ^a	14.361 \pm 2.678 ^a
C concentration (mmol/g)	3.810 \pm 0.102 ^a	3.616 \pm 0.0345 ^a
Total cations (cmol L ⁻¹)	14.990 \pm 8.352 ^a	11.240 \pm 5.882 ^b
Exchangeable acidity (cmol L ⁻¹)	0.133 \pm 0.009 ^a	1.967 \pm 1.279 ^b



Supplementary figure 4.1. Relative abundance of microbial communities determined from soils collected from the nutrient trials at the Ukulinga Research Farm. (a) P solubilizing, (b) N fixing, and (c) N cycling bacteria.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

5.1 General discussion and conclusion

Low nutrient availability specifically N and P (Luo *et al.*, 2019) and soil acidity (Roberts *et al.*, 2003) has been reported in savanna and grassland ecosystem soils (Pellegrini *et al.*, 2016). The imbalance between these nutrients in ecosystems may affect plant productivity and vegetation composition (Elser *et al.*, 2007) and crop production given the increasing population with high agricultural demands (Pang *et al.*, 2018). Legume establishment in nutrient-poor ecosystems has been reported to increase soil N due to their N fixing ability (Jhariya *et al.*, 2018). Atmospheric N fixation by legumes can facilitate soil N inputs for co-occurring plant species, promoting plant growth and productivity and are considered for sustainable nutrient management practices in nutrient-poor ecosystems (Pirhofer-Walzl *et al.*, 2012). However, legumes plants are mainly affected by these deficient nutrients as they required P as ATP as an energy driver during atmospheric N fixation (Mitran *et al.*, 2018). Insufficient P in soils in legume plants affects nodulation, nodule metabolism (Divito and Sadras, 2014) and consequently plant N accumulation (Yahiya and Fatna, 1995).

Chapter 3 aimed at assessing the effects of soil nutrient variability, potential of soil-borne microbes and associated enzymatic activities in remediating nutrient-poor soils. The presence of P solubilizing and N fixing bacteria such as *Pseudomonas sp.* and *Burkholderia contaminans* were identified in the experimental soils. The presence of P solubilizing bacteria in nutrient deficient and acidic soils enhances soil P concentration which is mostly not available for plant utilization due to toxic cations (Chen *et al.*, 2006). Microbes play a critical role in cycling of C, N and P nutrients which contributes to soil structure and function making it habitable for host plants (Aislabie *et al.*, 2013) and agricultural practices (Tosi *et al.*, 2020). Soil microbes can also regulate, and release associated enzymes such as phosphatase and β -Glucosaminidase which are important in nutrient mineralization in nutrient ecosystems (Caldwell *et al.*, 2005). Soil-borne microbes in nutrient cycling and mineralization using extracellular enzymes may play an important role in nutrient changes in KwaZulu-Natal (KZN) grassland and savanna ecosystem soils.

Chapter 4 examined the physiological adaptations of the legume, *V. nilotica*, growing in competition with *T. triandra* and in P deficient soils. Grass-legume interaction has been practiced due to its greater yield of herbage from grasses and legume plants (Haynes, 1980). During this interaction there is a transfer of atmospherically fix N by legume to non-legume species (Pirhofer-Walzl *et al.*, 2012). However, this interaction introduces soil nutrient competition between these species (Cramer *et al.*, 2010) affecting N assimilation by legumes. Nodulation in *V. nilotica* was inhibited by the presence of *T. triandra* in nutrient deficient soils. However, this legume acquired atmospheric N suggesting its symbiotic interaction with rhizobacteria. Similar to the results of this study, legume plants have been observed to shift N preference from atmospheric derived N to soil-derived N in nutrient poor soil conditions (Mohammadi *et al.*, 2012). Also, Ndzwana *et al.* (2018) reported switching of N sources

in *Vachellia sieberiana* growing with invasive *Chromolaena odorata* in P deficient savanna environment soils. Increasing root surface area for nutrient assimilation by increasing below-ground biomass is considered as one of survival strategies for legumes growing in nutrient-deficient soils (Horst *et al.*, 2001).

5.2 Recommendations for future work

This study may be used as a baseline study for further molecular and genetic studies. Moreover, to investigate the metabolic and energy-saving strategies that affect native legume physiological responses to grass competition in nutrient-poor ecosystem soils. Such research may also provide insights into plant-microbe symbiosis and plant physiological adaptations, which may, in turn, be applied successfully in ecosystem nutrient enrichment using native legumes.

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