EFFECTS OF PLANT GROWTH-PROMOTING RHIZOBACTERIA AND BIOCONTROL BACTERIA ISOLATED FROM KAVANGO ON *PENNISETUM GLAUCUM* AND *SORGHUM BICOLOR*

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Abstract

As a result of the ever increasing population growth, the demand on food is expected to rise significantly. In order to meet this increase in food demand, alternatives (PGPR and biocontrol bacteria) to environmentally damaging chemical augmenters can be utilized for the production of agricultural crops. This study was conducted to isolate and screen rhizospheric bacteria from grasses along the Kavango River for plant growth promoting and biocontrol abilities. Once validated, bacteria possessing these abilities were used as peat-based inoculants to evaluate their effects on the growth of *Pennisetum glaucum* and *Sorghum bicolor*. The bacteria were isolated from rhizospheres of *P. australis, Sporobolus sp., V. nigritana, P. glaucum, S. bicolor* and a grass known locally as ngwena. The isolates were screened for NH_3 production, protease activity, phosphate solubilisation, siderophore production, indole-3-acetic acid production, *nifH*-gene presence and antifungal-activity. Up to 14 bacteria considered as possessing plant growth enhancing (11) or biocontrol (3) traits were isolated from grasses located at Mashare and surrounding areas. The majority of the isolates exhibited more than one trait and were classified as follows; 3 phosphate solubilizers, 4 siderophore producers, 8 IAA producing isolates, 5 N_2-fixers, and 3 isolates with antifungal-activity. *S. maltophilia* LCS2-11 as single treatment and in combination treatment with *B. amyloliquefaciens* LSM1-61 and *P. stutzeri* ACM2-32, enhanced *P. glaucum* biomass statistically similarly to the commercial fertilizer's growth effects. The combination treatment T_9 (*B. amyloliquefaciens* LSM1-61: *E. cloacae* FCM2-50: *P. stutzeri* ACM2-32) enhanced *S. bicolor* biomass significantly ($p = 0.032$) compared to the water control. Single inoculants *S. maltophilia* LCS2-11, *E.*

cloacae FCM2-50, and *B. amyloliquefaciens* LSM1-61 and combination inoculants T_7, T_8 and T_9, enhanced *S. bicolor* root biomass as well as the commercial fertilizer. The results indicate that PGPR and biocontrol bacteria can be used as efficient plant growth enhancing agents.

Keywords: PGPR, biocontrol bacteria, inoculants, plant growth enhancing, *Pennisetum glaucum, Sorghum bicolor*

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
DNA	Deoxyribonucleic acid
et al.	et alia (and others)
FLP's	Fluorescent pseudomonads
GC	Guanine-cytosine
i.e.	id est (that is)
IAA	Indole-3-acetic acid
IaaH	IAM hydrolase
IAAld	Indole-3-acetaldehyde
IaaM	Tryptophan- 2-monooxygenase
IAM	Indole-3-acetamide
IAN	Indole-3-acetonitrile
IPDC	Indole-3-pyruvate decarboxylase
IPyA	Indole-3-pyruvate
ISR	Induced systematic resistance
MHB	Mycorrhization-helper bacteria
nif	Genes encoding for nitrogen-fixation enzyme nitrogenase
nifD	Gene encoding for the dinitrogenase α-subunit
nifH	Gene encoding for the dinitrogenase reductase
nifK	Gene encoding for the dinitrogenase β-subunit
OD_{600}	Absorbance/optical density measured at 600 nm wavelength
OD_{660}	Absorbance/optical density measured at 660 nm wavelength

PCR	Polymerase chain reaction
PGPR	Plant growth-promoting rhizobacteria
rpm	Revolutions per minute
sp.	Species (singular)
spp.	Species (plural)
TAM	Tryptamine
TSO	Tryptophan side-chain oxidase
v/v	% volume per volume

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Dedication

I dedicate this thesis to my family. I am extremely grateful for my parents Jakobine Haiyambo, a constant source of inspiration, and Jafet Haiyambo, whose words of encouragement I remember to this day, you are missed. To my siblings whom I will always look up to, your care and love is appreciated.

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Declaration

I, **Daniel H Haiyambo**, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

……. Date……

Daniel H. Haiyambo

CHAPTER 1: INTRODUCTION

1.1 Research overview

The world population is continually growing and is estimated to increase by a third in 2050; correspondingly the demand for agricultural products will have risen by 70% (Noble & Ruaysoongnern, 2010). In response to a growing population and the accumulative environmental damage caused by the use of agrochemicals, sustainable alternative methods for improving crop agricultural output must to be developed in order to meet food demands. Scientific approaches towards solving agricultural issues like the application of ecologically unfavourable fertilizers and the control of plant diseases can be accomplished by using microorganisms known as plant growth-promoting rhizobacteria (PGPR) and biocontrol bacteria (Akhtar & Siddiqui, 2011; Glick, 2012).

PGPR are able to influence plant growth because they are central participants of crucial metabolic processes which have an impact on soil functions (Nannipieri et al., 2003). Important environmental processes such as biogeochemical cycling of nutrients (carbon, nitrogen, phosphorus, and sulphur) and maintaining soil quality and plant health are accountable to various interactions within different microorganisms (Barea, Azcón, & Azcón-Aguilar, 2004). Disease-suppression capabilities of biocontrol bacteria are taken advantage of to improve plant health (Handelsman & Stabb, 1996). The mechanisms of biological control are mediated by the synthesis of bacterial allochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxication enzymes, among others (Glick, 1995; Compant, Duffy, Nowak, Clèment, & Barka, 2005).

1.2 PGPR and Biocontrol Bacteria enhance Plant Growth

There are many soil dwelling bacteria that play crucial roles in the enhancement of plant growth. These bacteria are able to colonize the rhizosphere and in some instances enter roots of plants, where they prompt a beneficial effect on the host plant (Kloepper, Leong, Teintze, & Schroth, 1980). Rhizobacteria that exert beneficial effects on plant development are referred to as plant growth-promoting rhizobacteria (PGPR) or biocontrol bacteria because their association with a plant often leads to increased rates of plant growth (Kloepper & Schroth, 1978). The rhizosphere is a habitat to many bacteria which are either detrimental, beneficial or have no effect to the plants' health, an estimated 2–5% of the rhizosphere bacteria are plant growth-promoting bacteria (Antoun & Prévost, 2005). Various groups of bacteria in association with the plant rhizosphere have been shown to be beneficial for the growth, yield and crop quality of plants (Orhan, Esitken, Ercisli, Turan, & Sahin, 2006).

After colonizing the root these bacteria prompt plant growth promotion or biological control of plant disease (Lee et al., 2010) and in some cases both effects are caused by individual species. Plant growth promotion is based on increasing nutrient cycling and producing biologically active substances such as plant hormones (Gül, Ozaktan, Kıdoğluc, & Tüzel, 2013). Whereas plant pathogen biological control is regulated by mechanisms such as competition of root colonization, producing bacterial allelochemicals, and induced resistance (Compant et al., 2005).

1.3 Subsistence farming in Kavango, Zambezi and the Northern Regions

The Kavango region (now separated into West and East Kavango) is located in the North East of Namibia and is home to the Kavango River which flows from the Southern areas of Angola through North Eastern Namibia and finally empties into the Okavango Delta of Botswana's Kalahari Desert. A relatively dense portion of the region's total human settlements is naturally located along the Kavango River. The majority of those settlements consist of rural households which rely on small-scale farming for domestic consumption; these activities commonly entail growing *Pennisetum glaucum* (mahangu) as the dominant crop while sorghum, maize and vegetables (melon, groundnut, beans, spinach and pumpkins) take up a smaller area of the farming land (Mendelsohn, 2009). Mahangu is a popular crop not only in the region, but in settlement areas across North and North Eastern Namibia, because it is a hardy crop that is able to grow in the Kalahari sands also known as arenosols, which most of the region consists of (Coetzee, 2004).

However, the subsistence farmers experience poor harvests from their farming activities which use no agrochemical inputs on what are already considered nutrient poor soils (Grönemeyer, Burbano, Hurek, & Reinhold-Hurek, 2011). Methods of small scale-farmers require clearing of vegetation for new fields and ploughing for established ones before the first rains. The decrease of plant biomass in farming areas coupled with no fertilizer usage has caused a decrease in the overall soil fertility (Mendelsohn, 2006). The decline of soil fertility and deteriorating soil structures, eventually results in a decrease in crop yields, therefore there is a need to develop improved soil fertility management practices (Namibia Resource Consultants & Vigne & Associates Consultants, n.d.).

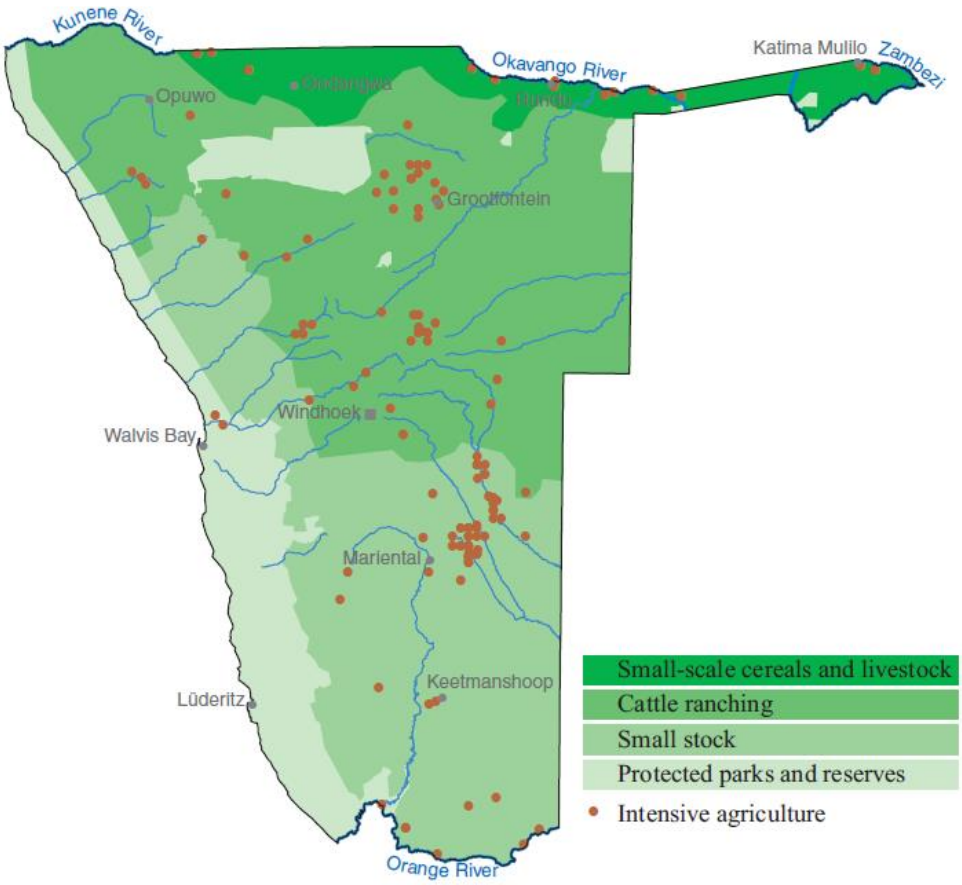


Figure 1. The distribution of farming methods in Namibia. Adapted from Farming Systems in Namibia by J Mendelsohn, 2006, p. 9. Copyright 2006 by RAISON (Research & Information Services of Namibia).

It would be beneficial for the welfare of Namibia, to promote the sustainable use of land assets in a way that improves crop production in order to aid inhabitants with their farming-dependent livelihoods. According to Tilston, Sizmur, Dixon, Otten, and Harris (2010), sustainable agriculture is achieved through managing natural resources, more so than synthetic inputs, to maximize production. It follows that the naturally occurring organisms, plant growth promoting bacteria and biocontrol bacteria, can be used under various treatments as biological fertilizers,

phytostimulators and biological pesticides to enhance plant growth in a sustainable manner (Gholami, Shahsavani, and Nezarat, 2009; Gül et al., 2013; Karakurt & Aslantas, 2010; Orhan, Esitken, Ercisli, Turan, & Sahin, 2006). Accordingly, this study focuses on screening rhizospheric bacteria which are well adapted to the specific soil environment of Mashare (East Kavango) for various plant enhancing capabilities and assessing the growth effects with regards to practical application of PGPR and biocontrol bacteria, on staple crops mahangu and sorghum. The plants were grown in soil from the Mashare area and peat was used as a carrier material for the bacterial inoculation suspensions.

1.4 Statement of the Research Problem

Most of the soils that subsistence farmers in Namibia use for crop agricultural lack sufficient amounts of nutrients such as nitrogen, iron and phosphorus to support optimal plant growth (Glick, 2012). Since the "Green revolution" in the 1950s, farmers all over the world have been using chemical products to obtain high yields from crops. This dependence on the utilization of chemical fertilizers along with herbicides, fungicides, and insecticides has recently been described as causing long term negative effects on the environment. However, in Namibia and more generally Africa's case the majority of subsistence farmers do not use chemical fertilizer because it is not economically viable (Grönemeyer et al., 2014), hence the farming communities experience low yield from their crop harvest. Studies have shown that crop yields of cereal subsistence farming, of which nearly half of the population are dependent on, are low and have been on the decline for 30 years (Mendelsohn, 2009). At present subsistence farming practices have to make ground on improving agricultural

productivity; nevertheless the lessons from green revolution have indicated that we should direct our attention to alternative agricultural crop enhancing techniques, this is where PGPR and biocontrol bacteria can play a role (Chianu, Nkonya, Mairura, Chianu, & Akinnifesi, 2011).

1.5 Objectives

The specific objectives of the study were:

1. To isolate rhizosphere bacteria from grasses along the Kavango River and screen the isolates for the plant growth promoting and fungal antagonistic abilities.
2. To evaluate the growth effects of treating *Sorghum bicolor* and *Pennisetum glaucum* seed with the isolated plant growth-promoting rhizobacteria and biocontrol bacterial suspensions.

1.6 Research Hypotheses

This study was done to test the hypotheses that:

1. Bacteria isolated from the rhizospheres of grasses along the Kavango River possess plant growth promoting and microorganism antagonistic capabilities.
2. PGPR and biocontrol bacteria from grasses along the Kavango River can enhance the growth of *S. bicolor* and *P. glaucum* via single bacterial suspension treatment and via combination bacterial suspension treatment.

1.7 Significance of the Study

The outcomes of this study will be significant in developing strategies for utilizing endemic plant-associated bacteria in order to improve sustainability and productivity of crop farming in Namibia. This will contribute to increasing agricultural productivity in Namibia, thus overcoming the challenge of feeding a growing population in an efficacious yet prudent manner, i.e. providing practical and affordable scientific solutions to local farmers.

CHAPTER 2: LITERATURE REVIEW

2.1 The Rhizosphere is a Habitat for Microorganisms

The organic material which is deposited in the soil by the growing root system of a plant provides a suitable environment for microorganisms to propagate around the roots (Dardanelli et al., 2010). The volume of soil affected by the presence of the roots of a plant is defined as the rhizosphere (Uren, 2007) and this region provides a habitat for a sizable number of microorganisms including bacteria, fungi, algae and protozoa (Saharan & Nehra, 2011). The region between the rhizosphere and endorhizosphere (root cortex) supports a continuous plane of microbial populations that are very much alike with reference to microbial ecology (Manoharachary & Mukerji, 2006). Amongst the microorganisms, bacteria prevail as the most abundant in numbers. Plants select bacteria that are able to utilize organic compounds from the root exudates thus creating a relatively low bacterial diversity environment around the plants roots (Saharan & Nehra, 2011).

The root exudates that are released are products of the plants natural processes for e.g. photosynthesis; these compounds consist of sugars and polysaccharides, amino acids, organic acids, fatty acids, sterols, growth factors, enzymes, flavonones, nucleotides and miscellaneous compounds (Uren, 2007). Antoun and Prévost (2005) report that exchanges of signal molecules and biochemical interactions between plants and soil microorganisms in the rhizosphere can influence plant growth and crop yields considerably. Since the bacteria are the most abundant microorganisms in the rhizosphere it is highly likely that they have a greater influence on plant physiology (Saharan & Nehra, 2011).

2.2 Plant Growth-promoting Bacteria and Biocontrol Bacteria

PGPR and biocontrol bacteria are originally free-living soil bacteria that colonize the rhizosphere or the tissues of living plants (Antoun & Prévost, 2005). Once the bacteria are in association with the plant, by colonizing the rhizosphere around the roots, the rhizoplane (root surface) or the root itself (Dardanelli et al., 2010), the bacteria are able to exert an indirect or direct growth enhancing effect on the plant (Kloepper & Schroth, 1978). The indirect mechanisms include production of antibiotics against pathogenic microorganisms, reduction of iron availability to phytopathogens, production of fungal cell wall-degrading enzymes, and competition with other microorganisms for sites on plant roots (Lucy, Reed & Glick, 2004). Whilst direct mechanisms of plant growth include increasing the availability of soluble phosphorus compounds for plant uptake, increasing iron availability to plants, nitrogen fixation, production of plant hormones and regulation of ethylene concentration (Lucy et al., 2004). Members of the genera *Arthrobacter, Azoarcus, Azospirillum, Bacillus, Burkholderia, Enterobacter, Gluconacetobacter, Herbaspirillum, Klebsiella, Paenibacillus, Pseudomonas*, and *Serratia* are well known to possess plant growth enhancing capabilities (Dardanelli et al., 2010).

Members of the *Azospirillum* group are free-living rhizobacteria and the best recognized genus of PGPR. Currently ten species have been classified according to their biochemical and molecular characteristics. Although initially isolated from cereals, *Azospirillum* has proven to be more successful as an inoculant in non-cereal species (Saharan & Nehra, 2011). *Azospirlla* are occasionally endophytic (Sumner, 1990) although they are generally root surface colonizing bacteria, this happens in a two-step process; the polar flagellum, a glycoprotein flagellin, mediates the adsorption

step thereafter a polysaccharide functions in the anchoring phase (Steenhoudt & Vanderleyden, 2000).

Bacteria from the genus *Azospirillum* promote the growth of plants by fixing nitrogen, they also produce phytohormones. As early as 1929, Sen (1929) suggested that the activity of nitrogen fixing bacteria such as *Azospirillum* could meet the nitrogen requirements of cereal crops. The combination of producing substances that improves root development for increased water and mineral uptake (Fages, 1994) and N_2-fixing capability results in an increase of yield from crops. Consistent increases in crop yield from *Azospirillum* inoculation were observed by Rodríguez Caćeres, Di Ciocco, and Carletti (2008) under water deficient and limited soil nutrient (low organic matter) conditions. *Azospirillum* inoculation formulations for crops are currently commercially available in Europe and Africa (Vessey, 2003).

An aerobic/microaerophilic *Azoarcus* sp. that was isolated from kaller grass (*Leptochloa fusca* L Kunth) proved to be very effective at nitrogen-fixing, (Reinhold, Hurek, Niemann, & Fendrik, 1986). Gnotobiotic studies showed that one strain *Azoarcus* colonizes rice roots endophytically; root invasion occurs in the elongation and differentiation zone at the root tip, followed by intercellular and intracellular colonization as reported by Reinhold-Hurek and Hurek (1997). The two species, *A. indigens* and *A. communis* and additional three unnamed groups, distinct at species level, have been identified as PGPR (Saharan & Nehra, 2011).

The spore-forming bacteria of *Bacillus* genus are an abundant group of bacteria in both the rhizosphere and soil. *Bacillus* species are well suited to their role as biocontrol bacteria and PGPR, offering protection against root pathogens because of their ability to form endospores, broad-spectrum activity antibiotic production (Quan,

Wang, & Fan, 2010). *Bacillus* species have been documented to produce 167 biological antagonistic compounds which are active against bacteria, fungi, protozoa, and viruses (Bottone & Peluso, 2003). Most of the antibiotics produced by *Bacillus* spp. are peptides that are effective against both Gram positive and Gram-negative bacteria and filamentous fungi (Quan et al., 2010).

Burkholderia species inhabit a wide variety of ecological niches including soil, water, in and on fungal mycelia and in the plant's shoot, root and rhizosphere (LiPuma, Spilker, Coenye, & Gonzalez, 2002; Quan et al., 2010; Salles, Van Veen, & Van Elsas, 2004). *Burkholderia* species are recognized for their biological and metabolic properties, which can be used for biological control of diseases from soil-borne plant pathogens and plant-growth promotion (Quan et al., 2010). *Burkholderia cepacia* complex (Bcc) are a group of at least nine species which are abundant in soil, these bacteria are genetically distinct and possess a wide array of beneficial properties including indole-3-acetic acid (IAA) production, nitrogen-fixation, and the production of antimicrobial compounds, including cepacin, cepaciamide, cepacidines, altericidins, pyrrolnitrin, quinolones, phenazine, siderophores, and a lipopeptide (Quan et al., 2010).

Several members from genera such as *Enterobacter, Klebsiella,* and *Serratia* of the Enterobacteriaceae family (Kennedy, Choudhury, & Kecskés, 2004) usually found residing in contaminated soil and water (Singleton & Sainsbury, 2006), possess plant growth enhancing properties. An *Enterobacter* isolate from maize roots produced indole-3-acetic acid, plant polymer hydrolyzing enzymes, pectinase and cellulase as well as ammonia in vitro (Ogbo & Okwonko, 2012). Xue et al. (2009) reported that *Enterobacter* sp. (Xy3) and an *Acinetobacter* strain (Xa6) proved to be successful

biocontrol inoculants in tomato plants, resulting in higher biocontrol efficacy and plant-yield increase compared to the control.

Strains of *K. pneumoniae* (Maczulak, 2011) and *K. oxytoxa* (Bao et al., 2013) are of agricultural importance because they are able to carry out biological nitrogen fixation in soil. Bacterial cells of non-motile *Klebsiella* species are surrounded by a capsule which helps in protection of the cell, nutrient uptake, and adhesion to surfaces (Maczulak, 2011; Singleton & Sainsbury, 2006). The capsules' adhesion qualities may aid in efficient colonization of the rhizosphere and inside the root as demonstrated in a study by Dong, Iniguez, and Triplett (2003), which showed *Klebsiella pneumoniae* 342 as an efficient colonizer in five host plants (*Medicago sativa*, *Medicago truncatula*, *Arabidopsis thaliana*, *Triticum aestivum*, and *Oryza sativa*). Endophytic *K. pneumoniae* strains 2028 and 342 are able to produce nitrogenase in maize under the appropriate plant cultivation conditions (Chelius & Triplett, 2001).

Serratia lives in the gastrointestinal tract but also does well in water and soil, on plants, and inside insects (Maczulak, 2011), bacteria from this genus have been documented to enhance plant growth and subdue plant disease. Guo, Ying, Guo, Ge, Gong, Zhang, and Sun (2004) showed that *Serratia* J2, *Pseudomonas*, and *Bacillus* BB11 suppressed wilt of tomato and increased the yield. Whereas Zahir, Ghani, Naveed, Nadeem, and Asghar (2009) reported that *Serratia proteamaculans* (M35) improved growth and yield of wheat (*Triticum aestivum L.*) by producing 1-aminocyclopropane-1-carboxylic acid-deaminase (ACC demaminase) which alleviated stress induced inhibitory levels of ethylene.

Pseudomonas species are common bacteria in agricultural soils, water, and plant surfaces and have various traits that brand them as suitable PGPR (Saharan &

Nehra, 2011). These bacteria usually live in commensal relationships with plants, using nutrients from the plant and occupying sites provided by the plant, while the bacteria produces of antibiotics, siderophores, hydrolytic enzymes, biosurfactants; competition for sites on the plant; aids in triggering ISR (induced systemic resistance) and act as mycorrhization-helper bacteria (MHB) all in favour of the plants health (Quan et al., 2010). *Fluorescent pseudomonads* (FLPs) are the most effective strains of *Pseudomonas* thus far (Saharan & Nehra, 2011). They produce a wide range of metabolites that are useful in the biocontrol of plant diseases (Ramette et al., 2011). *Pseudemonads* species also synthesize plant growth stimulating hormones and promote plant mechanisms involved in disease resistance (Dardanelli et al., 2010).

2.3 Antibiotics from Bacteria in the Rhizosphere

Rhizobacteria such as *Pseudomonas, Bacillus, Azospirillum, Rhizobium* and *Serratia* species plays a vital role in the management of plant diseases to increase crop productivity via various mechanisms (Fernando, Nakkeeran & Zhang, 2005). Biocontrol can be referred to as the use of one organism to restrict the growth and proliferation of another one that is not preferred. Bacteria that can be defined as biocontrol organisms are used to reduce pathogen survival or activity with the goal of reducing the disease occurrence caused by the pathogen, (Garrette, 1965).

One mechanism by which biocontrol bacteria are able to accomplish suppression of phytopathogens is by secreting extracellular metabolites known as antibiotics; which are compounds able to inhibit or kill susceptible microorganisms at low concentrations (Singleton & Sainsbury, 2006). Numerous antibiotics including 2,4 Diacetyl phloroglucinol, phenazine-1-carboxyclic acid, phenazine-1-carboxamide,

pyoluteorin, pyrrolnitrin, oomycinA, viscosinamide, butyrolactones, kanosamine, zwittermycin-A, aerugine, rhamnolipids, cepaciamide A, ecomycins, pseudomonic acid, azomycin, antitumor antibiotics FR901463, cepafungins and antiviral antibiotic karalicin have been documented as compounds produced by various plant growth enhancing bacteria (Fernando et al., 2005). As a way of preventing plant pathogens from increasing in number, the production of antibiotics is one of the most effective mechanisms for biocontrol bacteria to ensure good plant growth (Akhtar & Siddiqui, 2011).

A study by Handelsman and Stabb (1996) was one of the earliest to implicate antibiotics from fluorescent pseudomonads in successful biocontrol. A number of *Pseudomonas* and *Burkholderia* spp. are reported to produce broad spectrum Pyrrolnitrin and Pyoluteorin (Quan et al., 2010). Positive autoregulation mechanisms, GacA/GacS or GrrA/GrrS, RpoD, and RpoS, and N-acyl homoserine lactone are responsible for antibiotic production regulation (Bloemberg & Lugtenberg, 2001; Brodhagen, Henkels, & Loper, 2004; Chancey, Wood, & Pierson, 1999; Haas & Keel, 2003; Pierson, Wood, & Pierson, 1998). Furthermore, plant growth and development also influences antibiotic production by certain bacteria (Picard, Di Cello, Ventura, Fani, & Guckert, 2000).

Bacterial antibiotics are effective in direct antipathogenic activity, they also serve as trigger elements in ISR of the plant's defence system and contribute further to disease suppression by granting a competitive advantage to the biocontrol bacteria (Fernando et al., 2005). The plant's induced or acquired resistance is described as an increase in the level of resistance via external agents, without any modification of the plant genome occurring (Figueiredo, Seldin, Araujo, & Mariano, 2010). Studies by

Audenaert, Pattery, Cornelis, and Höfte (2002), Haas and Keel (2003), Leeman et al. (1995) and Vleesschauwer and Höfte (2009) have reported that antibiotics in combination with other elements operate in tandem to trigger ISR in plants.

The production of antibiotics by bacterial biocontrol agents can suppress soil borne plant pathogenicity (Raaijmakers, Vlami, & De Souza, 2002). Two studies (Haas et al., 1991; Wirthners, & Défago, 1992) have demonstrated that antibiotic production and plant disease suppression have a relationship which is positively correlated.

2.4 Fungal Cell Wall-Degrading Enzymes

A wide range of bacteria are able to reduce the level of fungal pathogenicity by producing what are known as cell wall-degrading enzymes, examples of these include glucanases, proteases, chitinases and cellulases which are all capable of hydrolyzing susceptible fungal cell walls. Among the beneficial rhizobacteria, members from the genera *Bacilli*, *Pseudomonads*, and *Burkholderia* have been best studied and are attributed to having protective effects which are largely due to production of antifungal metabolites (Quan et al., 2010). Bacteria that belong to the genus *Bacillus* are able to produce both antibiotics and different types of fungal cell wall-degrading enzymes, strains of *B. megaterium*, *B. cereus*, and *B. subtilis* have been used as biocontrol agents (Idris, Labuschagne, & Korsten, 2008; Kildea et al., 2008), and are available commercially as Serenade, EcoGuard, Kodiak, Yield Shield, and BioYield (Quan et al., 2010).

Pseudomonas stutzeri produces extracellular chitinases and laminarinas which are vital for the bacteria to act against the growth of *Fusarium solani* (Lim, Kim, &

Kim, 1991). The β-1, 3-glucanase that is synthesized by *Burkholderia cepacia* is able to disrupt the cell wall integrity of *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium ultimum* according to Fridlender, Inbar and Chet (1993). The plant growth-promoting rhizobacteria, *S. plymutica* IC14 synthesize proteases and incorporate other biocontrol traits in order to suppress *B. cinerea* and *Serratia sclerotiorum* (Kamensky, Ovadis, Chet, & Chernin, 2003). Regulation of cell wall-degrading enzyme production, specifically proteases and chitinases, as in the case of antibiotics and siderophores (Compant et al., 2005) involves GacA/GacS (Corbell & Loper, 1995; Gaffney et al, 1994; Natsch, Keel, Pfirter, Hass, & De´fago, 1994; Sacherer, De´fago, & Hass, 1994) or GrrA/GrrS regulatory systems (Ovadis et al., 2004) and colony phase variation (Lugtenberg, Dekkers, & Bloemberg, 2001).

2.5 Microbial Siderophores

Siderophores are low-molecular weight compounds that are produced by microorganisms to chelate ferric iron so that it can be taken up (Singleton & Sainsbury, 2006). The function of siderophores is to scavenge iron from the nearby environment and to make the mineral available to the cell (Neilands, 1995). Depending on the type of moiety donating the oxygen ligands for Fe (III) coordination, siderophores are grouped either as catecholates (catecholates and phenolates), hydroxamates or α-hydroxy carboxylates (Miethke & Marahiel, 2007).

Enzymes and transport mechanisms that function in siderophore biosynthesis, secretion, siderophore-delivered iron uptake, and iron release (Miethke & Marahiel, 2007) are closely regulated by microorganisms. Gene regulation of siderophore utilization and iron homeostasis in bacteria is mediated at the transcriptional level by

the ferric uptake repressor (Fur) or the diphtheria toxin regulator (DtxR) (Hantke, 2001). Fur is the global iron regulator in low GC content gram-positive bacteria and many gram-negative bacteria whereas DtxR performs a similar role in gram-positive bacteria with a high GC content (Miethke & Marahiel, 2007); there are no distinct similarities between the two sequences yet their structural forms are highly identical regarding metal binding and domain organization (Pohl et al., 2003; Pohl, Holmes, & Hol, 1998; Pohl, Holmes, & Hol, 1999).

2.6 Iron is acquired from Soil by Microbial Siderophores

In soils, iron is an abundant mineral found mainly in insoluble mineral complex forms. Soluble and useful Fe^{2-} and Fe^{3+} are released once these complexes are disbanded (Lindsay & Schwab, 1982). Organisms that depend on the soil as their sole source of mineral nutrients are susceptible to Fe deficiency, more so when conditions are aerobic with a neutral to alkaline pH (Alexander & Zuberer, 1991) and when there is competition from other microorganisms.

Microorganisms have adopted transport systems for siderophores which allow them to better compete for Fe in their natural habitat. It has also been shown that Fe-deficient plants can acquire Fe from siderophores produced by various microorganisms (Crowley, Wang, Reid, & Szaniszlo, 1991). When Fe (III) is acquired by the secreted siderophore molecule, it becomes available for cellular uptake which occurs in one of two ways; either the iron is reduced at the cell surface and released from the siderophore into the cell as a single ion or the complex consisting of the Fe (III) and siderophore is transferred into the cell (Miethke & Marahiel, 2007).

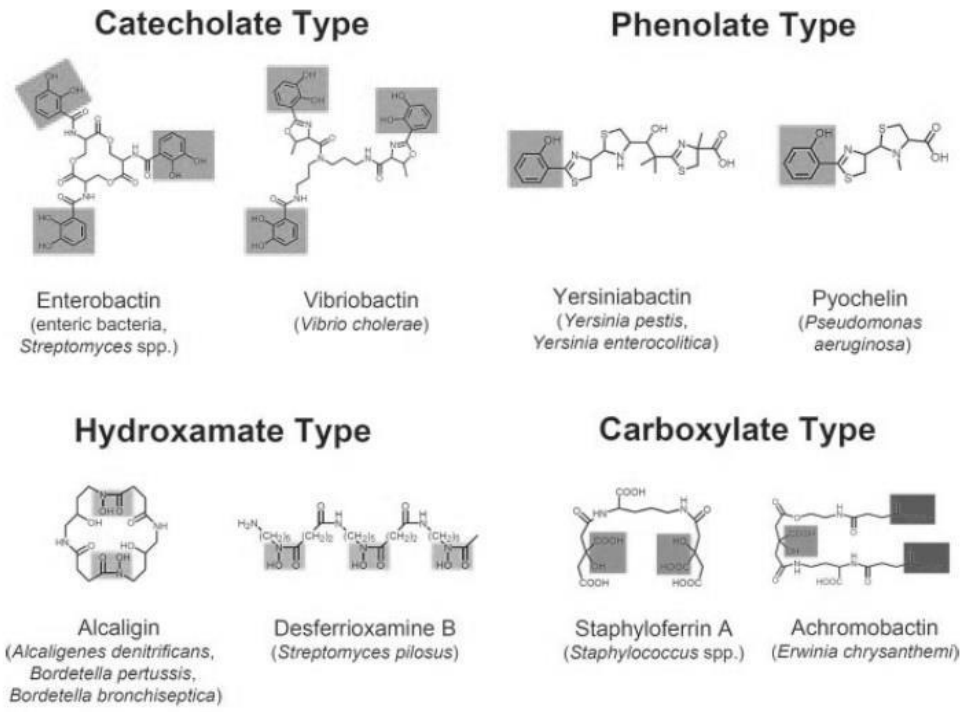


Figure 2. Examples of different siderophores and their natural producers. Adapted from "Siderophore-Based Iron Acquisition and Pathogen Control," by M. Miethke and M. A. Marahiel, 2007, *Microbiology and Molecular Biology Reviews, 71*(3), p. 415. Copyright 2007 by American Society for Microbiology.

2.7 Phosphate Solubilizing Bacteria

Phosphorus is one of the essential mineral nutrients that commonly limit the growth of crops. It is essential for plant growth and development, constituting approximately 0.2 % of the plant's dry weight (Sharma, Kumar, & Tripathi, 2011). Plants obtain phosphorus from the soil as phosphate anions; however phosphorus is commonly not available to plants as it may be insoluble through precipitation with ions such as Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+} (Sharma et al., 2011). Rodriguez and Fraga (1999) stated that under suitable conditions the insoluble compounds can be solubilized so that phosphorus becomes available for both plants and microorganisms.

Some PGPR offer a biological system capable of solubilizing the insoluble inorganic phosphorus of soil and makes it available to the plants (Saharan & Nehra, 2011). Phosphate solubilizing bacteria (PSB) produce phosphatases and organic acids that solubilize inorganic phosphate and converts the insoluble phosphates into soluble phosphate ions thus making soil phosphorus available to plants, (Kumar & Narula, 1999; Whitelaw, 2000; Gyaneshwar, Naresh Kumar, Parekh, & Poole, 2002).

Bacteria belonging to genera *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium* and *Erwinia* possess the ability to solubilize phosphate (Rodriguez & Fraga, 1999). Katznelson, Peterson, and Rouatt (1962) and Raghu and MacRae (1966) noted that a significantly greater concentrations of phosphate solubilizing bacteria are present in the rhizosphere as compared to non-rhizosphere soil. Nevertheless, numbers of phosphate solubilizing bacteria in the soil itself are generally low because of competition. Consequently, the amount of phosphate liberated by them is too low for considerable plant growth, hence inoculating PSB at higher concentrations than that found in soil is required to take advantage of phosphate solubilisation (Rodriguez & Fraga, 1999).

According to Elkoca, Kantar and Sahin (2008) formulations of *B. subtilis* (OSU-142) and P-solubilizing *B. megaterium* (M-3) inoculations significantly increased plant height, shoot, root and nodule dry weight, nitrogen content, chlorophyll content, pod number, seed yield, total biomass yield, and seed protein content in chickpea compared to the control treatments. Results in an experiment by Peix et al. (2001) showed that phosphorous was mobilized by *Mesorhizobium mediterraneum* strain PECA21in barley and chickpea when tricalcium phosphate was added to the soil

medium. Furthermore, the phosphorous content, dry matter, nitrogen, potassium, calcium, and magnesium content in both plants were considerably increased upon inoculation with strain PECA21 and addition of insoluble phosphates.

2.8 Nitrogen-fixing PGPR: Biological Fertilizers

Quite often nitrogen (N) is the most limiting factor in crop production, this is why farmers apply nitrogen fertilizer to their fields. The application of these fertilizers results in higher biomass and protein yields in plants. The nutritional quality of a crop is determined by amino acid composition of proteins, which is affected by nitrogen content, and in the case of cereals increasing the nitrogen quantity results in improved kernel quality (Blumenthal, Baltensperger, Cassman, Mason, & Pavlista, 2008).

Microorganisms known as diazotrophs contribute the most to fixed-nitrogen in the biosphere through a process called biological N_2-fixation (~ 60% of the total annual input) (Newton, 2007). Diazotrophs are generally grouped on the basis of their lifestyle, being either free-living, symbiotic (Fuentes-Ramirez & Caballero-Mellado, 2005), or in a loose association usually with plant roots (Newton, 2007). Increasing the use of PGPR as bio-fertilizers would reduce the need for chemical fertilizers and in turn decrease their adverse effects on the environmental (Kaymak, 2010). Microorganisms are gaining importance in agriculture to promote the circulation of plant nutrients and reduce the need for chemical fertilizers (Şahin, Çakmakçi, & Kantar, 2004). Coinoculation experiments of *Azospirillum brasilense Sp246* with *Bacillus* species indicated yield increases in barley cereal (Öztürk, Caglar, & Sahin, 2003). *Azospirillum* and other bacteria including *Enterobacter, Klebsiella, Burkholderia*, and *Stenotrophomonas* are well-known for nitrogen fixation capabilities

and association with crops (Chelius & Triplett, 2001; Fuentes-Ramirez, & Caballero-Mellado, 2005; Kaymak, 2010; Ramos et al., 2011). One study showed that although PGPR cannot satisfy all of the nitrogen requirements of plants, they do contribute significant amounts of nitrogen supply of plants (Kaymak, 2010).

A genomics survey by Raymond, Siefert, Staples, and Blankenship (2004), shows that approximately 5% of prokaryotes have nitrogen fixation-like genes; since the N_2-fixation genes are plasmid and chromosome based it would be difficult for the prokaryotes to loose *nif* genes (Fuentes-Ramirez & Caballero-Mellado, 2005). The oxygen sensitive enzyme nitrogenase adds hydrogen atoms to a nitrogen atom to form ammonia in bacteria. Nitrogenase consists of two proteins; an iron-containing protein (Fe protein) and an iron-molybdenum-protein complex (MoFe protein) (Maczulak, 2011). The nitrogenase reaction requires the bacterial cell to use energy in the form of adenosine triphosphate (ATP): $N_2 + 8H^+ + 8e^- + 16\ ATP + 16\ H_2O \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16$ phosphates. Nitrogenase activity also has a feedback inhibition mechanism, when ammonia builds up the enzyme stops working. Most likely the accumulation of the end product suppresses the gene expression of nitrogenase (Maczulak, 2011).

Of the four known genetically distinct nitrogenases the conventional molybdenum-based nitrogenase (Mo-nitrogenase) is the best studied. The vanadium-based enzyme (V-nitrogenase) and iron-based (Fe-nitrogenase) are closely related to the Mo-nitrogenase, the only difference being their heterometals (Mo, V, and Fe) (Newton, 2007). The fourth nitrogenase, from the thermophile *Streptomyces thermoautotrophicus*, is extensively different from the others; it is insensitive to O_2

and its makeup consists of components and polypeptides that are different from any of the known nitrogenases (Ribbe, Gadkari, & Meyer, 1997).

It was established that Mo-nitrogenase in *Klebsiella pneumoniae* is coded by a set of genes known as the *nif* genes (Cannon, Riedel, & Ausubel, 1979; MacNeil, Zhu, & Brill, 1981); the genes express polypeptides of two component proteins (MoFe protein and Fe protein), products required for maturation of these proteins, products for biosynthesis of the FeMo-cofactor, products for components of a specific electron–donor system, and products for the regulation of the expression of these genes (Newton, 2007). The 20 *nif* genes discovered in *K. pneumoniae* are arranged in eight adjacent transcriptional units on the chromosome, designations used for *K. pneumoniae nif* genes are also used for genes of other diazotrophs whose products have similar functions (Jacobson et al., 1989). Therefore, *nifH* (encodes the Fe protein polypeptide), *nifD* (encodes MoFe protein α-subunits), and *nifK* (encodes MoFe protein β-subunits) genes are used to label the *nif* structural genes of most diazotrophs (Newton, 2007).

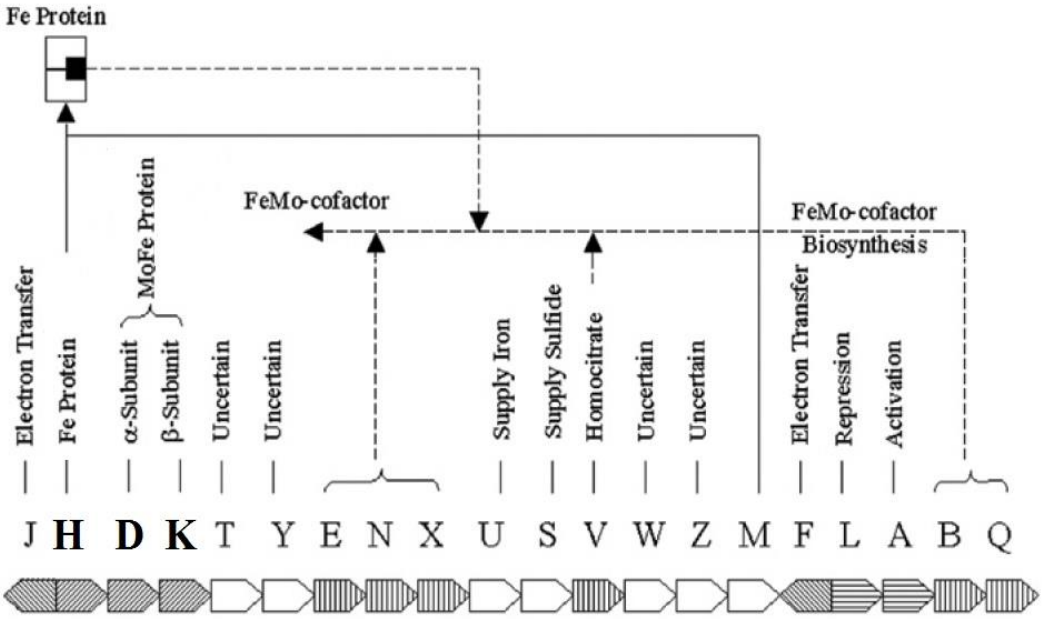


Figure 3. The nitrogen-fixation (*nif*) genes of *Klebsiella pneumonia* together with the putative functions of their products. Adapted from "Physiology, biochemistry, and molecular biology of nitrogen fixation". By Newton, 2007, *Biology of the Nitrogen Cycle*, p. 124. Copyright 2007 by Elsevier B.V.

2.9 Production of Plant Growth Regulators by PGPR

Hormones are organic compounds which act as chemical messengers, signalling the plant to respond to its environment accordingly. Plant hormones are effective at very low concentration; they are synthesized in one part of the plant and transported to another part where they induce physiological responses, e.g. growth (Saharan & Nehra, 2011) Plant hormones play important roles in many plant processes, interestingly certain microorganisms can biologically synthesize compounds that are similar or identical to the hormones produced by plant cells (Singleton & Sainsbury, 2006). Plant growth-promoting rhizobacteria produce plant hormones such as auxins,

cytokinins, gibberillins and are able to lower the concentration of ethylene levels via the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Jung, Kim, Woo, & Kim, 2007; Kang, et al., 2012; Kaymak, 2010; Ortíz-Castro, Valencia-Cantero, & López-Bucio, 2008; Viterbo, Landau, Kim, Chernin, & Chet, 2010). Plant hormones, especially auxins and cytokinins control many stages of plant growth and development, including cell elongation, cell division and tissue differentiation and specialization (Kaymak, 2010).

Auxins which are derivatives of tryptophan are produced mainly in meristems, some microorganisms synthesize similar or identical compounds (Singleton & Sainsbury, 2006). It was determined in the mid-1930s that indole-3-acetic acid (IAA) is the most abundant and the most physiologically relevant auxin in higher plants (Taiz & Zieger, 2002). The ability to synthesize phytohormones is widely distributed among plant associated bacteria, roughly 74- 80% of the bacteria isolated from plant rhizosphere produce IAA (Sahasrabudhe, 2011).

The fact that tryptophan is the main precursor for IAA biosynthesis has led to the identification of five pathways, using tryptophan as a precursor for IAA production in bacteria (Spaepen, Vanderleyden & Remans, 2007). The two step pathway, indole-3-acetamide (IAM) pathway is the best characterized pathway in bacteria. First tryptophan is converted to IAM by enzyme tryptophan-2-monooxygenase (IaaM) and in the second step IAM hydrolase (IaaH) converts IAM to IAA (Spaepen et al., 2007).

The indole-3-pyruvate (IPyA) pathway is the most common tryptophan-dependent pathway for IAA biosynthesis in plants. The pathway involves a conversion of tryptophan to IPyA by an aminotransferase, followed by a rate limiting step where IPyA is decarboxylated by indole-3-pyruvate decarboxylase (IPDC) to form indole-3-

acetaldehyde (IAAld) and the in the last step indole-3- acetaldehyde is oxidized to IAA by a specific dehydrogenase (Taiz & Zieger, 2002). Production of indole-3-acetic acid via the IPyA pathway has been documented in bacteria such as *Bradyrhizobium, Azospirillum, Rhizobium* and *Enterobacter cloacae*, and cyanobacteria (Spaepen et al., 2007).

In plants the tryptamine (TAM) pathway is similar to the IPyA pathway, the difference is that the deamination and decarboxylation reaction is reversed with different enzymes performing the functions (Taiz & Zieger, 2002). In contrast to the bacterial pathway, the last step of this pathway involves an amine oxidase that converts TAM directly into IAAld (Hartmann, Singh, & Klingmüller, 1983). The TAM pathway has been identified in *Bacillus cereus* and *Azospirillum brasilense* (Hartmann, Singh, & Klingmüller, 1983; Perley & Stowe, 1966).

One peculiar tryptophan-dependent IAA pathway is the Tryptophan side-chain oxidase (TSO) which has only been demonstrated in *Pseudomonas fluorescens CHA0*. In this pathway tryptophan is directly converted to IAAld (Oberhansli, Defago, & Haas, 1991). There are no signs nor is there any evidence that suggests that this pathway exists in plants (Spaepen et al., 2007).

In the indole-3-acetonitrile (IAN) pathway tryptophan is converted to indole-3-acetaldoxime and then to indole-3-acetonitrile followed by converting IAN to IAA (carried out by nitrilase) (Taiz & Zieger, 2002). However two different pathways are proposed for the steps involved in the formation of IAN from tryptophan. One pathway utilizes indolic glucosinolates (glucobrassicin) whilst the other utilizes indole-3 acetaldoxime (Bak, Tax, Feldmann, Galbraith, & Feyereisen, 2001; Zhao et al., 2001). Conversion of IAN to IAA has been discovered in *Bacillus amyloliquefaciens* FZB42

(Idris, Iglesias, Talon & Borriss, 2007) and *Azospirillum brasilense* (Carreno-Lopez, Campos-Reales, Elmerich & Baca, 2000).

Many PGPR produce the auxin, indole-3-acetic acid (IAA), and inoculation with auxin-producing rhizobacteria was shown to increase plant growth (Vessey, 2003). Certain PGPR produce cytokinins, for e.g. *Pseudomonas fluorescens* as reported by Bent, Tuzun, Chanway, and Enebak (2001). However more research is needed to accurately determine the role cytokinins produced by bacteria play in plant growth promotion (Kaymak, 2010).

Some PGPR produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase which is an enzyme that cleaves ACC, the immediate precursor to ethylene production in plants, irreversibly into ammonia and α-ketobutyrate. Glick, Penrose, & Li (1998) had come to a conclusion that ACC deaminase activity could decrease ethylene production in the roots of host plants thus resulting in root lengthening. PGPR that have ACC deaminase activity can enhance plant growth especially under stressed conditions by regulating accelerated ethylene production in response to biotic and abiotic stresses such as salinity, drought, and waterlogging (Saleem, Arshad, Hussain, & Bhatti, 2007).

2.10 Mahangu and Sorghum are important Cereals in Namibia

Along with maize, mahangu (*Pennisetum glaucum*) and sorghum (*Sorghum bicolor [L] Moench*], all members of the Poaceae (grass) family, are the most important crops for small scale subsistence farmers in the Northern and North Eastern regions of Namibia. The mahangu plant is adapted to growing in nutrient-poor soils and irregular rainfall conditions, but when conditions are favourable growth is rapid

and vigorous (Maiti & Bidinger, 1981). The nutritional value of mahangu is superior to that of other cereals like maize, wheat or rice and it has a higher content of proteins and lipids, and its amino acid balance is better (Labetoulle, 2000). Flour from mahangu is used to make probably the most important food in North and North Eastern Namibia, a firm porridge called yisima (Kavango name), oshithima (Oshiwambo name), or buhobe (Zambezi name). Mahangu meal is also used to make a traditional non-alcoholic fermented drink known as sikundu (Kavango name), oshikundu (Oshiwambo name); bread that is fried with vegetable oil called mungome (Kavango name), omungome (Oshiwambo name), and a readily eaten powder that is prepared with a little water, sugar and raw mahangu meal called sikuvira (Kavango name), oshikuvila (Oshiwambo name).

Sorghum also produces reasonable yields even with minimal management inputs; however yields are reduced by factors such as environmental stress and poor management. Of course, grain sorghum will grow better under optimum conditions and proper management of inputs for high yields (Espinoza & Kelley, n.d.). Because these plants are able to grow in relatively poor conditions they are key cereals for the people in the drier areas not only in Namibia but in West Africa, East Africa, some parts of Southern Africa and in the Indian subcontinent. The fast growing Okashana-1 is a variety of mahangu is an example of a cereal that has been developed for Namibian conditions (Mendelsohn, 2006).

Sorghum is grown in river plain soils which are exposed by retreating floods along the Kavango River. Sorghum meal is used for various food purposes and for producing beer (tombo) while plant parts may rarely be used for animal feed. If the

population continues to grow as predicted, the most important use for sorghum will tilt towards food purposes (Encyclopædia Britannica, 2013).

2.11 PGPR and Biocontrol Bacteria: Potential Use for Sustainable Agriculture in Namibia

Raising agricultural productivity requires the use of crop farming methods which take into account soil structure, water management and soil fertility management, unfortunately the farming methods that small scale subsistence farmers in Namibia are engaged in lack any of the mentioned features (Namibia Resource Consultants & Vigne & Associates Consultants, n.d.). In a Nation where the majority of the population depends on farming more than any other economic activity (Mendelsohn, 2006) it is of utmost importance to ensure a productive and reliable system of methods for the appropriate farming activities. Sustainable and productive agriculture in the near future requires a combination of water and crop management and optimal use of soil fertility via enhanced activity of the soil biota (Sandhu, Gupta, & Wratten, 2010).

The use of inorganic fertilizers may offer a short term solution but it is not an economically viable option for building up soil fertility (Namibia Resource Consultants & Vigne & Associates Consultants, n.d.). Additionally, the increased use of chemical inputs causes negative impacts on the non-target environment and development of pathogen resistance to the applied pesticide agents (Gerhardson, 2002). Hence, biological control is being considered as an alternative to chemical usage for the purpose of decreasing plant pathogenicity in agriculture systems (Dardanelli et al., 2010).

The use of plant growth-promoting rhizobacteria (PGPR) and biocontrol bacteria offers an attractive alternative to chemical fertilizer, pesticides, and supplements, since their application in enhancing plant growth and crop yields has been demonstrated over the past few decades (Saharan & Nehra, 2011). More suited to the situation at hand, bacterial inoculation has a much better stimulatory effect on plant growth in nutrient deficient soil than in nutrient rich soil (Egamberdiyeva, Juraeva, Gafurova, & Höflich, 2002).

2.12 Background of methodology used in this study

2.12.1 Isolation and plating media. The combined carbon medium integrates common factors of several N-free media, contains organic growth factors and an initial nitrogen source that promotes growth of *Azotobacter* sp., *Bacillus* spp. and diazotrophs (Bashan, Holguin, & Lifshitz, 1993). Synthetic malate medium (SM) medium is a complex medium that supports the growth of heterotrophic microaerophilic nitrogen-fixing bacteria (Reinhold et al., 1986). VM ethanol is a complex medium that is based on SM medium but is supplemented with peptone, yeast extract, salt, ammonium chloride and agar (Dworkin & Falkow, 2006).

2.12.2 Chrome azurol-agar for detecting siderophores. Schwyn and Neiland developed a universal siderophore assay using chrome azurol-S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators. However when there is a large presence of HDTMA (a cationic detergent that is added to stabilize the Fe-CAS indicator and gives the media a blue colour) many bacteria fail to grow on CAS agar (Alexander & Zuberer, 1991). When an iron chelating siderophore removes iron

from the CAS/HDTMA complex, the colour changes from blue to orange (Louden, Haarmann, & Lynne, 2011).

2.12.3 Colorimetric estimation of IAA concentration. Salkowski reagent is aqueous mixture of $FeCl_3$ and concentrated acid. The reagent is specific for indoles. The reagent causes two indole molecules to join together. Two nitrogen atoms from the resulting compound chelates with the Fe (III) to give an assortment of colours based on substituents on the indole. Indole-3 acetic acid gives a pinkish-rhedish colour which is best measured at around 530nm (Gordon & Weber, 1951).

2.12.4 Gene amplification via polymerase chain reaction. The Polymerase Chain Reaction (PCR) is a technology used to amplify segments of DNA. The technique was invented by Kary B. Mullis in 1985 (Okafor, 2007). Two synthetic oligonucleotides, which are complementary to two regions of the target DNA to be amplified, are added to the target DNA, in the presence of deoxynucleotides and a heat-stable DNA polymerase. The target DNA is repeatedly amplified in a series of temperature cycles. (Lackie, 2007)

The bacterial ribosome contains 16S rRNA gene (in the 30S subunit). The 16S rRNA is widely used for the taxonomic classification of prokaryotes. The gene occurs in all prokaryotes and contains highly conserved sequences (for classification of high end taxa) and more variable sequences (for classification of lower ranking taxa) (Singleton & Sainsbury, 2006).

The eukaryotic 80S ribosome contains 18S rRNA (in the 40S subunit), and 28S, 5.8S and 5S rRNA in the 60S subunit (Singleton & Sainsbury, 2006). The 18s rRNA genes that are highly conserved intraspecifically and variable between different species. Therefore the gene is used for taxonomy (Lackie, 2007).

The *nif* genes code for the proteins that are required for nitrogen fixation. The structural genes for nitrogenase *nifD and nifK* specify the two subunits of the MoFe protein, whilst *nifH* specifies the subunit of the Fe protein (Singleton & Sainsbury, 2006). When one of these genes are amplified from the genomic DNA of a bacteria, this is an indication that the bacteria is able to express the nitrogen fixing enzyme.

2.12.5 Design of formulation. The formulation is a vital aspect which influences the effectiveness of the bacterial inoculant. A carrier material is necessary to protect the bacterial inoculant in the soil. The carrier material provides a protective surface, pore space and a substrate (Trevors, van Elsas, Lee, & van Overbeck, 1992). Peat and soil rich in organic matter are widely used in legume inoculants (Arora, Khare, & Maheshwari, 2011). Bacterial inoculants applied individually or in combinations leads to improved plant growth. Beneficial bacterial inoculants applied in combination interact synergistically to improve plant growth (Figueiredo et al., 2010). Studies by Aseri, Jain, Panwar, Rao & Meghwal (2008), Dardanelli et al. (2008) and Zaidi and Khan (2005) show how combination inoculants facilitate improved plant growth**.**

CHAPTER 3: METHODOLOGY

3.1 Research Design

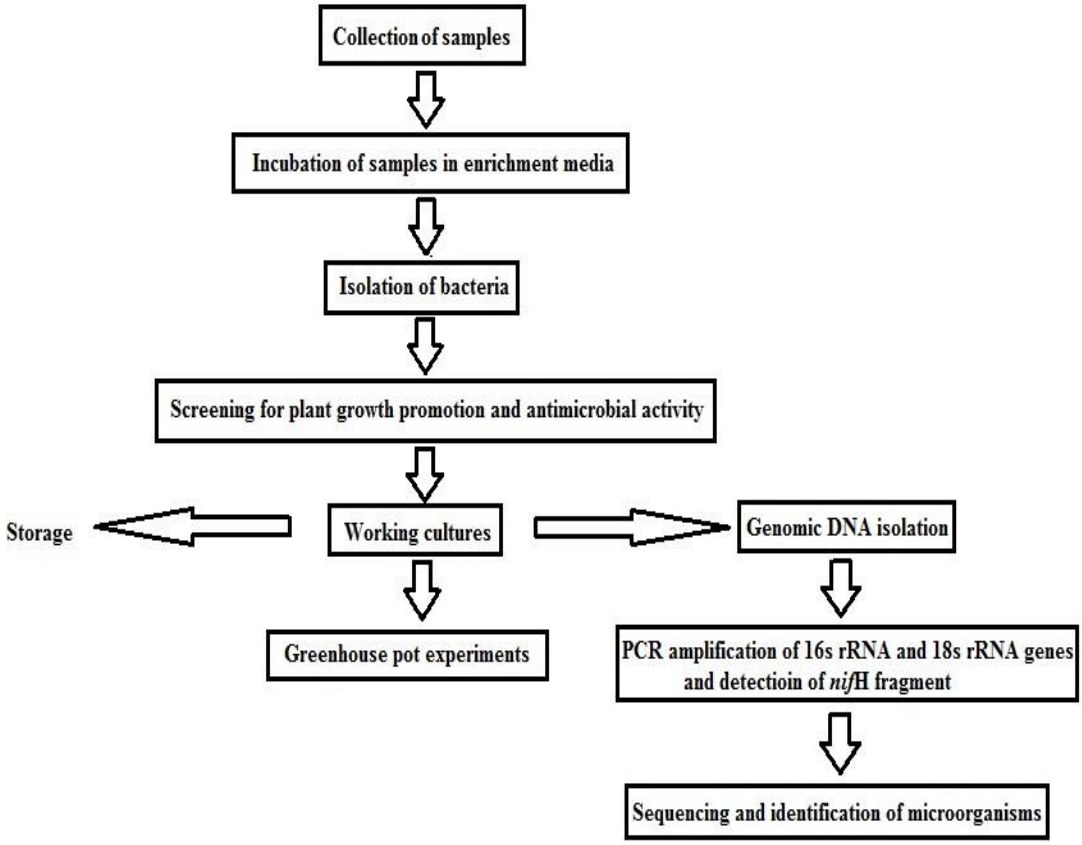


Figure 4. Schematic flow chart of research design that was used during this study.

The research design consisted of experiments that produced both qualitative and quantitative data. Plate assays, test tube assays that were used for plant growth promotion and biocontrol screening and DNA related experiments yielded qualitative data. Data from the colorimetric determination of IAA concentration and data from the pot experiments used to evaluate the effect of bacterial suspensions on *P. glaucum* and *S.bicolor* were both of quantitative nature.

3.2 Sampling Area

Mashare and its surrounding areas, located in the North eastern region of East Kavango, were identified as comprising of suitable sampling points. These points contain suitable vegetation while providing accessibility and availability of disturbed and pristine lands along the Kavango River. There were a total of 8 sampling points (see **Figure 5** and **Table 1**) half of those from pristine lands and the other half were from the fields of subsistence farmers. Collection points Leon and Dries consist of conserved woodland areas, whereas points Site 3 and Site 4 are uninhabited areas located close to flood plains. Collection points WMS, ATR, Lukas 2 and Field were chosen because these were the only farmer's fields where consent was given at the time of sampling.

Figure 5. Map of sampling sites around Mashare. Google earth (Version 7.1.2.2041) [software]. Mountain View, CA: Google Inc. (2013).

Table 1: GPS coordinates of sampling points.

Site name	Land type	Latitude coordinate	Longitude coordinate
Site 3	Pristine	17°53′38.49″ S	20°09′08.97″ E
Site 4	Pristine	17°52′40.49″ S	20°10′31.03″ E
Leon	Pristine	17°53′33.62″ S	20°14′56.13″ E
Dries	Pristine	17°52′30.82″ S	20°15′21.88″ E
WMS	Subsistence farming	17°55′00.13″ S	20°06′16.14″ E
ATR	Subsistence farming	17°53′49.75″ S	20°09′07.07″ E
Lukas 2	Subsistence farming	17°53′43.80″ S	20°14′05.26″ E
Field E	Subsistence farming	17°54′04.40″ S	20°14′14.34″ E

3.3 Collection of Samples

In this study a sample is defined as a root system of a plant along with its tightly adhering soil. Samples were collected by digging up a whole plant and removing the surrounding soil. Then the root system was aseptically detached from the plant, placed inside a Ziploc bag and then stored at 4°C. All samples were collected in April 2012 and October 2012.

The sampling method that was used in subsistence farming lands was convenience sampling coupled with a plant health scale that was based on the physical appearance of the plants. Plants were ranked on three levels: 0= wilted/pale yellow leaves, 1= wilted/green leaves and 2= not wilted/green leaves. Only those with a healthy appearance of no wilt and green leaves (level 2) were selected. This sampling method was chosen so as to minimize damage to crops and increase efficiency of sampling beneficial bacteria communities. Two *P. glaucum* and two *Sorghum* sp. samples were obtained from the four subsistence farmer's fields.

Sampling in pristine areas was done by systematic sampling method, where samples were selected along a 30-50 meters transect line at 10 meters intervals. Four

distinct grasses (*Sporobolus* sp., *Phragmites australis*, *Vetiveria nigritana*; ngwena) were collected from the four pristine points along the Kavango River.

3.4 Incubation of Samples in Enrichment Media

For each sample, 1g of root material was rinsed with 2ml sterile distilled water and transferred in 9ml phosphate-buffer saline solution, followed by vortexing. Subsequently, 1ml of the mixture was transferred into 9ml of both enrichment media i.e. combined carbon medium (Bashan, Holguin, & Lifshitz, 1993) and synthetic malate medium (SM) (Reinhold, Hurek, Niemann, & Fendrik, 1986). The mixtures were incubated at 30°C for 14 days.

3.5 Isolation of Bacteria

Serial dilutions up to dilution factor of 10^{-6} were prepared from the incubated tubes followed by spread plating on VM-ethanol agar medium (Reinhold-Hurek, & Hurek, 2000). The inoculated plates were incubated at 30°C for 5-7 days. After incubation single colonies were subcultured onto fresh agar plates and all isolated bacteria were maintained on VM–ethanol agar medium from henceforth.

3.6 Screening for Plant Growth-Promotion and Antimicrobial Capabilities

3.6.1 NH_3 production. Bacteria were inoculated into test tubes containing 2ml buffered peptone water and incubated for 48hours at 30°C. After the incubation period, 0.1ml Nessler's reagent was added to each inoculated tube by means of swirling and observations of the solution colour were recorded. Peptone water that was not inoculated with any bacterial culture was used as a negative control. The

development of a dark yellow to orange colour after adding Nessler's reagent was categorised as a positive test for NH_3 production.

3.6.2 Screening for proteolytic activity. Bacteria were subcultured onto skimmed milk agar and incubated at room temperature for 5 days. Development of clear zones around the bacterial colonies was indicative of protease activity. The diameters of digested casein zones were measured in millimeters and recorded. This test was done in triplicates.

3.6.3 Inorganic phosphate solubilisation. Testing for solubilisation of inorganic phosphate was carried out by growing bacterial isolates on Pikovskaya agar dyed with bromophenol blue (Gupta, Singal, Shankar, Kuhad, & Saxena, 1994) for 10 days at 30°C. The formation of clear zones around the bacterial colonies was indicative of inorganic phosphate solubilisation. The diameter of the clear zones around the colonies and the diameter of the colonies were measured in millimetres and recorded. This test was done in triplicates**.**

3.6.4 Siderophore production. Testing for bacteria ability to produce iron chelating siderophore complexes was done by growing bacteria on chrome azurol-S (Schwyn & Neilands, 1987) agar at 30°C for five days. The formation of orange halos around the bacterial colonies was indicative of siderophore activity. This test was done in triplicates.

3.6.5 Antifungal activity via plate assay. Fungi from soil that was collected from a mahangu field (17°53′57.90″ S; 20°14′04.39″ E) was used to test the bacterial isolates for antifungal activity. A mixture of 1g soil and 99ml sterile distilled water was prepared and vortexed for 5min. Serial dilutions (10^{-5} – 10^{-8}) were prepared from the soil water mixture and plated on Brain Heart Infusion Soil Extract Medium (Atlas,

2005). The plates were kept at room temperature for 7 days and fungal colonies were subcultured onto Sabouraud dextrose agar (SDA). Each of the isolated fungi were grown on the centre of SDA plates for 3 days at 30°C. Afterwards, two different bacterial isolates were streaked on two opposing sides 2cm away from the centre of the fungal growth, which was represented as a mark on the bottom of the plate. After a further 4 days of incubation at 30°C the antifungal activity of the isolated was visually determined by observing the presence or absence of fungal growth inhibition by the bacterial colonies.

3.6.6 Quantitative evaluation of indole-3 acetic acid production. Indole-3-acetic acid (IAA) production was determined by a two phase screening process. In the first step, bacteria were grown in VM-ethanol broth and adjusted to a concentration of OD_{600} =1. The broth cultures (OD_{600}=1) were used to prepare 1ml suspensions (10% v/v) in VM-ethanol broth supplemented with 5mM L-tryptophan and placed in a shaking incubator under dark conditions at 30°C for 48 hours. Cultures were centrifuged at 4 500 rpm for 15min followed by mixing the supernatant with Salkowski's reagent in the ratio of 1:2 and incubating at room temperature for 30 minutes. Absorbance values of the supernatant and Salkowski reagent mixtures were measured at 530nm. Non inoculated broth was kept as a control. This test was done in triplicates.

In the second step, selected bacterial isolates that produced high absorbance values were treated to similar screening procedure as in the first phase except the volume of 10% v/v suspensions were increased to 10ml and the cultures were centrifuged at 4 400 rpm instead of 4 500 rpm. The quantity of indole acetic acid produced by the bacterial isolates was determined by comparing absorbance values

with those from a standard curve, see **Appendix 3** (pure IAA was used to prepare standard concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 mg/L).

3.7 Genomic DNA Isolation

Bacterial isolates were grown in 10ml VM-ethanol broth for 24hours at 30°C followed by centrifugation (4 400 rpm for 7½ minutes) and removal of supernatant. The bacterial cells were then suspended in 200µl sterile distilled water. Genomic DNA of the bacterial cells was extracted with ZR Fungal/Bacterial DNA MiniPrep™ according to the manufacturer's instructions and kept at 4°C.

After 4 days of incubating fungal isolates in malt extract broth at 30°C, clusters of fungal growth were collected on filter paper through filtration and dried in sterile conditions. Dried fungal material was weighed (50mg) and suspended in 200µl Phosphate buffer saline. Genomic DNA of the fungal cells was extracted with ZR Fungal/Bacterial DNA MiniPrep™ according to the manufacturer's instructions and kept at 4°C.

3.8 Amplification of Nitrogenase *nifH* Gene Fragments

Nitrogen-fixation capability was tested for by detection of the *nifH* gene through a nested PCR. The nested PCR was carried out using outer primers FGPH19 (5′-TACGGCAARGGTGGNATHG-3′), PolR (5′-ATSGCCATCATYNTC RCCGGA-3′) and inner primers PolF (5′TGC GAYCCSAARGCBGACTC-3′) and AQER (5′-GACGATGTAGATYTCCTG-3′) according to Sato et al. (2009) in a Esco Swift™ MaxPro Thermal Cycler. For the first step of the nested PCR the 25µl PCR mixture consisted of 12.5µl DreamTaq Green PCR Master Mix 2× (ThermoScientific),

2µl Genomic DNA, 0.5µM of each primer and 10 µl Nuclease-free Water. The reaction was carried out as follows: initial denaturation at 94°C for 4 min, 30 cycles at 94°C for 1min, 55 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 5 min. The second step of the nested PCR the 25µl PCR mixture consisted of 12.5µl DreamTaq Green PCR Master Mix 2× (ThermoScientific), 2µl template from the first step, 0.5µM of each primer and 10 µl Nuclease-free Water. The reaction was carried out as follows: initial denaturation at 94°C for 4 min, 30 cycles at 94°C for 1min, 56 °C for 1 min and 72 °C for 2 min, final extension at 72 °C for 5 min and hold at 4°C. A strain of *Enterobacter* sp. MOP 1-1, Burbano Roa (2011) was used as a positive control. The products were separated by electrophoresis in 1.2% agarose gels stained with ethidium bromide and visualized under UV light.

3.9 PCR Amplification of 16S rRNA Genes

Universal primers were used to amplify highly conserved sequences found in the 16S rRNA gene of bacteria. Genomic DNA from each isolate was used as a template for amplifying the 16S RNA gene fragment by PCR using forward primer 27F (5′-GAGTTTGATCM TGGCTCAG-3′) and reverse primer 1492r (5′-GGTTACCTTGTTACGACTT-3′). The 50µl PCR mixture consisted of 25µl DreamTaq Green PCR Master Mix 2× (ThermoScientific), 2µl Genomic DNA, 1µM of each primer and 21 µl Nuclease-free Water. The reaction was carried out according to Grönemeyer et al. (2011) in a Esco Swift™ MaxPro Thermal Cycler. More specifically PCR cycle steps were as follows: initial denaturation at 95°C for 4 min, 35 cycles at 95°C for 1min, 50 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 10 min whereas the holding temperature was at 4°C. The products were

separated by electrophoresis in 1% agarose gels stained with ethidium bromide and visualized under UV light. Purification and sequencing of PCR fragments was carried out at Inqaba biotec (Pretoria, South Africa). The sequences were compared to nucleotide databases using the NCBI's Basic Local Alignment Search Tool program to identify the bacteria. Evolutionary analyses were conducted in MEGA5 using the Neighbor-Joining method with Jukes-Cantor substitution model and 1000 bootstrap replicates.

3.10 Amplification of 18S rRNA Genes

The highly conserved internal transcribed spacers (ITS) region of eukaryotes contain species variable 18S genes which are used for identification of the isolated fungi. Genomic DNA from the fungal isolates was used to amplify 18S rRNA gene fragment using forward primer ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′). The 25µl PCR mixture consisted of 12.5µl DreamTaq Green PCR Master Mix 2× (ThermoScientific), 4µl Genomic DNA, 0.5µM of each primer and 7.5 µl Nuclease-free Water.

The reaction was carried out in a Esco Swift™ MaxPro Thermal Cycler, the PCR cycle steps were as follows: initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 45 sec, 51 °C for 30 s and 72 °C for 2 min, final extension at 72 °C for 6 min and hold at 4°C. The products were separated by electrophoresis in 1.2% agarose gels stained with ethidium bromide and visualized under UV light. The PCR fragments were purified and sequenced at Inqaba biotec (Pretoria, South Africa) and the sequences were analysed similarly to the 16S rRNA gene sequences.

3.11 Storage of Bacterial Isolates

For each of the bacterial isolates, prepared cultures were transferred in VM-ethanol broth and incubated at 30 °C. After 24 hours, 50% glycerol was mixed with the inoculated broth in a ratio of 1:1, inside a 1.5ml eppendorf tube. The isolate cultures were labelled and stored as stock cultures in a -80°C freezer facility.

3.12 Pot Experiments

Bacterial isolates were selected on were on the basis of nitrogen-fixation capability, phosphate solubilization, siderophore production, high IAA production, and/or antifungal activities. Five Bacterial isolates that showcased desirable qualities individually and potentially in combination with other isolates possessing distinct plant-growth promoting profiles were selected for pot experiments. Namely, *Stenotrophomonas maltophilia* LCS2-11, *Pseudomonas stutzeri* ACM2-32, *Enterobacter cloacae* FCM2-50, *Bacillus subtilis* ASM1-59 and *Bacillus amyloliquefaciens* LSM1-61 were chosen as treatments, see **Table 2**. All the combination treatments were made up of an IAA producing biocontrol bacteria, a N_2–fixer and one phosphate solubilizing isolate (*P. stutzeri* ACM2-32).

3.12.1 Preparation of treatments. Bacterial isolates were grown in VM–ethanol broth at 28 ±2°C for 3 days. The bacteria cell concentration was adjusted to OD_{660} = 0.9 in 50ml broth volume, washed with sterile distilled water and resuspended in 50ml 0.85% NaCl. Based on the number of treatments and number of replicates, this procedure had to be done in duplicates for some of the isolates to ensure sufficient availability of culture volume.

Table 2: List of selected treatments.

Representative label	Treatment
T_1	LCS2-11 (*Stenotrophomonas maltophilia*)
T_2	ACM2-32 (*Pseudomonas stutzeri*)
T_3	FCM2-50 (*Enterobacter cloacae*)
T_4	ASM1-59 (*Bacillus subtilis*)
T_5	LSM1-61(*Bacillus amyloliquefaciens*)
T_6	ASM1-59: LCS2-11: ACM2-32
T_7	ASM1-59: FCM2-50: ACM2-32
T_8	LSM1-61: LCS2-11: ACM2-32
T_9	LSM1-61: FCM2-50: ACM2-32
T_{10}	Fertilizer
T_{11}	Peat + water
T_{12}	No peat

Starke Ayres palm peat was prepared according to the manufacturer's instructions and dried overnight at 60°C. Approximately 50g of dry palm peat was weighed out per aluminium foil container and sterilized via autoclaving. The palm peat was then transferred to Ziploc plastic bags, moistened with 5ml sterile distilled water per bag under sterile conditions and kept at 4°C.

The inoculum treatments were prepared according to Rose et al. (2011) with modifications. Treatments consisted of 50g palm peat carrier material and 20ml of bacteria solution, i.e. 3ml bacteria-0.85% NaCl suspension + 17ml sterile distilled water for single bacterial treatments and 3ml bacteria-0.85% NaCl suspension of each bacterial isolate + 11ml sterile distilled water for combination bacterial treatments, whereas 3ml 0.85% NaCl + 17ml sterile distilled water was the control. After the

addition of the bacteria solutions to the palm peat enclosed in Ziploc bags, the treatments were incubated for 3 days at 30°C before being applied to soil. The non-inoculum control treatments were a commercial fertilizer, Hygrotech Terra Nova applied at 200 kg/hectare and a treatment with no peat.

3.12.2 Application of treatments and planting of mahangu and sorghum seeds. Plant pots with dimensions of 15cm diameter x 12cm depth, each containing approximately 1.6kg of unprocessed soil collected from a small crop field (17°53′57.90″ S; 20°14′04.39″ E) were used throughout the course of this phase of the study. Using a sterile trowel, each of the treatments were transferred from the Ziploc bags and mixed with soil in separate plant pots. *P. glaucum* variety Okashana-1and *S. bicolor* seeds bought from Rundu Open Market were surface sterilized by soaking them in 70% ethanol for 5 minutes, then in 1.5 % sodium hypochlorite for 1 minute and rinsed three times in sterile distilled water. The seeds were dried for 2 hours in sterile conditions and subsequently planted into the pot containing treatments. For each seed type there were two replicates per treatment with one seed planted per pot. After 25 days, the dry mass was determined by drying plants in an oven at 50°C until the weight remained constant; the length and mass of shoots and roots were recorded to determine the effects of the treatments on the growth of mahangu and sorghum plants.

3.12.3 Particulars of greenhouse pot experiments. Proceedings of the pot experiments were carried out at the University of Namibia Main campus' (Windhoek) greenhouse facility for 25 days. The plant pots were arranged in a randomized block manner with two blocks per plant type. The plants were watered every day with an average atmospheric pressure of 1006.923 hPa, an average maximum temperature

(recorded everyday between 12:00 – 15:00) of 34.1°C and an average 13h 26m 58s length of daylight per day for the duration of the pot experiments (see **Annex 2**).

3.13 Statistical Analysis

The data were analysed using SPSS Statistics (SPSS, version 22.0.0.0, 2013). Depending on the nature of the data either analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance procedure was performed followed by post hoc Fisher's Least Significant Difference (LSD). All analyses were tested at 5% level of significance.

CHAPTER 4: RESULTS

4.1 Summary

A total of 14 bacteria possessing numerous plant health promoting traits were isolated from the rhizospheres of grasses located at Mashare and surrounding areas along the Kavango River and identified. Most of the isolates exhibited more than one trait. They were categorized as follows (see **Table 6**); 3 phosphate solubilizers, 4 siderophore producers, 3 with antifungal capability, 5 N_2-fixers, and 8 of the isolates produced IAA ranging from 3.63 ±0.54 to 8.98 ±0.46mg IAA /L.

Plant experiments from the greenhouse showed that a single inoculant consisting of *S. maltophilia* LCS2-11 and a combination inoculant consisting of the same isolate along with *B. amyloliquefaciens* LSM1-61 and *P. stutzeri* ACM2-32 was able to enhance mahangu plant biomass tantamount to the commercial fertilizer's growth effects. A treatment consisting of *E. cloacae* FCM2-50, *B. amyloliquefaciens* LSM1-61 and *P. stutzeri* ACM2-32 enhanced sorghum plant biomass significantly ($p = 0.032$) compared to the water control. Enhancement of sorghum root biomass by three different single inoculants (*S. maltophilia* LCS2-11, *E. cloacae* FCM2-50, and *B. amyloliquefaciens* LSM1-61) was statistically as good as enhancement by the commercial fertilizer. This was also the case with three different combination inoculant treatments. Compared to the water control, the lower root-shoot ratios in plants treated with inoculations suggest there was more nitrogen available as a result of the bacterial treatments.

4.2 Collected Samples and Isolated Bacteria

Apart from *P. glaucum* and *Sorghum* sp. samples acquired from the fields of subsistence farmers, root samples were also collected from *Sporobolus* sp.*, Phragmites australis*; ruu, mbu (local names), *Vetiveria nigritana*; erenge (local name) and a grass known locally as ngwena. The plants were identified with the help of local para-ecologist Robert Mukuya.

Isolation efforts yielded a total number of seventy five initial bacterial isolates, however at this stage the number of isolates was a misrepresentation of the total number of distinct bacteria isolated as some of the bacteria from differing sites may have appeared as repeated isolates. As expected, the number of distinctive isolates decreased upon further morphological examination and further inspection by gram staining. Eventually 28 bacterial isolates were isolated and maintained throughout the study period, 5 of which were isolated from sorghum, 16 from mahangu, 1 from *P. australis*, 3 from *Sporobolus* sp., 1 from ngwena, 1 from both *V. nigritana* and *Sporobolus* sp. and 1 from both mahangu and sorghum. The isolates were designated a working tag based on the origin details of the bacteria (see **Table 3**).

Table 3: Source of bacterial isolates

Isolate designation	Site name	Enrichment medium	Sample
G1	Dries	CC	*P. australis*
G3	Leon/Site 3/ Site 4	CC	ngwena
G7 I	Leon	CC/SM	*Sporobolus* sp.
G3 I	Leon	CC/SM	*Sporobolus* sp.
G6 I	Leon	CC/SM	*Sporobolus* sp.
11	Lukas 2	CC	Sorghum
14	Lukas 2	CC	Mahangu
20	Lukas 2	CC	Mahangu
21	Lukas 2	SM	Sorghum
23	Lukas 2	SM	Mahangu
26	Lukas2	SM	Mahangu
32	ATR	CC	Mahangu
35	ATR	CC	Mahangu
36	ATR	CC	Mahangu
37	ATR	CC	Mahangu
38	Lukas 2	CC	Mahangu
40	All fields	CC/SM	Mahangu/Sorghum
41	WMS	CC	Mahangu
45	WMS	CC	Mahangu
47	WMS	SM	Sorghum
48	WMS	SM	Sorghum
49	WMS	SM	Sorghum
50	Field E	CC	Mahangu
59	ATR	SM	Mahangu
61	Lukas 2	SM	Mahangu
65	Lukas 2	SM	Mahangu
66	Field E	CC	Mahangu
68	Leon	CC/SM	*V. nigritana*/ *Sporobolus* sp.

Note. CC= Combined carbon medium, SM= Synthetic malate medium.

4.3 Plant Growth-Promotion and Antimicrobial Capabilities

Test tube assays showed that the majority (85.7%) of the isolates were able to produce ammonia. These bacteria had the capacity to deaminate peptone in the test tubes. The differences in the intensity of expected colour formation for positive reactions was an indication of the differing quantities of ammonia production amongst the isolates.

The ability to secrete proteases can be considered as an antimicrobial feature and was assessed for characterization purposes of the bacterial isolates. Nearly half (12) of the isolates were able to secrete proteases which were responsible for the formation of clear zones around the bacterial colonies. A clear zone around the bacterial colonies was the result of hydrolyzation of the milk protein casein.

Bacterial isolates LCM1-14 (*Pseudomonas veronii*), LCM2-20, ACM2-32 (*Pseudomonas stutzeri*) and WSS2-47 (*Bacillus megaterium*) were the only phosphate solubilizers identified via Pikovskaya agar plate assay. The assessment of phosphate solubilisation ability was based on an index where the diameter of the PO_4^{-3} solubilized area is divided by the diameter of the bacteria colony as shown in **Table 4**. Isolates LCM1-14 (index = 3.1) and ACM2-32 (index = 3.5) had what are considered desirable index values, i.e. $\geq$3, while LCM2-20 and WSS2-47 had index values of 1.9 and1.4, respectively. All of the four PO_4^{-3} solubilizing bacteria originated from either sorghum (WSS2-47) or mahangu (LCM1-14, LCM2-20 and ACM2-32).

Table 4: Relative quantification of inorganic phosphate solubilized zone

Isolate	Colony diameter (mm)	Zone + colony diameter (mm)	Index value
LCM1-14	5.5	15.5	3.1
LCM1-20	6.5	10.5	1.9
ACM2-32	4	14	3.5
WSS2-47	4.5	6.5	1.4

Note. Index value calculated by dividing zone + colony diameter by colony diameter.

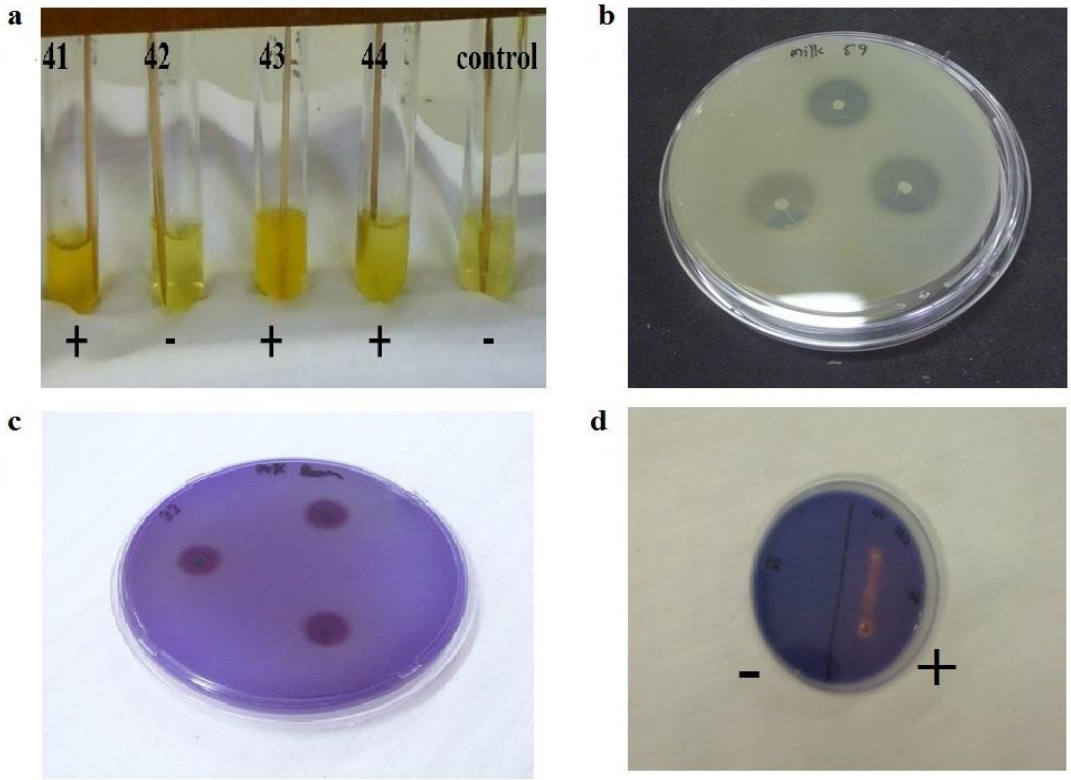


Figure 6. Illustrative screening assays. (a) NH_3 production assay via peptone water and Nessler's reagent. (b) Proteolytic activity assay on skimmed milk agar. (c) Phosphate solubilisation assay on Pikovskaya agar dyed with bromphenol blue. (d) Siderophore assay on CAS agar.

Chrome azurol-S (CAS) agar was used to test the isolates for mobilization of iron. The qualitative method depends on the formation of an iron siderophore complex which is accompanied with a colour change. Five isolates, LCS2-11, LCM1-14, LCM2-20, WSS2-47 and LSM1-65, tested positive for siderophore production. As with phosphate solubilisation, only isolates from mahangu and sorghum crops had the ability to chelate iron.

Isolates ASM1-59 (*Bacillus subtilis*) and LSM1-61 (*Bacillus amyloliquefaciens*) showed antagonistic activity against isolated soil fungi *Aspergillus allahabadii* and *Eupenicillium levitum.* Another isolate GRDB1 (*Bacillus* sp.) had also demonstrated antifungal activity although the isolate was able to suppress the growth of unidentified airborne fungal contaminants. Therefore based on their antifungal capabilities the three isolates were considered as potential biocontrol bacteria.

The first step of IAA production screening showed that exactly half (14) of the isolates produce IAA; see **Table 5** for results. Those that were labelled as IAA non-producers had average absorbance values of less than 0; negative values indicated that the detection level was less than that of the blank. From the 14, the isolates with absorbance values of ≥ 0.02 were considered as sufficient IAA producers. Seven isolates (LCM1-14, LCM2-20, LSS1-21(*Peanibacillus validus*), FCM2-50, ASM1-59, FCM1-66(*Bacillus licheniformis*), and LSE-68 (*Bacillus* sp.) and 2 potentially effective isolates (LSM1-61 and LCS2-11) were selected for IAA quantification experiment.

The *Enterobacter cloacae* strain (FCM2-50) produced the highest value of 8.98 ±0.46mg IAA /L, whilst *Stenotrophomonas maltophilia* (LCS2-11) produced the lowest value of 3.63 ±0.54 mg IAA/L amongst the selected isolates. The IAA

concentration value calculated for *Bacillus subtilis* (ASM1-59) is not as reliable as the other values simply because of the significantly bigger error bar. However the range of the error bars can be considered as similar to concentration values of the other isolates. Isolate LCS2-11 understandably produced the lowest IAA concentration value, as preliminary screening showed that the isolate is a relatively low IAA producer.

Table 5:Screening for IAA production

Isolate	Replicate (A at OD_{600})			Average (A at OD_{600})	IAA Producer
	1	2	3		
LCS2-11	0.02	-0.006	0.012	0.008667	+
LCM1-14	0.09	0.022	0.026	0.046	+
LCM2-20	0.019	0.026	0.021	0.022	+
LSS1-21	0.02	0.028	0.068	0.038667	+
LSM1-23	-0.037	-0.035	0.08	0.002667	+
LSM1 -26	0.016	0.011	0.046	0.024333	+
ACM2-32	0.032	-0.044	0.014	0.000667	+
ACM1-36	0.013	-0.037	0.002	-0.00733	-
ACM1-35	0.027	0.014	-0.014	0.009	+
ACM1-37	-0.023	-0.019	-0.025	-0.02233	-
LCM1-38	-0.025	-0.025	0.043	-0.00233	-
A-40	-0.028	0.014	-0.014	-0.00933	-
WCM1-41	0.004	-0.005	-0.001	-0.00067	-
WCM1-45	-0.021	0.012	0.029	0.006667	+
WSS2-47	0.047	-0.024	-0.028	-0.00167	-
WSS2-48	-0.072	0.00	-0.023	-0.03167	-
WSS2-49	0.002	-0.024	-0.034	-0.01867	-
FCM2-50	0.002	0.043	0.034	0.026333	+
ASM1-59	0.039	0.06	0.027	0.042	+
LSM1-61	-0.004	0.001	0.015	0.004	+
LSM1-65	-0.023	-0.032	0.00	-0.0275	-
FCM1-66	0.022	0.026	0.01	0.019333	+
LSE-68	0.001	0.045	0.03	0.025333	+
GRDB-1	0.008	-0.012	0.00	-0.002	-
GSLB-3	0.007	-0.018	-0.021	-0.01067	-
GXLB-3	-0.027	0.002	0.002	-0.00767	-
GXLB-6	-0.003	0.021	-0.046	-0.00933	-
GXLB-7	-0.023	0.006	-0.013	-0.01	-

Note: A = Absorbance, IAA = indole-3 acetic acid, + = positive test, - = negativetest.

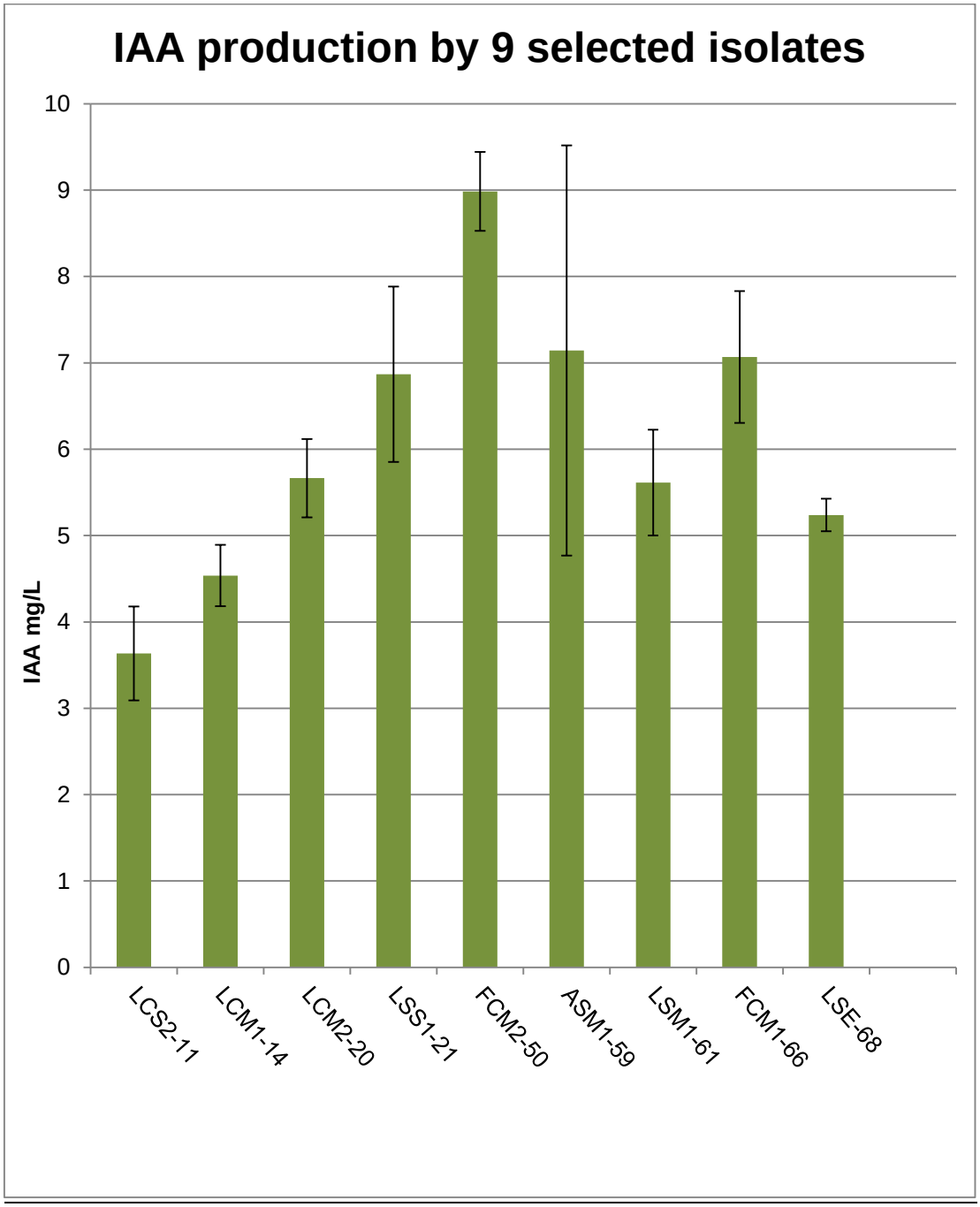


Figure 7. Mean average indole-3-acetic acid (IAA) produced (milligrams per litre) by nine selected bacterial isolates. Error bars denote one standard error around the mean.

4.4 Detection of *nifH* Gene Fragment

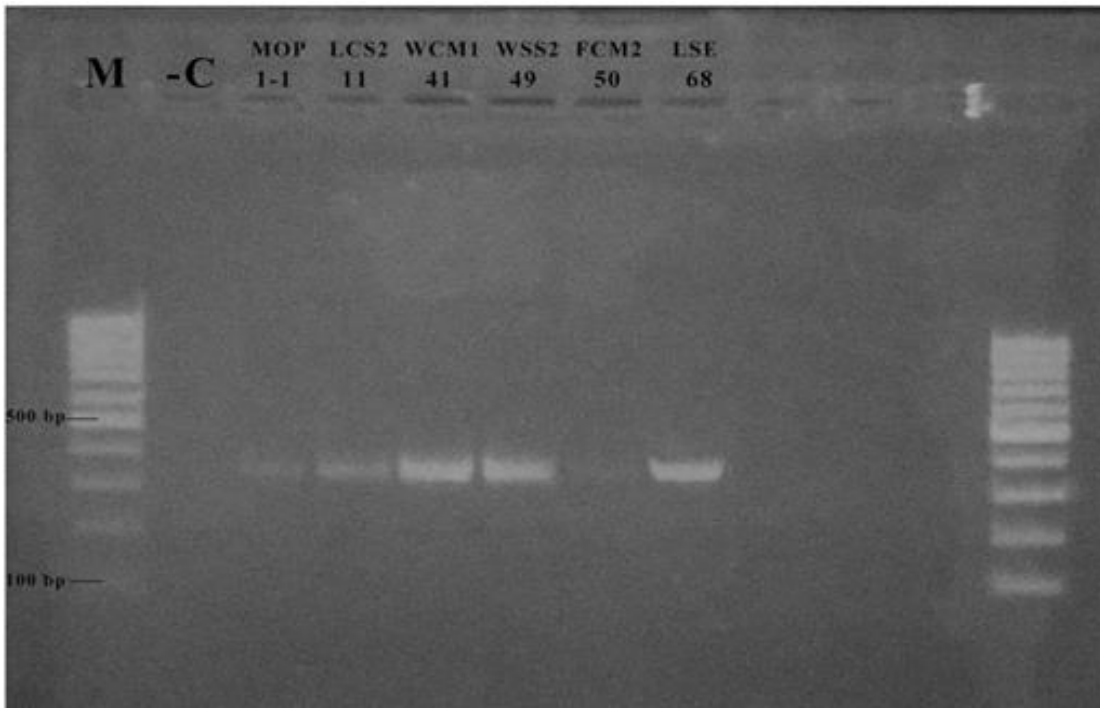


Figure 8. A 1.2% agarose gel consisting of amplified *nifH* fragments from genomic DNA of 5 isolates and *Enterobacter* sp. MOP 1-1.

Nitrogenase *nifH* gene fragments were amplified from the genomic DNA of 5 isolates. The amplified fragments were as expected ~320 base pairs long. The *nifH* gene amplified by nested PCR with primer pairs FGPH19/PolR and PolF/AQER, was detected in LCS2-11(*Stenotrophomonas maltophilia*), WCM1-41 (*Bacillus cereus*), WSS2-49 (*Bacillus cereus*), FCM2-50 (*Enterobacter cloacae*) and LSE-68 (*Bacillus* sp. b29[2010]). Analysed sequences of *nifH* gene fragments (**Table 7**) ranging from 271-289 base pairs show that four of the amplified fragments are similar to *nifH* sequences of diverse organisms. The sequence of the FCM2-50 fragment only revealed matches to the genome belonging to its source organism, *Enterobacter cloacae*.

4.5 16S rRNA Gene and 18S rRNA Gene Analyses

Out of the 28 bacterial isolates, 11 were classified as potential plant-growth promoting rhizobacteria and 3 were classified as biocontrol bacteria. The isolates were identified via blasting sequenced data from amplified ~1500bp fragments of 16s rRNA genes against nucleotide databases, thereby identifying each isolate as the organism with the highest similarity to its sequence. The 16s rRNA sequences used for blasting were 599-845 base pairs in length. According to the 16S rRNA gene sequences, 5 clusters were represented in all of the 14 bacterial isolates. Gammaproteobacteria are represented in the three Orders of Xanthomonadales, Enterobacteriales and Pseudomonadales. The biggest cluster is made up of Bacillales (Firmicutes) and the last clusters represents Order Actinomycetales from the Actinobacteria.

Table 8 shows that most of the isolated beneficial bacteria (64.3%) are members of the Firmicutes group. Results indicate that the nitrogen-fixing isolate WSS2-49 could potentially be a new *Bacillus* species. As with most of the beneficial bacteria WSS2-49 was isolated from a sample acquired from one of the of subsistence farmers fields. Phylogenic analysis (**Figure 9**) suggests that WSS2-49 is closely related to *B. cereus* isolates, thus it was considered a strain of that specified species.

Isolate LSM1-65 identified as *Kocuria* sp. MI-46a is a member of the Actinobacteria. The remaining four beneficial isolates were identified as *Pseudomonas stutzeri* (Pseudomonadales), *Pseudomonas veronii* (Pseudomonadales), *Stenotrophomonas maltophilia* (Xanthomonadales), and *Enterobacter cloacae* (Enterobacteriales) all of which belong to the Proteobacteria group. The isolated soil fungi F4 (*Aspergillus allahabadii*) and F5 (*Eupenicillium levitum*) were identified by blasting sequences of amplified 18s rRNA fragments, 600-700 bp long (**Figure 10**).

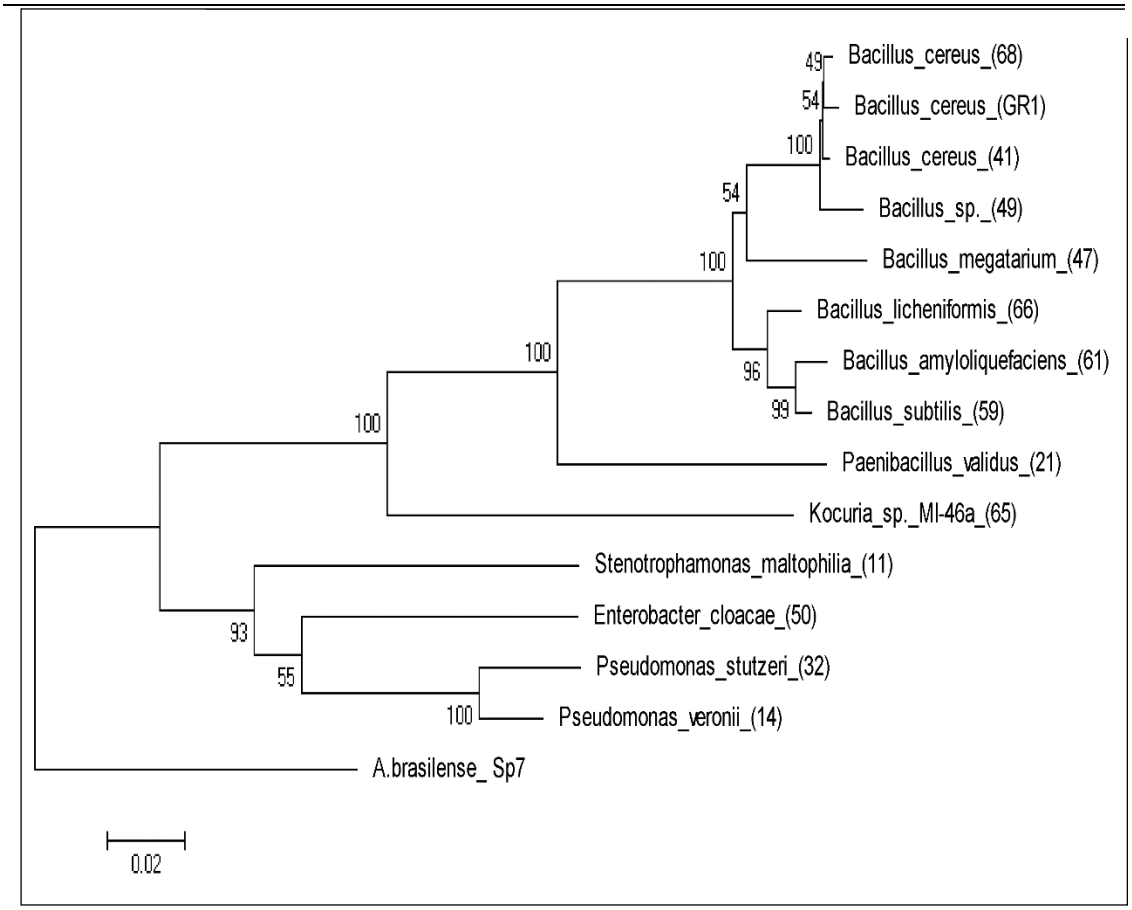
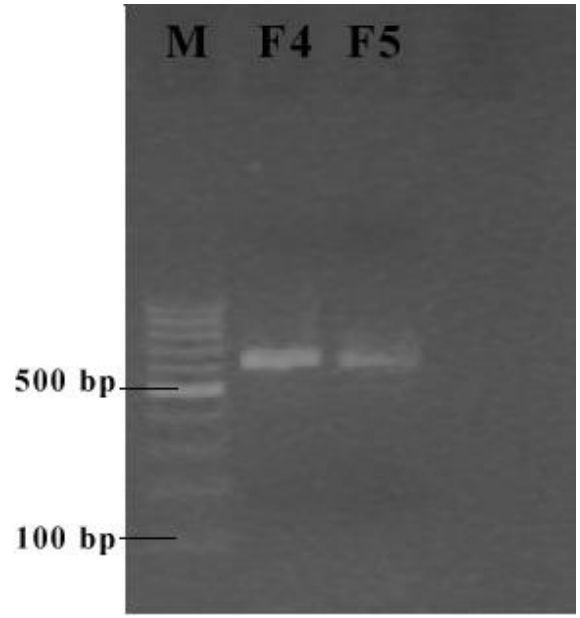
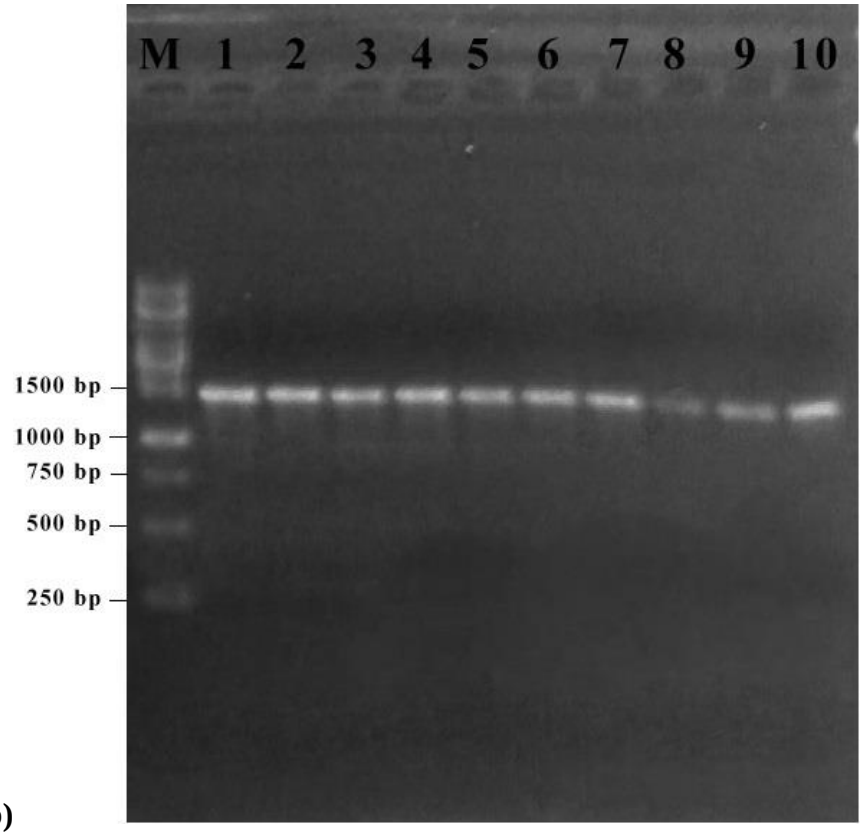


Figure 9. Phylogenic relationships of the beneficial isolates from grasses along the Kavango River. The 16s rRNA gene sequence of *Azospirillum brasilense* was retrieved from NCBI database and was used as the outgroup for rooting The numbers at the branches are % confidence values for bootstraps (1000 replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site.



a)



b)

Figure 10. Agarose gels. (a) Amplified 18s rRNA fragments from isolated foil fungi F4 and F5 in a 1.2% agarose gel. (b) Amplified 16s rRNA fragments from 10 isolates in a 1% agarose gel.

Table 6: Plant growth promoting trait profiles of bacterial isolates

Isolate	NH_3 production	Proteolytic activity	Siderophore production	PO_3^{-4} solubilisation	Antifungal activity	IAA production	*nifH*
Stenotrophomonas maltophilia LCS2-11	+	+	+	-	-	+	+
Pseudomonas veronii LCM1-14	+	-	+	+	-	+	-
Paenibacillus validus LSS1-21	+	-	-	-	-	+	-
Pseudomonas stutzeri ACM2-32	+	-	-	+	-	-	-
Bacillus cereus WCM1-41	+	-	-	-	-	-	+
Bacillus megaterium WSS2-47	+	+	+	+	-	-	-
Bacillus cereus WSS2-49	+	+	-	-	-	-	+
Enterobacter cloacae FCM2-50	+	-	-	-	-	+	+
Bacillus subtilis ASM1-59	+	+	-	-	+	+	-
Bacillus amyloliquefaciens LSM1-61	+	+	-	-	+	+	-
Kocuria sp. LSM1-65	+	+	+	-	-	-	-
Bacillus licheniformis FCM1-66	+	-	-	-	-	+	-
Bacillus cereus LSE-68	-	-	-	-	-	+	+
Bacillus cereus GR1	+	+	-	-	+	-	-

Note. Unidentified isolate LCM2-20 tested positive for Siderophore production, PO_3^{-4} solubilisation and IAA production.

Table 7: BLAST results from *nifH* sequences amplified from genomic DNA of five N_2-fixing isolates

Sequence origin	Highest 3 similarities	Score	E-value	Identity	Accession number
LCS2-11	*Ideonella dechloratans* nitrogenase Fe protein (*nifH*) gene, partial cds	405	1.00e-109	94%	EU542578.1
	Ideonella sp. Long 7 dinitrogenase reductase (*nifH*) gene, partial cds	383	5.00e-103	94%	AY231580.1
	*Azohydromonas australic*a *nifH* gene for iron protein of nitrogenase, partial cds, strain:IAM 12664	381	2.00e-102	92%	AB188121.1
WCM1-41	*Ideonella dechloratans* nitrogenase Fe protein (*nifH*) gene, partial cds	414	2.00e-112	94%	EU542578.1
	Azohydromonas lata nifH gene for iron protein of nitrogenase, partial cds, strain:IAM 12599	398	2.00e-107	93%	AB188122.1
	Azohydromonas lata nifH gene for Fe protein of nitrogenase, partial cds	398	2.00e-107	93%	AB201627.1
WSS2-49	*Dechlorosoma suillum* PS, complete genome	472	1.00e-129	98%	CP003153.1
	Azospira oryzae strain 6a3 nitrogenase iron protein (*nifH*) gene, partial cds	472	1.00e-129	98%	U97115.2
	Rhodoblastus acidophilus partial *nifH* gene for nitrogenase iron protein, strain ATCC 25092	333	5.00e-88	89%	AJ563959.1
FCM2-50	*Enterobacter cloacae* subsp. dissolvens SDM, complete genome	508	8.00e-141	100%	CP003678.1
	Enterobacter cloacae subsp. cloacae ATCC 13047, complete genome	490	3.00e-135	99%	CP001918.1
	Enterobacter cloacae subsp. cloacae ENHKU01, complete genome	451	1.00e-123	97%	CP003737.1
LSE-68	*Rhizobium rosettiformans* W3 dinitrogenase reductase (*nifH*) gene, partial cds	492	8.00e-136	99%	GQ241353.1
	*Rheinheimer*a sp. E48 nitrogenase Fe protein (*nifH*) gene, partial cds	446	6.00e-122	95%	KF430615.1
	Bosea thiooxidans KNUC165 nitrogenase (*nifH*) gene, partial cds ...	427	2.00e-116	99%	DQ431163.1

Table 8: BLAST results of 16s rRNA from 14 isolates and of 18s rRNA from soil fungi (F4 and F5).

Isolate	Most related reference	Identity	Accession	Organism
LCS2-11	*Stenotrophomonas maltophilia* strain JN256	100%	KF150491.1	Proteobacteria; Gammaproteobacteria
LCM1-14	*Pseudomonas veronii* strain NK7	100%	KF675203.1	Proteobacteria; Gammaproteobacteria
LSS1-21	*Paenibacillus validus* strain SB 3263	99%	GU191921.1	Firmicutes; Bacilli
ACM2-32	*Pseudomonas stutzeri* strain 1	100%	KF171338.1	Proteobacteria; Gammaproteobacteria
WCM1-41	*Bacillus cereus* strain JN244	100%	KF687092.1	Firmicutes; Bacilli
WSS2-47	*Bacillus megaterium* strain SRRNINew52	100%	KF724029.1	Firmicutes; Bacilli
WSS2-49	*Bacillus cereus* strain DkBoA3-1	98%	KF025652.1	Firmicutes; Bacilli
FCM2-50	*Enterobacter cloacae* strain GL7	99%	KC853285.1	Proteobacteria; Gammaproteobacteria
ASM1-59	*Bacillus subtilis strain Y38*	100%	KF641817.1	Firmicutes; Bacilli
LSM1-61	*Bacillus amyloliquefaciens* strain DMKUB24	99%	KF673349.1	Firmicutes; Bacilli
LSM1-65	*Kocuria* sp. MI-46a	99%	DQ180950.1	Actinobacteria; Actinobacteridae
FCM1-66	*Bacillus licheniformis* strain 55N2-3	99%	JN366736.1	Firmicutes; Bacilli
LSE-68	*Bacillus* sp. b29(2010)	100%	GU361639.1	Firmicutes; Bacilli
GRDB1	*Bacillus* sp. WBUNB004_A	99%	JX126128.1	Firmicutes; Bacilli
F4	*Aspergillus allahabadii* strain CBS 124597	100%	GQ342626.1	Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae
F5	*Eupenicillium levitum* strain NRRL 705	99%	AF033436.1	Eurotiomycetes; Eurotiomycetidae; Eurotiales; Aspergillaceae

4.6 Growth of Inoculated Mahangu and Sorghum seeds

A Shapiro-Wilk's test ($p > 0.05$)(Shapiro & Wilk, 1965; Razali &Wah, 2011) for data (**Table 10 and Table 11**) and visual examination of their normal Q-Q plots showed that measurements for both plants, except sorghum shoot length and shoot dry mass, were normally distributed. Subsequent ANOVA tests showed that there were significant differences between treatments for mahangu shoot dry mass ($p = 0.019$), mahangu root lengths ($p = 0.004$), mahangu plant dry mass ($p = 0.021$), sorghum root dry mass ($p = 0.037$) and sorghum plant dry mass ($p = 0.006$), see **Appendix 1** for all statistical analyses.

4.6.1 Growth effects on inoculated mahangu. In the case of mahangu, none of the inoculants significantly promoted plant growth compared to the control with respect to shoot dry mass. Contrastingly, the water control treatment had produced mean shoot dry mass measurements that were significantly larger ($p = 0.05$) than any of the inoculant treatments. Although not significant, the water control treatment had a greater mahangu shoot dry mass mean (0.03452g) than the fertilizer treatment. The mean for the fertilizer treatment was also significantly larger than all inoculant treatments. It is however worth mentioning that treatments T_1 - *S. maltophilia* LCS2-11 ($p = 0.038$), T_3- *E. cloacae* FCM2-50 ($p = 0.041$) and T_8- *B. amyloliquefaciens* LSM1-61: *P. stutzeri* ACM2-32: *S. maltophilia* LCS2-11 ($p= 0.041$) had relatively smaller differences to the fertilizer mean as compared to the other inoculant treatments with regards to mahangu shoot dry mass.

There was no significant difference between the means of the fertilizer treatment and inoculation treatments T_1- *S. maltophilia* LCS2-11 ($p = 0.168$), T_4-*B. subtilis* ASM1-59 ($p = 0.166$), T_7-*B. subtilis* ASM1-59: *E. cloacae* FCM2-50: *P.*

stutzeri ACM2-32 (p = 0.062) and T_9- *B. amyloliquefaciens* LSM1-61: *E. cloacae* FCM2-50: *P. stutzeri* ACM2-32(p=0.144) with relation to mahangu root length. Effects of treatments T_1, T_4 and T_9 showed that there was no significant difference to the water control regarding mahangu root lengths. The rest of the inoculant treatments had a statistically inferior influence on growth of mahangu root length compared to the water treatment. Mean mahangu plant dry mass of treatments T_1 (p = 0.055) and T_8 (p = 0.052) were statistically similar to that of the fertilizer treatment. Compared to the average root: shoot ratio **(Table 9)** of the control (T_{11} = 0.181625) treatments T_3 (0.168968), T_6 (0.164356), T_9 (0.170159) and as expected the fertilizer treatment (0.149988) had a lower average root: shoot ratio for mahangu plants.

4.6.2 Growth effects on inoculated sorghum. There was no statistically significant difference between the fertilizer and treatments T_1 (p = 0.105), T_3 (p = 0.089), T_5-*B. amyloliquefaciens* LSM1-61 (p = 0.122), T_7 (p = 0.082), T_8 (p = 0.057) and T_9 (p = 0.196) with respect to the root dry mass of sorghum. The difference of 0.06995g between the fertilizer treatment and the water control treatment in relation to sorghum root dry mass means was significant (p = 0.044). Results showed that there was a statistically significant difference between the T_{12}- no peat control treatment and all peat based treatments with exception to the fertilizer treatment. The T_{12} treatment and the water control T_{11} had growth effects that were significantly different (p= 0.003) pertaining to sorghum root dry mass. In fact this pattern was also a common occurrence in mahangu measurements, where T_{12} measurements were significantly greater than the majority of the peat based treatments. All treatments with exception to treatment T_4 increased average sorghum plant dry mass compared to the water control, with T_9

($p = 0.032$) calculated to be the only treatment to have significantly increased mean sorghum plant dry mass.

The different treatments produced a variation of root: shoot ratios for sorghum plants. The average root: shoot ratio of T_4- *Bacillus subtilis* (0.862899) and the no peat treatment (0.950799) were greater than that of the water control treatment (T_{11} = 0.583731). The rest of the treatments had a smaller average root: shoot ratio compared to the water control**.**

Table 9: Average root: shoot ratio's of mahangu and sorghum plants

Treatment	**Mahangu root : shoot ratio**	**Sorghum root : shoot ratio**
T_1 (*S. maltophilia* LCS2-11)	0.253291	0.392904
T_2 (*P. stutzeri* ACM2-32)	0.228307	0.312816
T_3 (*E. cloacae* FCM2-50)	0.168968	0.415245
T_4 (*B. subtilis* ASM1-59)	0.251418	0.862899
T_5 (*B. amyloliquefaciens* LSM1-61)	0.255553	0.326741
T_6 (ASM1-59: LCS2-11: ACM2-32)	0.164356	0.498623
T_7 (ASM1-59: FCM2-50: ACM2-32)	0.283707	0.33403
T_8 (LSM1-61: LCS2-11: ACM2-32)	0.227771	0.393982
T_9 (LSM1-61: FCM2-50: ACM2-32)	0.170159	0.282745
T_{10} (Water)	0.181625	0.583731
T_{11} (Fertilizer)	0.149988	0.201807
T_{12} (No peat)	0.181832	0.950799

Table 10. Comparison of treatment effects on plant mass, root mass, shoot mass, root length and shoot length of mahangu

Treatment	Plant mass (g)	Root mass (g)	Shoot mass (g)	Root length (cm)	Shoot length (cm)
T_1	0.28 ±0.15[a]	0.06 ±0.02	0.23 ±0.13[ab]	23.54 ±8.8[c]	25.0 ±6.08
T_2	0.13 ±0.06[abc]	0.02 ±0.01[ab]	0.11 ±0.06[abc]	19.15 ±1.2[abc]	19.35 ±5.73[a]
T_3	0.27 ±0.06[ab]	0.04 ±0.01[a]	0.23 ±0.05[ab]	20.38 ±4.0[abc]	26.83 ±1.38
T_4	0.22 ±0.15[ab]	0.04 ±0.02	0.18 ±0.13[ab]	23.5 ±1.27[c]	21.25 ±6.58[a]
T_5	0.06 ±0.02[abc]	0.01 ±0.01[abc]	0.05 ±0.01[abc]	10.8 ±3.82[abc]	13.0 ±0.14[abc]
T_6	0.24 ±0.04[ab]	0.03 ±0.01[a]	0.21 ±0.04[ab]	19.3 ±1.13[abc]	22.3 ±1.84[a]
T_7	0.25 ±0.09[ab]	0.05 ±0.01	0.2 ±0.07[ab]	21.48 ±1.45[ac]	22.4 ±7.92[a]
T_8	0.28 ±0.21[a]	0.05 ±0.03	0.23 ±0.19[ab]	16.78 ±1.52[abc]	23.93 ±9.02
T_9	0.19 ±0.06[ab]	0.03 ±0.01[a]	0.17 ±0.05[ab]	23.2 ±1.56[c]	20.93 ±5.76
T_{10}	0.57 ±0.06	0.09 ±0.01	0.48 ±0.07	29.0 ±4.38	35.2 ±3.25[a]
T_{11}	0.51 ±0.07	0.07 ±0.04	0.45 ±0.03	28.53 ±2.58	26.35 ±5.16
T_{12}	0.38 ±0.08	0.06 ±0.02	0.32 ±0.06	31.6 ±0.28	26.7 ±1.84

Data is presented as mean ±SD

[a] = mean difference between treatment and peat + water is significant at the 0.05 level.

[b] = mean difference between treatment and fertilizer is significant at the 0.05 level.

[c] = mean difference between treatment and no peat is significant at the 0.05 level.

T_1 = LCS2-11 **(***Stenotrophomonas maltophilia***)**

T_2 = ACM2-32 (*Pseudomonas stutzeri*)

T_3 = FCM2-50 (*Enterobacter cloacae*)

T_4 = ASM1-59 (*Bacillus subtilis*)

T_5 = LSM1-61(*Bacillus amyloliquefaciens*)

T_6 = ASM1-59: LCS2-11: ACM2-32

T_7 = ASM1-59: FCM2-50: ACM2-32

T_8 = LSM1-61: LCS2-11: ACM2-32

T_9 = LSM1-61: FCM2-50: ACM2-32

T_{10} = Water

T_{11} = Fertilizer

T_{12} = No peat

Table 11. Comparison of treatment effects on plant mass, root mass, shoot mass, root length and shoot length of sorghum

Treatment	Plant mass (g)	Root mass (g)	Shoot mass (g)	Root length (cm)	Shoot length (cm)
T_1	0.31 ±0.04[b]	0.09 ±0.01[c]	0.22 ±0.03	19.75 ±3.89	35.4 ±2.55
T_2	0.22 ±0.06[b]	0.05 ±0.01[bc]	0.17 ±0.05	18.05 ±5.59	32.7 ±4.24
T_3	0.29 ±0.02[b]	0.09 ±0.03[c]	0.21 ±0.01	22.7 ±3.82	36.15 ±0.21
T_4	0.17 ±0.16[b]	0.07 ±0.05[bc]	0.11 ±0.11	22.05 ±3.18	26.75 ±10.96
T_5	0.39 ±0.18[b]	0.09 ±0.02[c]	0.3 ±0.15	21.18 ±1.66	32.65 ±3.04
T_6	0.23 ±0.11[b]	0.07 ±0.01[bc]	0.16 ±0.1	18.95 ±0.35	32.2 ±5.09
T_7	0.34 ±0.08[b]	0.08 ±0.00[c]	0.26 ±0.08	24.05 ±0.92	39.78 ±3.64
T_8	0.34 ±0.30[b]	0.08 ±0.04[c]	0.26 ±0.25	24.3 ±9.76	37.0 ±11.31
T_9	0.45 ±0.04[ab]	0.10 ±0.03[c]	0.35 ±0.01	24.73 ±5.06[a]	34.93 ±1.31
T_{10}	0.18 ±0.23[b]	0.07 ±0.09[bc]	0.11 ±0.14	14.3 ±11.03	24.8 ±16.69
T_{11}	0.83 ±0.16[ac]	0.14 ±0.07[a]	0.69 ±0.09	22.95 ±2.47	49.95 ±2.05
T_{12}	0.39 ±0.08[b]	0.19 ±0.05[a]	0.20 ±0.02	23.3 ±3.25	34.33 ±2.58

Data is presented as mean ±SD

[a] = mean difference between treatment and peat + water is significant at the 0.05 level.

[b] = mean difference between treatment and fertilizer is significant at the 0.05 level.

[c] = mean difference between treatment and no peat is significant at the 0.05 level.

T_1 = LCS2-11 **(***Stenotrophomonas maltophilia***)**

T_2 = ACM2-32 (*Pseudomonas stutzeri*)

T_3 = FCM2-50 (*Enterobacter cloacae*)

T_4 = ASM1-59 (*Bacillus subtilis*)

T_5 = LSM1-61(*Bacillus amyloliquefaciens*)

T_6 = ASM1-59: LCS2-11: ACM2-32

T_7 = ASM1-59: FCM2-50: ACM2-32

T_8 = LSM1-61: LCS2-11: ACM2-32

T_9 = LSM1-61: FCM2-50: ACM2-32

T_{10} = Water

T_{11} = Fertilizer

T_{12} = No peat

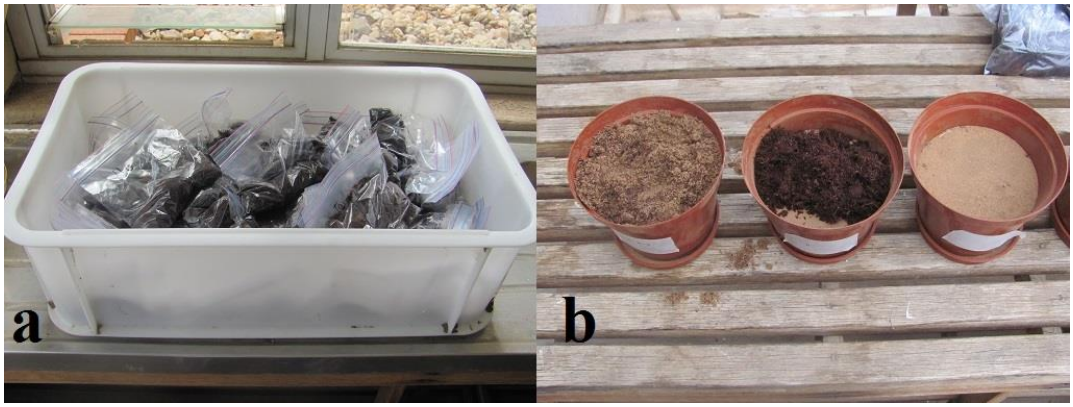


Figure 11. Pot medium preparation. (a) Numerous treatments consisting of peat and bacterial suspensions mixtures in Ziploc bags. (b) From left: Pot with mixed soil and treatment, pot with unmixed soil and treatment and pot with soil and no treatment.

Figure 12. Greenhouse pot experiments. Clockwise from top left: Pot experiment arrangement at day 1; plant growth after 18 days; harvested sorghum plants; and visual comparison of sorghum plants with seven different treatments.

CHAPTER 5: DISCUSSION

5.1 Brief Description of Traits Belonging to the Bacteria Isolated along the Kavango River

The fourteen isolates were identified via 16S rRNA sequences. Out of the 14 isolates 8 belong to the *Bacilli* genus and one of the isolates was identified as *Paenibacillus validus*. Podile and Kishore (2006) reported that *Bacillus* and *Pseudomonas* spp. are the predominant PGPR in the diverse genera of rhizosphere bacteria. The genome of a root-associated *Pseudomonas* strain (*P. stutzeri* A1501) was determined to contain genes involved in utilization of carbon sources, N_2-fixation, denitrification, degradation of aromatic compounds, multiple pathways of protection against environmental stress, and other functions all of which give the bacteria an advantage in the colonization of the root (Yan et al., 2008).

The ubiquitously occurring *Stenotrophomonas* spp. are mainly found in soils and on or inside plants. *S. maltophilia* and *S. rhizophila* are known to be involved in beneficial interactions with plants (Ryan et al., 2009). *S. maltophilia*, an emerging global opportunistic human pathogen (Looney, Narita & Mühlemann, 2009), is adapted to their environment because they have flagellar motility, a high bacterial growth rate, vitamin B1 synthesis, the exudation of NADH dehydrogenase, bacterial lipopolysaccharides (lPSs), particularly the o-antigen and production of extracellular enzymes (proteases, lipases, nucleases, chitinases and elastases) (Ryan et al., 2009).

Kocuria spp. are aerobic, coccoid, non-encapsulated, non-halophilic and non-endospore-forming Actinobacteria that inhabit mammalian skin, soil, the rhizoplane and fresh water (Kim et al., 2004). The isolate *Kocuria* sp. LSM1-65 was considered

as a PGPR because of its siderophore producing feature. Elsewhere, a PGPR *Kocuria sp*. (*K. turfanensis* strain 2M4) isolated from the rhizospheric soil of *Suaeda fruticosa* (Goswami, Pithwa, Dhandhukia, & Thakker, 2014) was found to be a high producer of IAA, phosphate solubilizer and siderophore producer.

Enterobacter bacteria are prevalent in the rhizosphere as well as the endorhizosphere; they have been isolated from the rhizosphere of cucumbers (Schütz, et al., 2003) and from various plant species as endophytes (Lodewyckx et al., 2002). *Enterobacter* spp. are a part of the enterobacteria group which have a rhizosphere colonization advantage via metabolic diversity, allowing them to efficiently utilize organic carbons and they have high productive rates for continued survival (*r*-strategists) when resources are temporarily abundant (Barraquio et al., 2000).

Interestingly, most of the isolates deemed as possible plant enhancing bacteria are known human pathogens. These bacteria belong to groups that are capable of causing a number of illnesses from diarrhoea (*Bacilli*), urinary tract infections (*Enterobacter* spp.), and pneumonia (*Pseudomaonas* spp.), to nosocomial infections in immuno-suppressed patients *(S. maltophilia)* and various infections in immunocompromised hosts (*Kocuria* spp.) (Chamberlain, 2009; Looney et al., 2009; Purty et al., 2013). The bacteria seem to have characteristics that allow them to act as human pathogens and as nonpathogenic but rather beneficial associates to plants (Naz & Bano, 2012). The mechanisms behind the colonisation characteristics, whether human or plant tissue, are similar; they include both recognition and adhesion (Cao, Baldini, & Rahme, 2001). The pathogenic strains come into being via induced stress factors and selective pressures, a good example is illustrated by drug resistant *Pseudomonas* strains in antibiotic exposed hospitals (Carvalho-Assef et al., 2010) and

infectious *Stenotrophomonas* strains in clinical environments with high mutation frequencies as compared to agricultural strains (Turrientes et al., 2010).

The vital aspect to avoid risking human health is to perform proper screening so that the clinical strains are distinguished from the agricultural ones. It is necessary to utilize a multidisciplinary approach to gain a better understanding of the interactions that take place between the microbes as pathogens and plant associates, the host plant, and the environment (Fletcher, Leach, Eversole, & Tauxe, 2013). The threat that PGPR and biocontrol bacteria pose as human pathogens is not a new danger, we face similar threats daily in various forms. The fact that most of the diseases caused by PGPR and biocontrol bacteria related strains are of an opportunistic nature means that a normally healthy person is at a little risk.

5.2 Insight of the Plant Growth Promoting and Biocontrol capabilities Demonstrated by the Isolates

The production of ammonia is a common (85.7% of the initial isolates and 92.8% of the PGPR and biocontrol isolates produce ammonia) and important trait of PGPR. Ammonia production indirectly influences plant growth as the deamination of protein derivatives from dead organisms consequently increase the availability of nitrogen in the soil which in turn benefits plant growth (Joseph, Patra, & Lawrence, 2007). Protease production was common to *Bacillus* spp. (71.4 %). S. *maltophilia* LCS2-11 and *Kocuria* sp. LSM1-65 were the other protease producing PGPR isolates. *Kocuria varians* and *S. maltophilia* are reported to produce extracellular proteolytic enzymes which may be required to hydrolyze major nutrients thus possibly playing a role in nutrient availability (Nwagu & Amadi, 2013; Windhorst et al., 2002; Miyaji et

al., 2005). *B. subtilis, B. licheniformis and B. amyloliquefaciens* are known to sectrete a neutral metalloprotease and an alkaline serine protease (subtilisin) (Piggot, 2009). Bacteria that produce proteases are likely to reduce competition from other microorganisms and are additionally advantaged by increasing nutrient availability from the hydrolyzing activity.

The two closely related isolates *B. subtilis* ASM1-59 and *B. amyloliquefaciens* LSM1-61 exhibited antifungal activity against isolated soil fungi *Aspergillus allahabadii* and *Eupenicillium levitum. Aspergillus* and *Penicillium* appear abundantly in soil as a result of their prolific sporulation (Carlile, Watkinson, & Gooday, 2001). The development of antibiotics and extracellular enzymes in *Bacillus* spp. begins during a phase known as the transition state, where the exponentially growing bacteria encounters nutrient limitation and undergoes a series of responses including secretion of degradative enzymes, synthesis of antibiotics, development of motility, development of competence, and biofilm formation all of which help the bacteria to survive (Piggot, 2009). Members of the *Bacillus* genus are commonly occurring and have been employed successfully in pharmaceutical and metabolite industries. The native antibiotic producing strains, *B. subtilis* ASM1-59 and *B. amyloliquefaciens* LSM1-61 have potential to be used not only in the aforementioned industries but also for the agricultural industry.

Phosphate solubilizing bacteria present a more ecologically safer alternative to large scale fertilizers. Plants absorb fewer amounts of phosphate than what is applied as fertilizer, the rest is leached and converted into insoluble complexes (Mckenzie & Roberts, 1990). According to Rodriguez and Fraga (1999) strains from *Pseudomonas, Bacillus* and *Rhizobium* are amongst the most powerful phosphate solubilizers. A

Pseudomonas strain isolated from rhizospheric soil of grasses growing spontaneously in Spanish soil was shown to actively solubilize phosphates in vitro using bicalcium phosphate as a phosphorus source (Peix et al., 2003). Isolates *Pseudomonas stutzeri* ACM2-32 and *Pseudomonas veronii* LCM1-14 were the strongest phosphate solubilizers.

Siderophore activity by *Pseudomonas spp* is attributed to pyoverdine and quinolobactin (Matthijs et al., 2004; Matthijs et al., 2007; Saha, Saha, Donofrio, & Bestervelt, 2012). S. *maltophilia* LCS2-11 is believed to secrete siderophores of the catechol-type. A study by Garcia, De Rossi, Alcaraz, Vay, and Franco (2012) demonstrated that all of the 31 clinical *S. maltophilia* isolates produced catechol-type siderophores and no hydroxamate-type siderophores. *Bacillus* spp. (*Bacillus megaterium* WSS2-47) are documented to produce hydroxamate-type siderophores (schizokinen, N-deoxyschizokinen and arthrobactin) under limited iron conditions (Hu & Boyer, 1996; Ollinger, Song, Antelmann, Hecker, & Helmann, 2006). Siderophore producing *Kocuria* spp. were isolated from studies by Goswami et al. (2014) and Kaplan et al. (2013), similarly *Kocuria* sp. LSM1-65 was amongst the rhizosphere isolates that produced siderophores on CAS-agar in this study.

IAA production in media supplemented with tryptophan was exhibited by 14 of the isolates. Seven of those were selected for the quantification assay. An external source of tryptophan, as in the rhizosphere, initiates IAA production by microbial populations in the stationary phase (Ryu, & Patten, 2008). Phytohormones produced by *Bacillus* and *Paenibacillus* in the rhizosphere may affect the host plant (Govindasamy et al., 2011). Researchers have reported variable amounts of IAA (1.1-32.2mg/L) produced by bacteria in tryptophan supplemented media (Khalid, Arshad,

& Zahir, 2004; Torres-Rubio, Valencia-Plata, Bernal-Castillo, & Martínez-Nieto, 2000). The IAA produced by plant growth-promoting rhizobacteria (PGPR) has been discovered to enhance the host plant's root system development (Ryu, & Patten, 2008). Auxin allows elongation by promoting the intake of water, through stimulation of hydrogen secretion from the cells into the cell walls, thereby lowering the pH of the cell wall and increases the elasticity of the cell wall (Hannahlu, 2013). Low concentrations of IAA stimulate root growth and have little effect on the shoot, while higher concentrations stimulate shoot growth and inhibit root growth (Hannahlu, 2013).

The similarity in *nifH* sequences between the *nifH* positive isolates and the variety of organisms displayed in **Table 7** can be ascribed to the fact that the N_2-fixation genes and their structure products are highly conserved among various species (Masepohl & Forchhammer, 2007). The exclusivity of the nitrogen fixation capability makes diazotrophic PGPR vital components of bacterial inoculation formulations. Other than chemical fertilizers, N_2-fixing bacteria offer the only practical option to overcoming the challenge of nitrogen deficiency in agricultural soils.

5.3 PGPR and Biocontrol bacteria Community

The specific group of PGPR and biocontrol bacteria isolated in this study comprises mostly of Bacilli bacteria. Bhromsiri C. and Bhromsiri (2010) isolated a group plant growth promoting bacteria from rice and vetiver grass in Thailand. The majority of the isolates were *S. maltophilia.* Other constituents were from the genera *of Rhizobium, Agrobacterium, Bacillus, Paenibacillus, Serratia, Klebsiella, Alcaligenes and Azospirillum.* Other studies managed to isolate a majority of multiple

bacilli species of bacilli from the soil and rhizosphere (Kumar, Amaresan, Bhagat, Madhuri & Srivastava, 2011; Xie, Cai, Guang, & Steinberger, 2003). However, Beneduzi, et al. (2013) demonstrated that a diverse population of plant growth promoting bacteria was present in the rhizosphere, root and stem of sugarcane from Southern Brazil. In all cases the native bacterial strains are adapted to the specific activities taking place in soil of each area. The activities taking place in the soil cause measurable shifts in the composition of the bacterial community. Seemingly, the species with the competitive adaptations toward flourishes in the soil environment (Madigan, Martinko, Dunlap, & Clark, 2012). These adaptations are vital to the survival of the bacteria thus allowing them to contribute significantly to growth of plants (Beneduzi et al., 2013). The generally hot climate of northern Namibia may also contribute to the community of pgpr and biocontrol bacteria. The spore forming Bacilli are able to resist desiccation thus are widely abundant in the soil.

5.4 Growth of inoculated cereals in greenhouse experiment

Bacterial treatments did not enhance the growth of *P. glaucum* okashana-1 compared to the water control treatment. On the other hand, results show that single bacterial suspension treatments *E. cloacae* FCM2-50, *S. maltophilia* LCS2-11 and *B. amyloliquefaciens* LSM1-61 were able to enhance root growth of *S. bicolor*.

The water control treatment for mahangu had produced unexpectedly better growth features compared to the other treatments. However, the credibility of the data from the control treatment may not be so high because this would propose that the fertilizer would be an ineffectual mahangu growth enhancer, as the commercial fertilizer treatment had lesser growth effects compared to the control. A more reliable

measure in this case would be comparison of the inoculant treatments to the fertilizer treatment. The high IAA producing isolates *E. cloacae* FCM2-50, *B. subtilis* ASM1-59 and *B. amyloliquefaciens* LSM1-61 are believed to be the main facilitators behind root growth stimulation. *Enterobacter* spp. are also known to possess ACC deaminase activity, causing seedling root elongation by decreasing plant ethylene inhibition of roots (Li, Ovakim, Charles, & Glick, 2000). Inoculation of mahangu seeds with *S. maltophilia* LCS2-11 and a combination of *B. amyloliquefaciens* LSM1-61, *S. maltophilia* LCS2-11 and *P. stutzeri* ACM2-32 produced mean plant dry mass that were comparable to that of the fertilizer.

. There is little doubt that the N_2-fixing isolates play a critical role in the accumulation of plant biomass, because they provide an environment where the plant acquires nitrogen for assimilation into many metabolites. A plant which grows in an environment that has high nitrogen content will have higher nitrogen concentration and a relatively better growth rate (Pilbeam, 2010). The root-shoot ratio of the plant is also determined by nitrogen availability. If there is a deficiency of nitrogen during the vegetative phase plants experience an overall growth rate decrease and the root fraction increases i.e. the shoot growth is decreased more than root growth so that the proportionately larger root system is given volumetric preference to increase nutrient acquisition and uptake (Pilbeam, 2010). A high root-shoot ratio for the treatment with no peat compared to the water control suggests lower nitrogen availability in the no peat treatments compared to the water control. The peat may have contributed to the nitrogen content while the plants in no peat treatments had to invest into more into root growth in search of food in the nutrient poor arenosol soils.

5.5 Limitations

Twelve of the fourteen isolates considered as PGPR and Biocontrol bacteria were isolated from mahangu (8) and sorghum (4) while a mere two were isolated from grasses of pristine areas. There are undoubtedly more PGPR and Biocontrol bacteria residing in the rhizospheres of grasses which may be more adapted to conditions of anthrosol type soils located closer to the Kavango River banks. The media used for enrichment and isolation may not have supported or favoured the growth of beneficial microorganisms with alternative metabolisms e.g. facultative anaerobes. Another limiting factor was the control treatment for mahangu plants, as one of the reference points for other data an error subjected to this treatment could have distorted observations for the effects of bacterial inoculation on the growth of mahangu plants.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

Fourteen bacterial isolates possessing plant growth promoting and microorganism antagonistic capabilities were isolated from the rhizospheres of grasses located at Mashare and surrounding areas along the Kavango River. Some of the PGPR and biocontrol bacteria isolated from grasses along the Kavango River demonstrated plant enhancing effects on *S. bicolor* when inoculated as both single and combination bacterial suspension treatments. Findings from this study acknowledge the possibility of using bacterial inoculants as inexpensive, effective and ecologically friendly alternatives for increased agricultural crop productivity.

Therefore, based on the results presented in this study it is recommends that further study efforts should be directed towards the discovery and use of native plant-associated bacteria for improved plant growth. The development of bacterial inoculant can be achieved by employing a systematic approach that combines; identifying the most suitable bacteria for enhancing plant growth; resource investment into biotechnological and molecular sciences to improve the efficacy of bacteria; selecting the most suitable carrier material for delivering the bacteria; raising awareness and improving skills in farming communities (Reddy et al., 2011). Bacterial inoculants can alleviation of challenges concerning the importing of chemical fertilizers and affordability of fertilizers for small scale farmers (Office of the President, National Planning Commission, 2012). Public policy makers are urged to consider investing human and financial resources into native bacterial inoculant development so that the benefits deriving from this technology can be realised.

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Appendices

Appendix 1

Statistical analyses data generated from SPSS software.

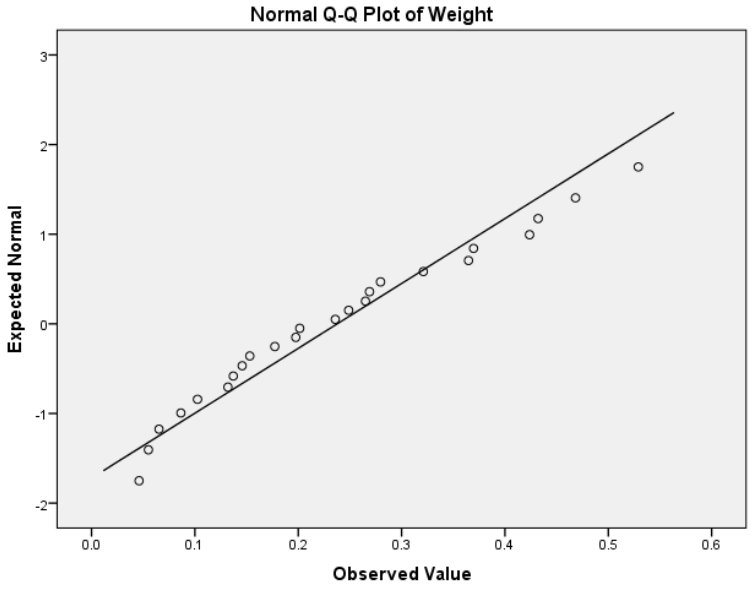
Mahangu shoot mass

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mass	.105	24	.200*	.952	24	.304

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: Mass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	1.703[a]	13	.131	15.518	.000
Block	6.151E-6	1	6.151E-6	.001	.979
Treatment	.346	11	.031	3.729	.019
Error	.093	11	.008		
Total	1.796	24			

a. R Squared = .948 (Adjusted R Squared = .887)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Mass

LSD

Treatment	Treatment	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T_{10} (Fertilizer)	T_1	.2169500*	.09187457	.038	.0147354	.4191646
	T_2	.3368750*	.09187457	.004	.1346604	.5390896
	T_3	.2127500*	.09187457	.041	.0105354	.4149646
	T_4	.2703250*	.09187457	.013	.0681104	.4725396
	T_5	.3955250*	.09187457	.001	.1933104	.5977396
	T_6	.2394750*	.09187457	.024	.0372604	.4416896
	T_7	.2487500*	.09187457	.020	.0465354	.4509646
	T_8	.2124250*	.09187457	.041	.0102104	.4146396
	T_9	.2793500*	.09187457	.011	.0771354	.4815646
	T_{11}	-.0345250	.09187457	.714	-.2367396	.1676896
	T_{12}	.1214250	.09187457	.213	-.0807896	.3236396
T_{11} (WATER)	T_1	.2514750*	.09187457	.019	.0492604	.4536896
	T_2	.3714000*	.09187457	.002	.1691854	.5736146
	T_3	.2472750*	.09187457	.021	.0450604	.4494896
	T_4	.3048500*	.09187457	.007	.1026354	.5070646
	T_5	.4300500*	.09187457	.001	.2278354	.6322646
	T_6	.2740000*	.09187457	.012	.0717854	.4762146
	T_7	.2832750*	.09187457	.010	.0810604	.4854896
	T_8	.2469500*	.09187457	.021	.0447354	.4491646
	T_9	.3138750*	.09187457	.006	.1116604	.5160896
	T_{10}	.0345250	.09187457	.714	-.1676896	.2367396
	T_{12}	.1559500	.09187457	.118	-.0462646	.3581646
T_{12} (No peat)	T_1	.0955250	.09187457	.321	-.1066896	.2977396
	T_2	.2154500*	.09187457	.039	.0132354	.4176646
	T_3	.0913250	.09187457	.342	-.1108896	.2935396
	T_4	.1489000	.09187457	.133	-.0533146	.3511146
	T_5	.2741000*	.09187457	.012	.0718854	.4763146
	T_6	.1180500	.09187457	.225	-.0841646	.3202646
	T_7	.1273250	.09187457	.193	-.0748896	.3295396
	T_8	.0910000	.09187457	.343	-.1112146	.2932146
	T_9	.1579250	.09187457	.114	-.0442896	.3601396
	T_{10}	-.1214250	.09187457	.213	-.3236396	.0807896
	T_{11}	-.1559500	.09187457	.118	-.3581646	.0462646

Based on observed means.

The error term is Mean Square(Error) = .008.

*. The mean difference is significant at the 0.05 level.

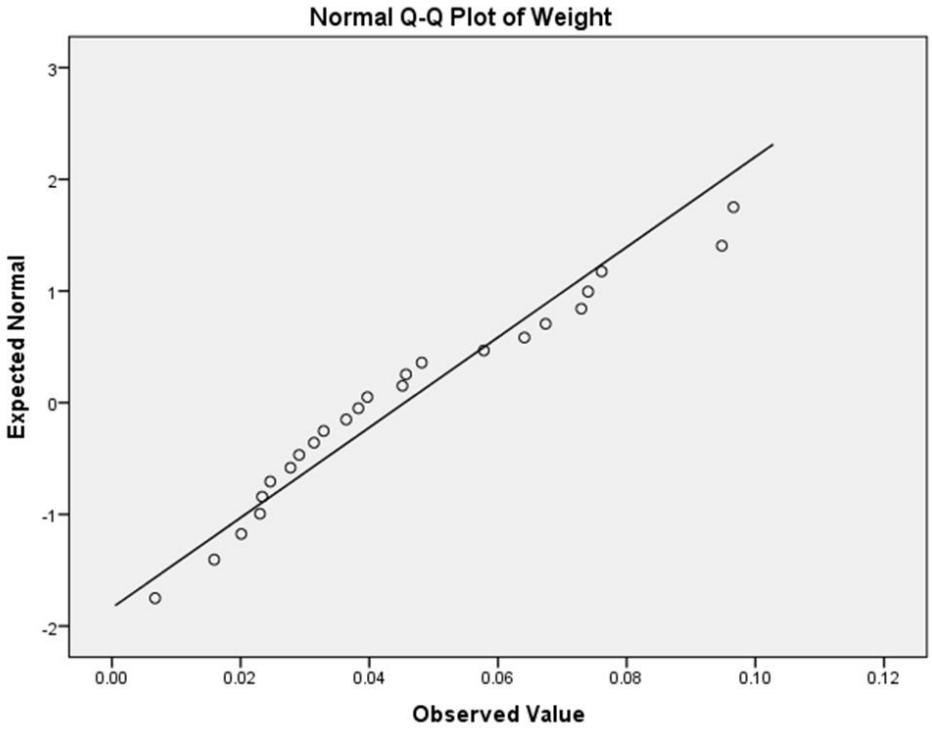
Mahangu root mass

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mass	.134	24	.200*	.942	24	.177

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: Mass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	.059[a]	13	.005	11.215	.000
Block	.000	1	.000	.403	.538
Treatment	.009	11	.001	2.114	.115
Error	.004	11	.000		
Total	.064	24			

a. R Squared = .930 (Adjusted R Squared = .847)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Mass

LSD

Treatment	Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T_{10} (Fertilizer)	T_1	.0125250	.02016880	.547	-.0318662	.0569162
	T_2	.0487000*	.02016880	.034	.0043088	.0930912
	T_3	.0283750	.02016880	.187	-.0160162	.0727662
	T_4	.0269500	.02016880	.208	-.0174412	.0713412
	T_5	.0547500*	.02016880	.020	.0103588	.0991412
	T_6	.0354000	.02016880	.107	-.0089912	.0797912
	T_7	.0135250	.02016880	.516	-.0308662	.0579162
	T_8	.0205750	.02016880	.330	-.0238162	.0649662
	T_9	.0400250	.02016880	.073	-.0043662	.0844162
	T_{11}	-.0173000	.02016880	.409	-.0616912	.0270912
	T_{12}	.0083000	.02016880	.689	-.0360912	.0526912
T_{11} (WATER)	T_1	.0298250	.02016880	.167	-.0145662	.0742162
	T_2	.0660000*	.02016880	.007	.0216088	.1103912
	T_3	.0456750*	.02016880	.045	.0012838	.0900662
	T_4	.0442500	.02016880	.051	-.0001412	.0886412
	T_5	.0720500*	.02016880	.004	.0276588	.1164412
	T_6	.0527000*	.02016880	.024	.0083088	.0970912
	T_7	.0308250	.02016880	.155	-.0135662	.0752162
	T_8	.0378750	.02016880	.087	-.0065162	.0822662
	T_9	.0573250*	.02016880	.016	.0129338	.1017162
	T_{10}	.0173000	.02016880	.409	-.0270912	.0616912
	T_{12}	.0256000	.02016880	.231	-.0187912	.0699912
T_{12} (No peat)	T_1	.0042250	.02016880	.838	-.0401662	.0486162
	T_2	.0404000	.02016880	.070	-.0039912	.0847912
	T_3	.0200750	.02016880	.341	-.0243162	.0644662
	T_4	.0186500	.02016880	.375	-.0257412	.0630412
	T_5	.0464500*	.02016880	.042	.0020588	.0908412
	T_6	.0271000	.02016880	.206	-.0172912	.0714912
	T_7	.0052250	.02016880	.800	-.0391662	.0496162
	T_8	.0122750	.02016880	.555	-.0321162	.0566662
	T_9	.0317250	.02016880	.144	-.0126662	.0761162
	T_{10}	-.0083000	.02016880	.689	-.0526912	.0360912
	T_{11}	-.0256000	.02016880	.231	-.0699912	.0187912

Based on observed means.

The error term is Mean Square(Error) = .000.

*. The mean difference is significant at the 0.05 level.

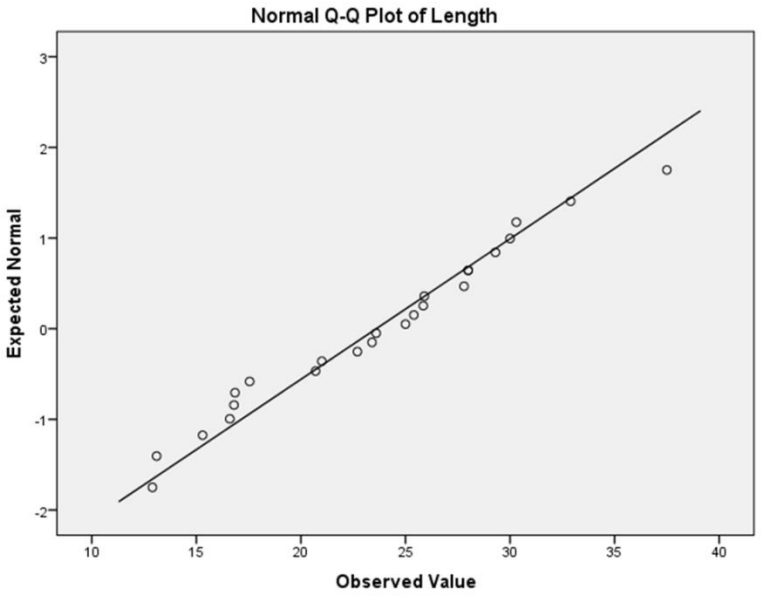
Mahangu shoot lengths

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Length	.118	24	.200*	.971	24	.684

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: Length

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	14007.176[a]	13	1077.475	37.127	.000
Block	16.918	1	16.918	.583	.461
Treatment	620.859	11	56.442	1.945	.143
Error	319.231	11	29.021		
Total	14326.408	24			

a. R Squared = .978 (Adjusted R Squared = .951)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Length

LSD

Treatment	Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T_{10} (Fertilizer)	T_1	1.3500	5.38712	.807	-10.5070	13.2070
	T_2	7.0000	5.38712	.220	-4.8570	18.8570
	T_3	-.4750	5.38712	.931	-12.3320	11.3820
	T_4	5.1000	5.38712	.364	-6.7570	16.9570
	T_5	13.3500*	5.38712	.031	1.4930	25.2070
	T_6	4.0500	5.38712	.468	-7.8070	15.9070
	T_7	3.9500	5.38712	.479	-7.9070	15.8070
	T_8	2.4250	5.38712	.661	-9.4320	14.2820
	T_9	5.4250	5.38712	.336	-6.4320	17.2820
	T_{11}	-8.8500	5.38712	.129	-20.7070	3.0070
	T_{12}	-.3500	5.38712	.949	-12.2070	11.5070
T_{11} (WATER)	T_1	10.2000	5.38712	.085	-1.6570	22.0570
	T_2	15.8500*	5.38712	.013	3.9930	27.7070
	T_3	8.3750	5.38712	.148	-3.4820	20.2320
	T_4	13.9500*	5.38712	.025	2.0930	25.8070
	T_5	22.2000*	5.38712	.002	10.3430	34.0570
	T_6	12.9000*	5.38712	.036	1.0430	24.7570
	T_7	12.8000*	5.38712	.037	.9430	24.6570
	T_8	11.2750	5.38712	.060	-.5820	23.1320
	T_9	14.2750*	5.38712	.023	2.4180	26.1320
	T_{10}	8.8500	5.38712	.129	-3.0070	20.7070
	T_{12}	8.5000	5.38712	.143	-3.3570	20.3570
T_{12} (No peat)	T_1	1.7000	5.38712	.758	-10.1570	13.5570
	T_2	7.3500	5.38712	.200	-4.5070	19.2070
	T_3	-.1250	5.38712	.982	-11.9820	11.7320
	T_4	5.4500	5.38712	.333	-6.4070	17.3070
	T_5	13.7000*	5.38712	.027	1.8430	25.5570
	T_6	4.4000	5.38712	.431	-7.4570	16.2570
	T_7	4.3000	5.38712	.442	-7.5570	16.1570
	T_8	2.7750	5.38712	.617	-9.0820	14.6320
	T_9	5.7750	5.38712	.307	-6.0820	17.6320
	T_{10}	.3500	5.38712	.949	-11.5070	12.2070
	T_{11}	-8.5000	5.38712	.143	-20.3570	3.3570

Based on observed means.

The error term is Mean Square(Error) = 29.021.

*. The mean difference is significant at the 0.05 level.

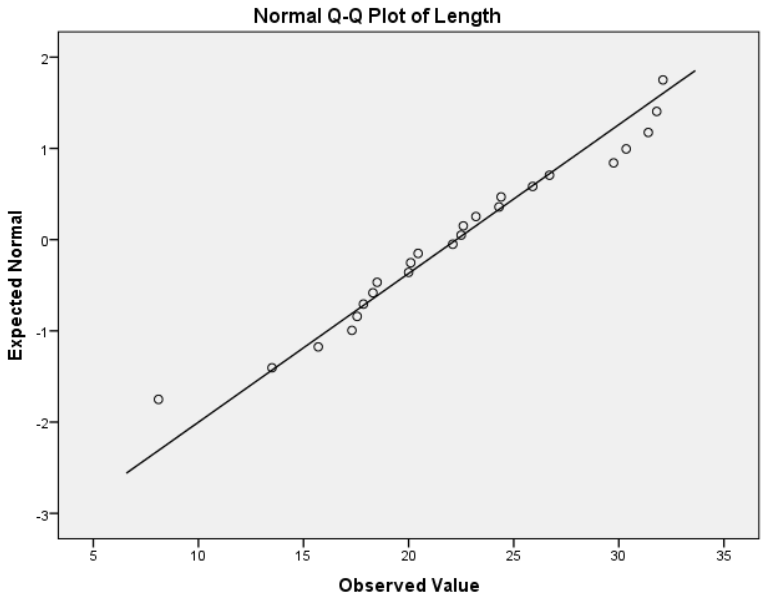
Mahangu root lengths

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Length	.097	24	.200*	.966	24	.578

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: Length

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	12640.256[a]	13	972.327	84.640	.000
Block	18.815	1	18.815	1.638	.227
Treatment	719.908	11	65.446	5.697	.004
Error	126.366	11	11.488		
Total	12766.623	24			

a. R Squared = .990 (Adjusted R Squared = .978)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Length

LSD

Treatment	Treatment	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T_{10} (Fertilizer)	T_1	5.000	3.3894	.168	-2.460	12.460
	T_2	9.375*	3.3894	.018	1.915	16.835
	T_3	8.150*	3.3894	.035	.690	15.610
	T_4	5.025	3.3894	.166	-2.435	12.485
	T_5	17.725*	3.3894	.000	10.265	25.185
	T_6	9.225*	3.3894	.020	1.765	16.685
	T_7	7.050	3.3894	.062	-.410	14.510
	T_8	11.750*	3.3894	.005	4.290	19.210
	T_9	5.325	3.3894	.144	-2.135	12.785
	T_{11}	-.475	3.3894	.891	-7.935	6.985
	T_{12}	-3.075	3.3894	.384	-10.535	4.385
T_{11} (WATER)	T_1	5.475	3 3894	.135	-1.985	12.935
	T_2	9.850*	3.3894	.014	2.390	17.310
	T_3	8.625*	3.3894	.027	1.165	16.085
	T_4	5.500	3.3894	.133	-1.960	12.960
	T_5	18.200*	3.3894	.000	10.740	25.660
	T_6	9.700*	3.3894	.015	2.240	17.160
	T_7	7.525*	3.3894	.048	.065	14.985
	T_8	12.225*	3.3894	.004	4.765	19.685
	T_9	5.800	3.3894	.115	-1.660	13.260
	T_{10}	.475	3.3894	.891	-6.985	7.935
	T_{12}	-2.600	3.3894	.459	-10.060	4.860
T_{12} (No peat)	T_1	8.075*	3.3894	.036	.615	15.535
	T_2	12.450*	3.3894	.004	4.990	19.910
	T_3	11.225*	3.3894	.007	3.765	18.685
	T_4	8.100*	3.3894	.036	.640	15.560
	T_5	20.800*	3.3894	.000	13.340	28.260
	T_6	12.300*	3.3894	.004	4.840	19.760
	T_7	10.125*	3.3894	.012	2.665	17.585
	T_8	14.825*	3.3894	.001	7.365	22.285
	T_9	8.400*	3.3894	.031	.940	15.860
	T_{10}	3.075	3.3894	.384	-4.385	10.535
	T_{11}	2.600	3.3894	.459	-4.860	10.060

Based on observed means.

The error term is Mean Square(Error) = 11.488.

*. The mean difference is significant at the 0.05 level.

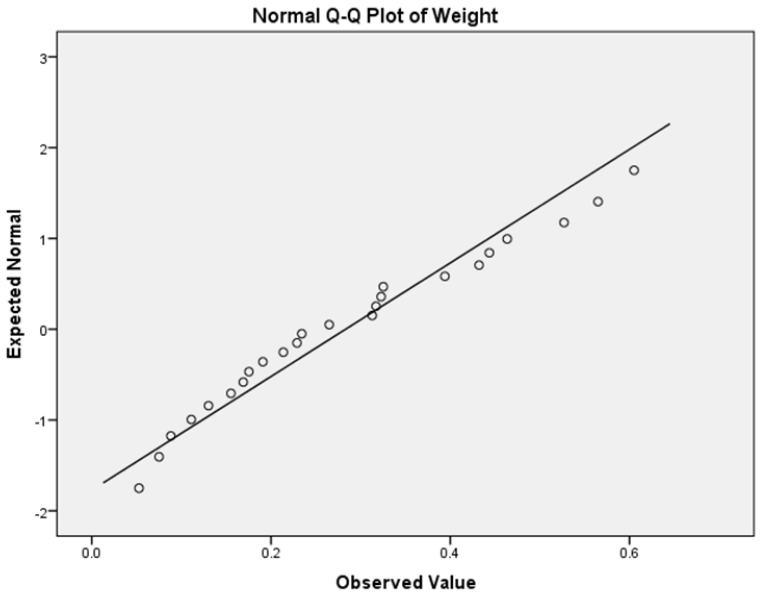
Mahangu plant mass

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mass	.120	24	.200*	.952	24	.293

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: Mass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	2.387[a]	13	.184	16.019	.000
Block	.000	1	.000	.020	.889
Treatment	.462	11	.042	3.660	.021
Error	.126	11	.011		
Total	2.513	24			

a. R Squared = .950 (Adjusted R Squared = .891)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Mass

LSD

Treatment	Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T_{10} (Fertilizer)	T_1	.2294750	.10707082	.055	-.0061863	.4651363
	T_2	.3855750*	.10707082	.004	.1499137	.6212363
	T_3	.2411250*	.10707082	.046	.0054637	.4767863
	T_4	.2972750*	.10707082	.018	.0616137	.5329363
	T_5	.4502750*	.10707082	.001	.2146137	.6859363
	T_6	.2748750*	.10707082	.026	.0392137	.5105363
	T_7	.2622750*	.10707082	.032	.0266137	.4979363
	T_8	.2330000	.10707082	.052	-.0026613	.4686613
	T_9	.3193750*	.10707082	.012	.0837137	.5550363
	T_{11}	-.0518250	.10707082	.638	-.2874863	.1838363
	T_{12}	.1297250	.10707082	.251	-.1059363	.3653863
T_{11} (WATER)	T_1	.2813000*	.10707082	.024	.0456387	.5169613
	T_2	.4374000*	.10707082	.002	.2017387	.6730613
	T_3	.2929500*	.10707082	.019	.0572887	.5286113
	T_4	.3491000*	.10707082	.008	.1134387	.5847613
	T_5	.5021000*	.10707082	.001	.2664387	.7377613
	T_6	.3267000*	.10707082	.011	.0910387	.5623613
	T_7	.3141000*	.10707082	.014	.0784387	.5497613
	T_8	.2848250*	.10707082	.022	.0491637	.5204863
	T_9	.3712000*	.10707082	.005	.1355387	.6068613
	T_{10}	.0518250	.10707082	.638	-.1838363	.2874863
	T_{12}	.1815500	.10707082	.118	-.0541113	.4172113
T_{12} (No peat)	T_1	.0997500	.10707082	.372	-.1359113	.3354113
	T_2	.2558500*	.10707082	.036	.0201887	.4915113
	T_3	.1114000	.10707082	.320	-.1242613	.3470613
	T_4	.1675500	.10707082	.146	-.0681113	.4032113
	T_5	.3205500*	.10707082	.012	.0848887	.5562113
	T_6	.1451500	.10707082	.202	-.0905113	.3808113
	T_7	.1325500	.10707082	.242	-.1031113	.3682113
	T_8	.1032750	.10707082	.355	-.1323863	.3389363
	T_9	.1896500	.10707082	.104	-.0460113	.4253113
	T_{10}	-.1297250	.10707082	.251	-.3653863	.1059363
	T_{11}	-.1815500	.10707082	.118	-.4172113	.0541113

Based on observed means.

The error term is Mean Square(Error) = .011.

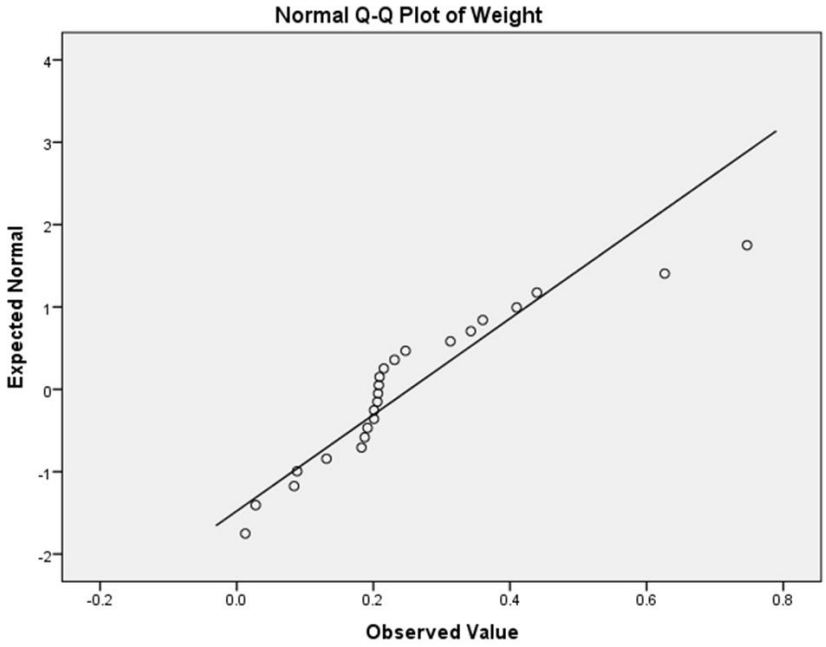
*. The mean difference is significant at the 0.05 level.

Sorghum shoot mass

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mass	.222	24	.004	.870	24	.005

a. Lilliefors Significance Correction



Oneway

ANOVA

abs_dif

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	565.715	11	51.429	1.118	.423
Within Groups	552.000	12	46.000		
Total	1117.715	23			

Kruskal-Wallis Test

Ranks

	Treatment	N	Mean Rank
Mass	1	2	13.00
	2	2	8.50
	3	2	12.00
	4	2	4.50
	5	2	14.50
	6	2	10.00
	7	2	14.50
	8	2	12.50
	9	2	19.50
	10	2	23.50
	11	2	7.00
	12	2	10.50
	Total	24	

Test Statistics[a,b]

	Mass
Chi-Square	11.960
df	11
Asymp. Sig.	.367

a. Kruskal Wallis Test

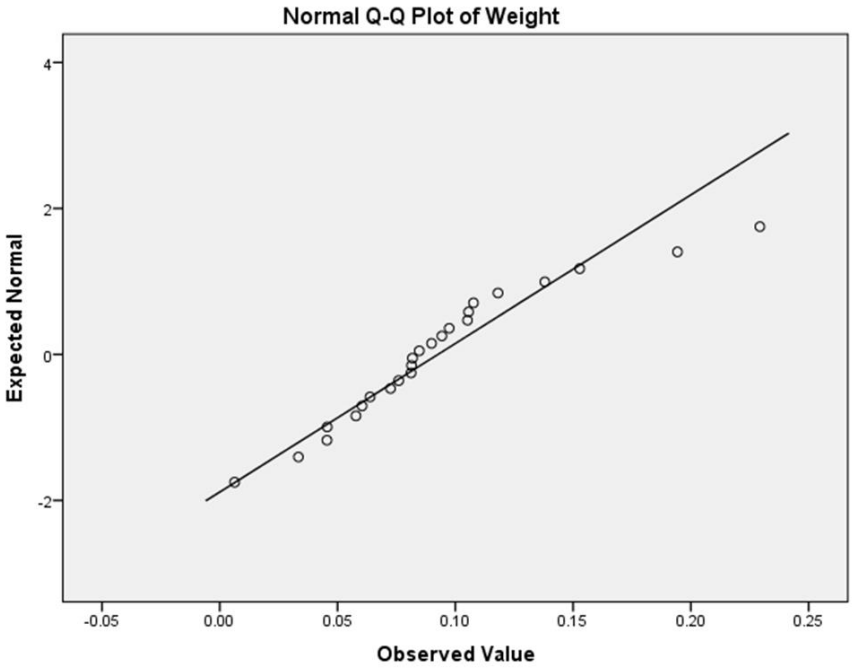
b. Grouping Variable: Treatment

Sorghum root mass

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mass	.171	24	.067	.924	24	.071

a. Lilliefors Significance Correction



Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: Mass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	.251[a]	13	.019	20.402	.000
Block	.013	1	.013	13.554	.004
Treatment	.032	11	.003	3.101	.037
Error	.010	11	.001		
Total	.261	24			

a. R Squared = .960 (Adjusted R Squared = .913)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Mass
LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T_{10} (Fertilizer)	T_1	.0543500	.03076409	.105	-.0133613	.1220613
	T_2	.0904500*	.03076409	.013	.0227387	.1581613
	T_3	.0574000	.03076409	.089	-.0103113	.1251113
	T_4	.0767000*	.03076409	.030	.0089887	.1444113
	T_5	.0515250	.03076409	.122	-.0161863	.1192363
	T_6	.0756500*	.03076409	.032	.0079387	.1433613
	T_7	.0588500	.03076409	.082	-.0088613	.1265613
	T_8	.0654500	.03076409	.057	-.0022613	.1331613
	T_9	.0423500	.03076409	.196	-.0253613	.1100613
	T_{11}	.0699500*	.03076409	.044	.0022387	.1376613
	T_{12}	-.0489500	.03076409	.140	-.1166613	.0187613
T_{11} (WATER)	T_1	-.0156000	.03076409	.622	-.0833113	.0521113
	T_2	.0205000	.03076409	.519	-.0472113	.0882113
	T_3	-.0125500	.03076409	.691	-.0802613	.0551613
	T_4	.0067500	.03076409	.830	-.0609613	.0744613
	T_5	-.0184250	.03076409	.561	-.0861363	.0492863
	T_6	.0057000	.03076409	.856	-.0620113	.0734113
	T_7	-.0111000	.03076409	.725	-.0788113	.0566113
	T_8	-.0045000	.03076409	.886	-.0722113	.0632113
	T_9	-.0276000	.03076409	.389	-.0953113	.0401113
	T_{10}	-.0699500*	.03076409	.044	-.1376613	-.0022387
	T_{12}	-.1189000*	.03076409	.003	-.1866113	-.0511887
T_{12} (No peat)	T_1	.1033000*	.03076409	.006	.0355887	.1710113
	T_2	.1394000*	.03076409	.001	.0716887	.2071113
	T_3	.1063500*	.03076409	.005	.0386387	.1740613
	T_4	.1256500*	.03076409	.002	.0579387	.1933613
	T_5	.1004750*	.03076409	.008	.0327637	.1681863
	T_6	.1246000*	.03076409	.002	.0568887	.1923113
	T_7	.1078000*	.03076409	.005	.0400887	.1755113
	T_8	.1144000*	.03076409	.003	.0466887	.1821113
	T_9	.0913000*	.03076409	.013	.0235887	.1590113
	T_{10}	.0489500	.03076409	.140	-.0187613	.1166613
	T_{11}	.1189000*	.03076409	.003	.0511887	.1866113

Based on observed means.
The error term is Mean Square(Error) = .001.

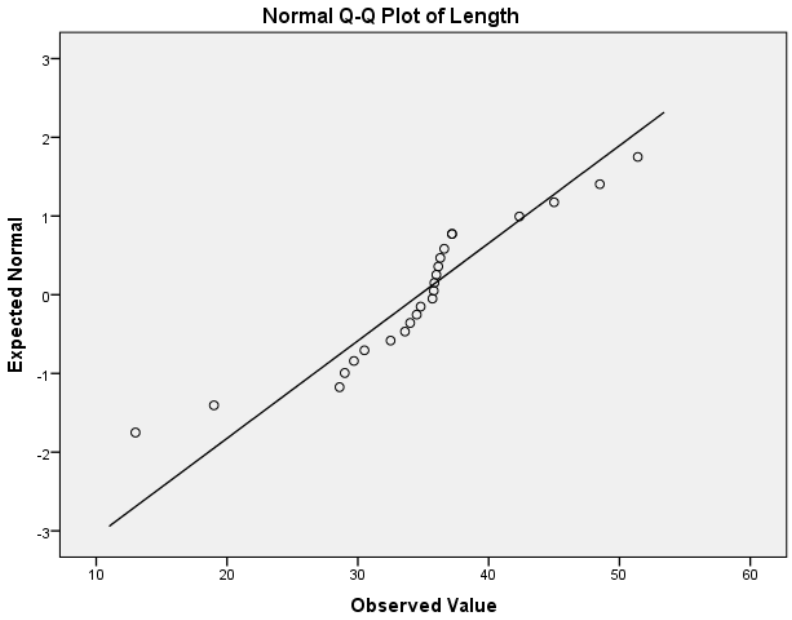
*. The mean difference is significant at the 0.05 level.

Sorghum shoot lengths

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Length	.213	24	.006	.909	24	.034

a. Lilliefors Significance Correction



ANOVA

abs_dif

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	192.730	11	17.521	.383	.938
Within Groups	548.250	12	45.688		
Total	740.980	23			

Kruskal-Wallis Test

Ranks

	Treatment	N	Mean Rank
Length	1	2	13.75
	2	2	8.50
	3	2	16.00
	4	2	6.00
	5	2	8.50
	6	2	8.00
	7	2	20.25
	8	2	13.00
	9	2	11.50
	10	2	23.50
	11	2	9.50
	12	2	11.50
	Total	24	

Test Statistics[a,b]

	Length
Chi-Square	12.030
df	11
Asymp. Sig.	.361

a. Kruskal Wallis Test

b. Grouping Variable: Treatment

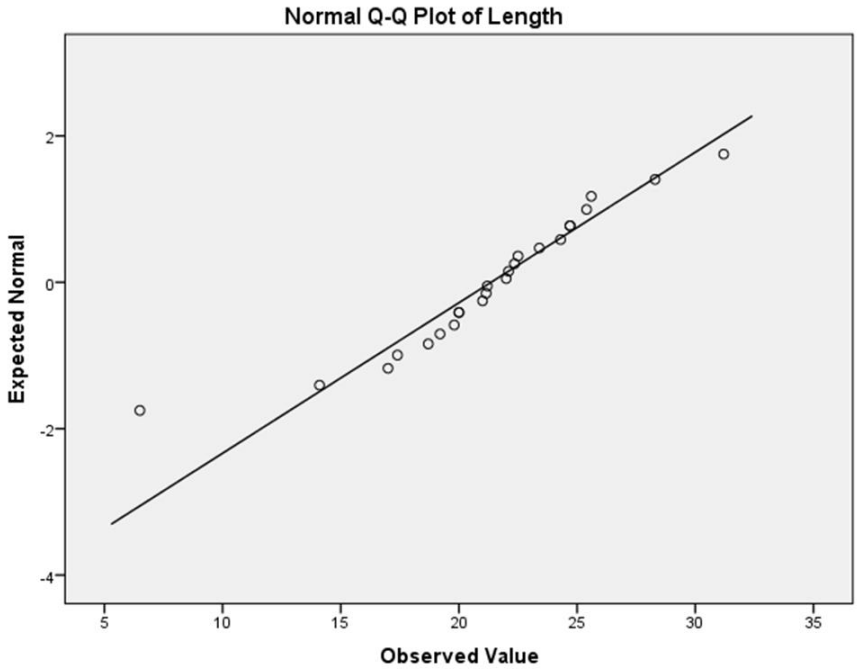
Sorghum root lengths

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Length	.126	24	.200*	.933	24	.112

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction



Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: Length

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	11247.218[a]	13	865.171	38.845	.000
Block	88.935	1	88.935	3.993	.071
Treatment	210.001	11	19.091	.857	.599
Error	244.998	11	22.273		
Total	11492.215	24			

a. R Squared = .979 (Adjusted R Squared = .953)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Length
LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T_{10} (Fertilizer)	T_1	3.200	4.7194	.512	-7.187	13.587
	T_2	4.900	4.7194	.321	-5.487	15.287
	T_3	.250	4.7194	.959	-10.137	10.637
	T_4	.900	4.7194	.852	-9.487	11.287
	T_5	1.775	4.7194	.714	-8.612	12.162
	T_6	4.000	4.7194	.415	-6.387	14.387
	T_7	-1.100	4.7194	.820	-11.487	9.287
	T_8	-1.350	4.7194	.780	-11.737	9.037
	T_9	-1.775	4.7194	.714	-12.162	8.612
	T_{11}	8.650	4.7194	.094	-1.737	19.037
	T_{12}	-.350	4.7194	.942	-10.737	10.037
T_{11} (WATER)	T_1	-5.450	4.7194	.273	-15.837	4.937
	T_2	-3.750	4.7194	.444	-14.137	6.637
	T_3	-8.400	4.7194	.103	-18.787	1.987
	T_4	-7.750	4.7194	.129	-18.137	2.637
	T_5	-6.875	4.7194	.173	-17.262	3.512
	T_6	-4.650	4.7194	.346	-15.037	5.737
	T_7	-9.750	4.7194	.063	-20.137	.637
	T_8	-10.000	4.7194	.058	-20.387	.387
	T_9	-10.425*	4.7194	.049	-20.812	-.038
	T_{10}	-8.650	4.7194	.094	-19.037	1.737
	T_{12}	-9.000	4.7194	.083	-19.387	1.387
T_{12} (No peat)	T_1	3.550	4.7194	.468	-6.837	13.937
	T_2	5.250	4.7194	.290	-5.137	15.637
	T_3	.600	4.7194	.901	-9.787	10.987
	T_4	1.250	4.7194	.796	-9.137	11.637
	T_5	2.125	4.7194	.661	-8.262	12.512
	T_6	4.350	4.7194	.376	-6.037	14.737
	T_7	-.750	4.7194	.877	-11.137	9.637
	T_8	-1.000	4.7194	.836	-11.387	9.387
	T_9	-1.425	4.7194	.768	-11.812	8.962
	T_{10}	.350	4.7194	.942	-10.037	10.737
	T_{11}	9.000	4.7194	.083	-1.387	19.387

Based on observed means.
The error term is Mean Square(Error) = 22.273.

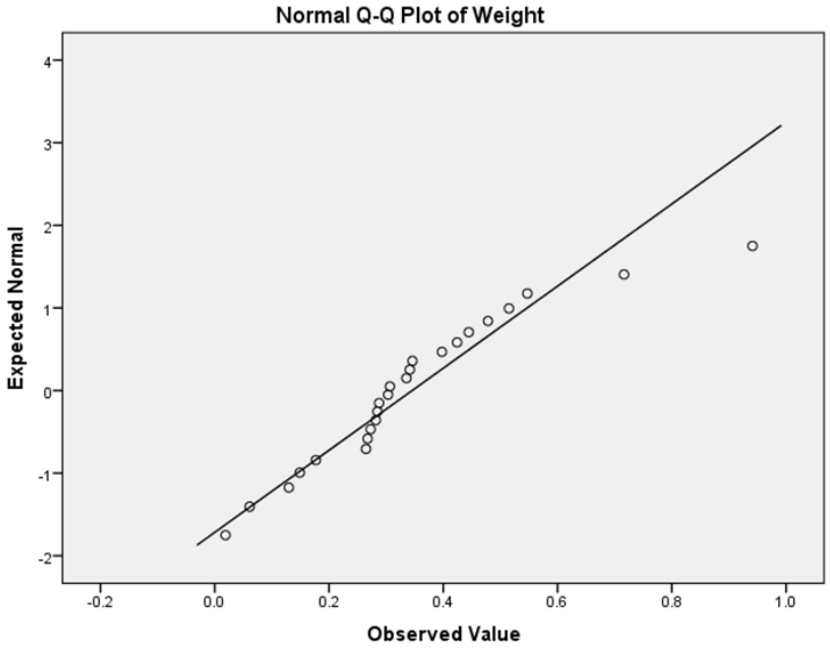
*. The mean difference is significant at the 0.05 level.

Sorghum plant mass

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mass	.166	24	.087	.920	24	.058

a. Lilliefors Significance Correction



Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: Mass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	3.665[a]	13	.282	23.637	.000
Block	.122	1	.122	10.226	.008
Treatment	.679	11	.062	5.172	.006
Error	.131	11	.012		
Total	3.796	24			

a. R Squared = .965 (Adjusted R Squared = .925)

Post Hoc tests

Multiple Comparisons

Dependent Variable: Mass

LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T_{10} (Fertilizer)	T_1	.5170750*	.10921631	.001	.2766915	.7574585
	T_2	.6080250*	.10921631	.000	.3676415	.8484085
	T_3	.5390750*	.10921631	.000	.2986915	.7794585
	T_4	.6559250*	.10921631	.000	.4155415	.8963085
	T_5	.4376750*	.10921631	.002	.1972915	.6780585
	T_6	.6027250*	.10921631	.000	.3623415	.8431085
	T_7	.4862250*	.10921631	.001	.2458415	.7266085
	T_8	.4904750*	.10921631	.001	.2500915	.7308585
	T_9	.3776750*	.10921631	.005	.1372915	.6180585
	T_{11}	.6464250*	.10921631	.000	.4060415	.8868085
	T_{12}	.4388000*	.10921631	.002	.1984165	.6791835
T_{11} (WATER)	T_1	-.1293500	.10921631	.261	-.3697335	.1110335
	T_2	-.0384000	.10921631	.732	-.2787835	.2019835
	T_3	-.1073500	.10921631	.347	-.3477335	.1330335
	T_4	.0095000	.10921631	.932	-.2308835	.2498835
	T_5	-.2087500	.10921631	.082	-.4491335	.0316335
	T_6	-.0437000	.10921631	.697	-.2840835	.1966835
	T_7	-.1602000	.10921631	.170	-.4005835	.0801835
	T_8	-.1559500	.10921631	.181	-.3963335	.0844335
	T_9	-.2687500*	.10921631	.032	-.5091335	-.0283665
	T_{10}	-.6464250*	.10921631	.000	-.8868085	-.4060415
	T_{12}	-.2076250	.10921631	.084	-.4480085	.0327585
T_{12} (No peat)	T_1	.0782750	.10921631	.488	-.1621085	.3186585
	T_2	.1692250	.10921631	.150	-.0711585	.4096085
	T_3	.1002750	.10921631	.378	-.1401085	.3406585
	T_4	.2171250	.10921631	.072	-.0232585	.4575085
	T_5	-.0011250	.10921631	.992	-.2415085	.2392585
	T_6	.1639250	.10921631	.162	-.0764585	.4043085
	T_7	.0474250	.10921631	.673	-.1929585	.2878085
	T_8	.0516750	.10921631	.645	-.1887085	.2920585
	T_9	-.0611250	.10921631	.587	-.3015085	.1792585
	T_{10}	-.4388000*	.10921631	.002	-.6791835	-.1984165
	T_{11}	.2076250	.10921631	.084	-.0327585	.4480085

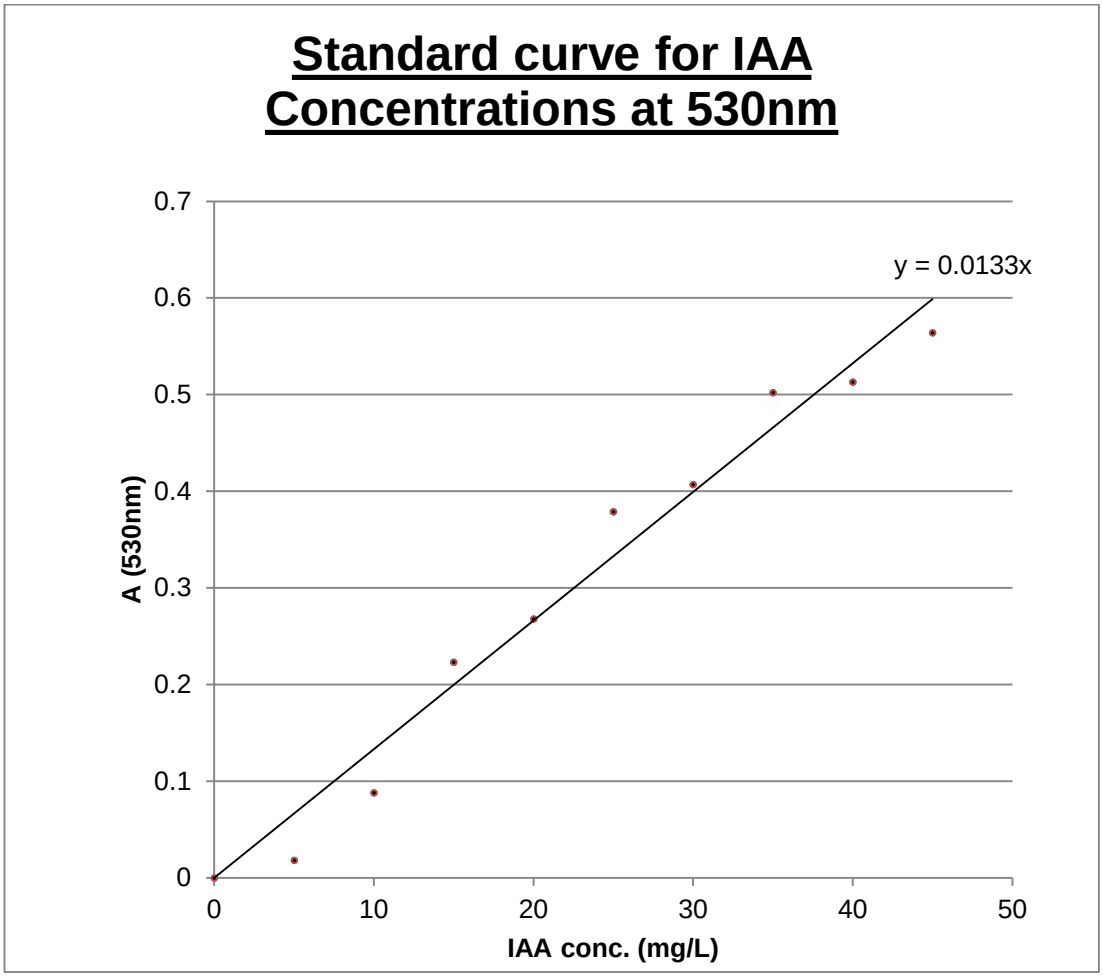
Based on observed means.

The error term is Mean Square(Error) = .012.

*. The mean difference is significant at the 0.05 level.

Appendix 2

IAA Standard curve



Annexure

Annex 1

Media

Combined Carbon Medium (Bashan, Holguin, & Lifshitz, 1993)

Solution A:

K_2HPO_4,	0.8 g
KH_2PO_4,	0.2 g
NaCl,	0.1 g
$Na_2FeEDTA$,	28 mg
$Na_2MoO_4 \cdot 2H_2O$,	25 mg
Yeast extract,	100 mg
Mannitol,	5 g
Sucrose,	5 g
Acetic acid	0.5 m1 (60%, v/v)
Distilled water,	900 ml

Solution B:

$MgSO_4 \cdot 7H_2O$,	0.2 g
$CaCl_2$,	0.06 g
Distilled water,	100 ml

The solutions should be autoclaved separately, cooled, and mixed. To this new solution, filter-sterilized biotin (5 mg/1) and p-aminobenzoic acid (10 mg/l) should be added and the final pH adjusted to 7.0.

Malt Extract Broth (Difco)

Malt Extract	6.0 g
Maltose	1.8 g
Dextrose	6.0 g
Yeast Extract	1.2 g
Distilled water	1 litre

Mix all components in distilled water and sterilize.

SM broth (Reinhold, Hurek, Niemann, & Fendrik, 1986)

DL-malic acid,	5.0 g
KOH,	4.5 g
KH_2PO_4,	0.6 g
K_2HPO_4,	0.4 g
$MgSO•7H_20$,	0.2 g
NaCl,	0.1 g
$CaC1_2$,	0.02 g
$MnSO_4•H_20$,	0.01 g
$Na_2MoO4•2H_20$,	0.002 g
Fe (III)-EDTA (0.66% [wt/vol] in water),	10 ml
Biotin,	0.1mg
Distilled water,	1 litre
Vitamin solution	1 ml

Vitamin solution:

D-biotin	200 mg
Calcium pantothenate	40 mg
Myoinositol	200 mg
Niacinamide	40 mg
P-aminobenzoicacid	20 mg
Pyridoxine hydrochloride	40 mg
Riboflavin	20 mg
Thiamine dichloride	4 mg
Distilled water	1 litre

Mix all components of vitamin solution in distilled water and filter sterilize. Mix all components of SM broth in distilled water and autoclave, adjust pH to 6.8 after autoclaving and supplement with 1ml vitamin solution.

VM-ethanol medium (Reinhold-Hurek, & Hurek, 2000)

K_2HPO_4	0.6 g
KH_2PO_4	0.4 g
NH_4Cl	0.5 g
$MgSO_4•7H_2O$	0.2 g
NaCl	1.1 g
$CaCl_2•2H2O$	0.026 g
$MnSO_4•H_2O$	0.01 g
$Na_2MoO4•2H_2O$	0.002 g
Fe(III)-EDTA	0.066 g
Yeast extract	1 g
Tryptone	3 g
Agar	15 g
Vitamin solution	1 ml
Ethanol	6 ml

Mix all components except vitamin solution and ethanol in distilled water and autoclave. Adjust pH to 6.8 after autoclaving and supplement with vitamin solution and ethanol.

Skimmed milk agar

Solution A:

Nutrient agar powder	23 g
Distilled water	900 ml

Solution B:

Skimmed milk powder	20 g
Distilled water	100 ml

Mix components of solutions and sterilize separately. After autoclaving, cool solutions to 45-50 °C and aseptically transfer milk solution to nutrient agar solution.

Pikovskaya agar (HiMedia Laboratories)

Yeast Extract	0.50 g
Dextrose	10.00 g
Calcium Phosphate	5.00 g
Ammonium Sulphate	0.50 g
Potassium Chloride	0.20 g
Magnesium Sulphate	0.10 g
Manganese Sulphate	0.0001 g
Ferrous Sulphate	0.0001 g
Agar	15 g
bromophenol blue	20mg

Mix all components in distilled water and sterilize.

Brain Heart Infusion Soil Extract Medium (Atlas, 2005)

Yeast extract	20.0g
Pancreatic digest of casein	16.0g
Brain heart, solids from infusion	8.0g
Peptic digest of animal tissue	5.0g
NaCl	5.0g
Na_2HPO_4	2.5g
Glucose	2.0g
Soil extract	250.0ml
Vitamin solution	1.0ml

Soil Extract:

Soil	1.0g
Na_2CO_3	1.0g
Distilled water	400ml

Combine soil extract components, sterilize and filter mixture through paper before use.

Mix components of brain heart solution, except glucose, yeast extract, and Vitamin solution, in 800ml of water and autoclave. Add yeast extract and glucose to 200.0mL of water, filter sterilize and add to cooled brain heart solution. Supplement with 1.0mL of Vitamin solution.

Annex 2

Table 12: Atmospheric pressure, temperature and daylight details during pot experiments.

Day	Atmospheric pressure	Temperature		Length of Daylight	
		GH	WHK	h: m: s	Decimal
24-Nov	1007.6 hPa	38° C	33.5° C	13h 19m 55s	13.319 h
25-Nov	1006 hPa	36° C	30.2° C	13h 20m 42s	13.345 h
26-Nov	1008.1 hPa	38° C	30.0° C	13h 21m 27s	13.358 h
27-Nov	1007.1 hPa	34° C	32.9° C	13h 22m 11s	13.37 h
28-Nov	1006.3 hPa	41° C	34.0° C	13h 22m 53s	13.381 h
29-Nov	1005 hPa	34° C	34.7° C	13h 23m 34s	13.393 h
30-Nov	1003.1 hPa	34° C	31.1° C	13h 24m 13s	13.404 h
1-Dec	1007.1 hPa	26° C	25.2° C	13h 24m 51s	13.414 h
2-Dec	1012.6 hPa	28° C	28.3° C	13h 25m 27s	13.424 h
3-Dec	1011.9 hPa	33° C	31.2° C	13h 26m 01s	13.434 h
4-Dec	1008.4 hPa	37° C	33.0° C	13h 26m 34s	13.443 h
5-Dec	1006.6 hPa	39.5° C	32.2° C	13h 27m 05s	13.451 h
6-Dec	1007.7 hPa	36° C	31.9° C	13h 27m 35s	13.46 h
7-Dec	1008.4 hPa	30° C	29.7° C	13h 28m 02s	13.467 h
8-Dec	1011.3 hPa	27° C	24.4° C	13h 28m 28s	13.474 h
9-Dec	1009.5 hPa	28° C	26.2° C	13h 28m 52s	13.481 h
10-Dec	1007.5 hPa	32° C	30.0° C	13h 29m 15s	13.488 h
11-Dec	1006.3 hPa	33° C	31.7° C	13h 29m 35s	13.493 h
12-Dec	1005 hPa	35° C	32.2° C	13h 29m 54s	13.498 h
13-Dec	1005.5 hPa	35° C	32.5° C	13h 30m 11s	13.503 h
14-Dec	1006.2 hPa	35° C	32.8° C	13h 30m 26s	13.507 h
15-Dec	1006.1 hPa	34° C	33.2° C	13h 30m 39s	13.511 h
16-Dec	1005.1 hPa	36° C	32.6° C	13h 30m 50s	13.514 h
17-Dec	1004.2 hPa	36° C	33.5° C	13h 30m 59s	13.516 h
18-Dec	1003.3 hPa	33° C	33.7° C	13h 31m 06s	13.518 h
19-Dec	1004.1 hPa	37° C	29.4° C	13h 31m 12s	13.52 h
25 Days	1006.92 hPa	34.1° C	31.2° C	13h 26m 58s	13.449 h

Note. Nov = November, Dec = December, hPa = hectopascal, °C = degree Celsius, h:m:s = hour: minute: second, GH = greenhouse, WHK = Windhoek.
Atmospheric pressure, WHK temperature and daylight values courtesy of Namibia Weather Network via http://www.namibiaweather.info/

Annex 3

ZR Fungal/Bacterial DNA MiniPrep™ procedure used for DNA extraction:

1. Add 50-100 mg (wet weight) fungal or bacterial cells that have been resuspended in up to 200 µl of water or isotonic buffer (e.g., PBS) or up to 200 mg of tissue to a ZR BashingBead™ Lysis Tube. Add 750 µl Lysis Solution to the tube.

2. Place in a vortex and process at maximum speed for 10 minutes.

3. Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at 10,000 x g for 1 minute.

4. Transfer up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at ~7,000 x g for 1 minute. Snap off the base of the Zymo-Spin IV™ Spin Filter prior to use.

5. Add 1,200 µl of Fungal/Bacterial DNA Binding Buffer to the filtrate in the Collection Tube from Step 4.

6. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.

7. Discard the flow through from the Collection Tube and repeat Step 6.

8. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute.

9. Add 500 µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x g for 1 minute.

10. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml microcentrifuge tube and add 100 µl DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute the DNA.

Annex 4

Hygrotech Terra Nova fertilizer nutrient profile:

N	3.5%
P	1.35%
K	2.25%
Ca	4.0%
S	0.5%
Zn	330ppm
Mn	600ppm
Cu	55ppm
Fe	1100ppm
B	45ppm

Research article

A survey for plant-growth-promoting rhizobacteria and symbionts associated with crop plants in the Okavango region of Southern Africa

Jann Grönemeyer, Dirk Berkelmann, Tabo Mubyana-John, Daniel Haiyambo, Percy Chimwamurombe, Bruce Kasaona, Thomas Hurek & Barbara Reinhold-Hurek*

Abstract: Regions in the Okavango catchment and delta such as highlands of Angola, the Kavango region of Namibia, and the Okavango Delta region of Botswana, although rich in plant diversity and density, have not produced significant yields when cropped by small scale farmers in the region. This phenomenon may be due to many factors among which is low nitrogen and other crop nutrients availability. This region due to its richness in flora may harbour bacteria which play a major role in plant nutrient availability. However, some of these rhizobacteria can be isolated and re-inoculated on crop plants to improve crop yields. Thus a survey on root nodulation of local pulses such as *Vigna unguiculata, V. subterranea*, and *Phaseolus vulgaris,* in the Chitembo area of Angola and the Kavango region of Namibia was carried out. Nodulated plants and putatively symbiotic bacteria were detected from a range of sites for all species. In Namibia, isolation of putative plant-growth-promoting rhizobacteria (PGPRs) was done on cereal crops and from other indigenous plants on farmland and from pristine areas. In Botswana, phosphate solubilizing bacteria were isolated from the roots of grasses in the floodplains and assayed for their ability to solubilize soil phosphate with the intention of using them to increase yields in sorghum (*Sorghum vulgare*). In total, 46 bacterial strains were isolated from nodules of legumes from Namibia, while 37 strains were isolated from Angola. Additionally, 32 strains of plant associated rhizobacteria were obtained from cereals or natural plants from the Kavango region in Namibia, and further ten isolates were selected from the Seronga region in Botswana. The large number of bacteria generated by this survey may contain some bacteria that may promote plant growth and improve soil fertility.

Keywords: bambara groundnut; cowpea; nodule; *Pennisetum glaucum*; phosphate solubilizing bacteria; *Rhizobium*; sorghum.

Pesquisa sobre rizobactérias promotoras do crecimento de plantas e simbiontes associados com cereais, na região do Okavango no sul da África

Resumo: Regiões na bacia e no delta do Okavango, como os planaltos de Angola, a região de Kavango da Namíbia e a região do delta do Okavango de Botsuana, embora ricas em diversidade e densidade de plantas, não produziram safras significativas quando cultivadas por agricultores de pequena escala na região. Este fenômeno pode ser devido a vários fatores, entre os quais a baixa disponibilidade de nitrogênio e outros nutrientes agrícolas. Essa região, devido à sua riqueza em flora, pode abrigar bactérias que desempenham um papel importante na disponibilidade de nutrientes nas plantas. No entanto, algumas destas rizobactérias podem ser isoladas e reinoculadas em culturas para melhorar seu rendimento. Assim, foi realizado um estudo sobre a nodulação das raízes de plantas locais, como a "*Vigna unguiculata*", "*V. subterranea*" e "*Phaseolus vulgaris*", em Chitembo na área Angolana e no Kavango na região da Namíbia. Plantas noduladas e bactérias putativamente simbióticas foram detectadas a partir de uma variedade de locais para todas as espécies. Na Namíbia, o isolamento de rizobactérias putativas promotoras do crescimento de plantas (PGPRs) foi feito em culturas de cereais e de outras plantas indígenas em terras agrícolas e em áreas intocadas. Em Botsuana, bactérias solubilizadoras de fosfato foram isoladas das raízes de gramíneas nas várzeas e analizadas por sua capacidade de solubilizar fosfato do solo, com a intenção de usá-las para aumentar a produtividade na cultura do sorgo (*Sorghum vulgare*). No total, 46 cepas de bactérias foram isoladas de nódulos de leguminosas na Namíbia, enquanto 37 foram isoladas em Angola. Além disso, 32 cepas de plantas associadas a rizobactérias foram obtidas a partir de cereais ou plantas naturais da região de Kavango na Namíbia e outras 10 cepas isoladas foram selecionadas da região de Seronga em Botsuana. O grande número de bactérias geradas por essa pesquisa pode conter algumas bactérias que podem promover o crescimento das plantas e melhorar a fertilidade do solo.

Palavras-chave: Amendoim bambara; bactérias de solubilização de fosfato; feijão-caupi; milheto; nódulo *Rhizobium*; *Pennisetum glaucum*; sorgo.

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Introduction

The predominant agriculture paradigm based on improved varieties of common staple crops in high-input systems has not succeeded addressing food insecurity and malnutrition e.g. in Sub-Saharan Africa (Rudebjer et al. 2013), where over 200 million people (28% of the population) were undernourished in 2005-2007 (FAO, 2010). Yield variability and risk for crop failure in Africa's rainfed agriculture systems contribute to factors that explain difficulties in adopting new technologies (Ogada et al. 2010). These risks are predicted to increase in many areas in Southern Africa due to climate change, as projections suggest a decline in land area suitable for cultivation of crops (Lane & Jarvis 2007). On the other hand, demand for staple crops like maize is currently increasing in Africa due to changes in eating habits, with limited production and increasing imports.

Low crop productivity is often a problem faced in smallholder farming systems in Sub-Saharan and Southern Africa. Subsistence agriculture with low agrochemical inputs is widespread in the greater Okavango catchment regions of the Okavango basin in Angola, Namibia and Botswana. For example in smallholder's farms of the Kavango region of Namibia, fields are not irrigated, herbicides as well as pesticides and fertilizers are not used, and crop yields are very low (Pröpper et al. 2010).

Low yields are often associated with declining soil fertility and low input by biologically fixed nitrogen (see below), depending on biological and environmental factors (Dakora & Keya 1997). The availability of nitrogen affects the productivity of crops and cereals worldwide in all ecosystems. Nitrogen fertilizer is the most widely used resource, of which one third is lost through emission of greenhouse gasses and leaching, causing adverse environmental impacts. Unfortunately, the majority of African small farmers are not able to afford the high mineral fertilizer prices (Yanggen et al. 1998) albeit there is a growing need for mineral N fertilizers (World bank, 2008). Low cost and sustainable technical solutions compatible with the socioeconomic conditions of small farmers are needed to solve soil fertility and yield problems (Chianu et al. 2011).

Nitrogen is unique among the other essential elements because N_2 from the atmosphere can be fixed by biological nitrogen fixation (BNF), exclusively carried out by prokaryotes that possess the enzyme nitrogenase. This potential can be employed in nitrogen-fixing symbioses between Fabaceae and rhizobia, but also in other crop systems for a more sustainable agricultural practice. The most important N_2-fixing agents in agricultural systems are the symbiotic associations between crop and forage/fodder legumes and rhizobia forming root nodule symbioses. Estimates of N fixed annually are > 100 kg N/ha/year in good-practice farmer's fields (Herridge et al. 2008). However, there is only little published knowledge on rhizobial symbionts of crops from Namibia, Angola, or Botswana (Pule-Meulenberg & Dakora 2007). Given the high cost of fertilizer in Africa and the limited market infrastructure for farm inputs, current research and extension efforts need to be directed to integrated nutrient management, in which legumes play a crucial role. Inoculation with compatible rhizobia resistant to harsh environmental conditions can make BNF a key source for farmers with little income (Smaling et al. 2008).

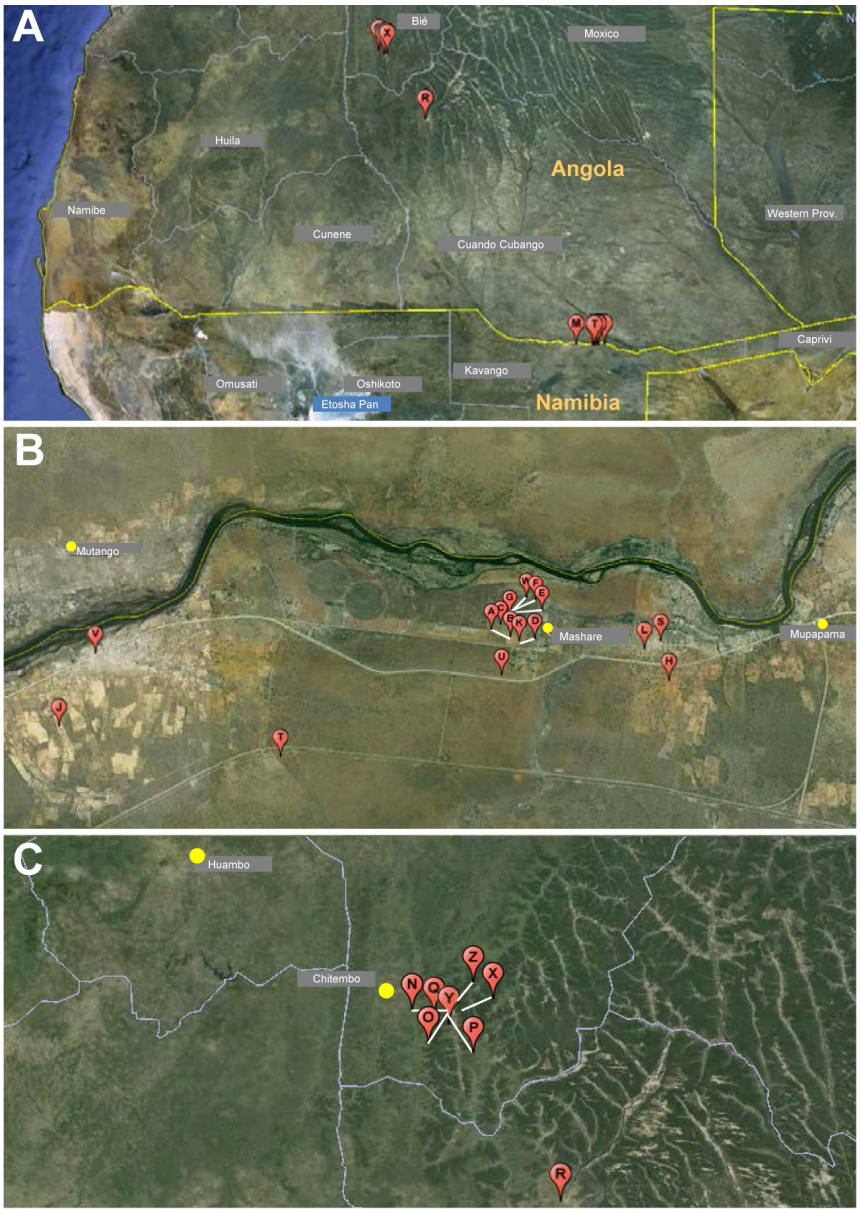

Fig. 1: Overview of the areas of the survey for nodulated grain legumes in Southern Africa. (A) Overview of sampling areas in Angola and Namibia. (B) Close-up of the sampling areas in the Kavango province of Namibia, near Kaiango, Mashare and Mupapama. (C) Close-up of the sampling area in the province of Bié near Kuseke in Angola.Flowchart of the implemented analysis scheme.

Additionally, roots support the growth of a variety of microorganisms that may have strong effects on growth or health of plants, not based on BNF. These beneficial bacteria (plant-growth-promoting rhizobacteria, PGPR) may enhance root or plant growth, nutrient uptake, stress resistance or resistance to pathogens by a variety of mechanisms (Adesemoye & Kloepper 2009, Desbrosses et al. 2009). Studies on or inoculants for traditional cereal crops in the Kavango area are not available. In a first survey, we isolated and identified endophytes from roots of Namibian maize, *Sorghum bicolor* and *Pennisetum glaucum*, which are affiliated to known PGPRs or might even represent new species (Grönemeyer et al. 2012).

Here, we report on a survey for putative symbionts of grain legumes and putative PGPRs of local cereals in the Okavango region, with focus on Namibia and extension to Botswana and Angola. In Botswana where phosphate is one of

Table 1. Survey of grain legumes for nodulation by rhizobia in Namibia and Botswana. Locations represent sites shown in Figure 1. Samples labeled with "soil" represent trapping experiments from collected soil samples, using the plant species mentioned. NAM, Namibia; ANG, Angola. MADI: Mashare Agricultural Development Institute, test fields for varieties.

Country	Location	Coordinates	Plant species	Land use form	Plant number	Isolates
NAM	A	S17.895486 E20.211047	*Arachis hypogaea*	MADI, not irrigated, local race	1	1
NAM	B	S17.895126 E20.210972	*Arachis hypogaea*	MADI, not irrigated, local race	2	1
NAM	C	S17.893389 E20.209500	*Vigna unguiculata*	MADI, irrigated, local race	3, 4	2
NAM	E	S17.893125 E20.210531	*Vigna unguiculata*	MADI, not irrigated, race from Kenia	6	1
NAM	F	S17.892806 E20.210467	*Vigna unguiculata*	MADI, not irrigated, dryland conditions, race as 6	7	2
NAM	G	S17.892661 E20.210467	*Arachis hypogaea*	MADI, field as 7, race as 1	8	0
NAM	H	S17.90074 E20.23303	*Vigna subterranea* *Vigna unguiculata*	Subsistence farmer`s field, dryland agriculture, Kalahari sands	9 10	1 1
NAM	I	S17.89546 E20.33150	*Vigna unguiculata*	Subsistence farmer`s field, dryland agriculture, old floodplain soils	14	1
NAM	J	S17.90719 E20.14718	*Arachis hypogaea*	Subsistence farmer`s field, dryland agriculture, Kalahari sands	16	1
NAM	K	S17.895881 E20.212164	*Vigna unguiculata* *Arachis hypogaea*	MADI, dryland conditions, local race, Kalahari sands	20-25 26-29	2 4
NAM	H	S17.90074 E20.23303	*Vigna unguiculata* *Vigna subterranea*	Subsistence farmer`s field, dryland agriculture, Kalahari sands	34, 35 36-38	5 5
NAM	M	S17.898225 E19.900658	*Vigna subterranea* *Arachis hypogaea* *Vigna unguiculata*	Subsistence farmer`s field, dryland agriculture, old floodplain soil, field legumes only	54-56, 60 57-59, 61 62	6 2 0
ANG	N	S13.69958 E17.06752	*Vigna unguiculata*	Subsistence farmer`s field, dryland agriculture	40, 41	4
ANG	O	S13.70122 E17.06739	*Phaseolus vulgaris*	Subsistence farmer`s field, dryland agriculture	42	2
ANG	P	S13.71203 E17.06544	*Phaseolus vulgaris* *Vigna unguiculata*	Subsistence farmer`s field, dryland agriculture	43, 44 45, 46	4 5
ANG	Q	S13.640244 E16.983867	*Vigna unguiculata* *Phaseolus vulgaris*	Subsistence farmer`s field, dryland agriculture Novel field, next to above, older field	47-48, 50-51 49	5 3
ANG	R	S14.662819 E17.665467	*Phaseolus vulgaris*	Small field in Menongue	52, 53	4
NAM (soil)	H	S17.90073 E20.23300	*Vigna subterranea*	Subsistence farmer`s field, dryland agriculture, Kalahari sands		4
NAM (soil)	S	S17.89518 E20.23199	*Vigna subterranea* *Vigna unguiculata*	Subsistence farmer`s field, dryland agriculture, old floodplain soil		1 1
NAM (soil)	T	S17.91146 E20.17834	*Vigna subterranea*	Bushveld, Kalahari sands		4
NAM (soil)	U	S17.89968 E20.20953	*Vigna unguiculata* *Vigna subterranea*	Bushveld, Kalahari sands		1 2
NAM (soil)	V	S17.89690 E20.15219	*Vigna unguiculata*	Subsistence farmer`s field, dryland agriculture, old floodplain soil		2
NAM (soil)	W	S17.89274 E20.21068	*Vigna subterranea*	Irrigation agriculture, old floodplain soil, maize field		1
ANG (soil)	X	S13.70896 E17.11225	*Vigna subterranea*	Fallow after subsistence farming		2
ANG (soil)	Y	S13.69081 E17.0633	*Vigna subterranea*	Subsistence farmer`s field, dryland agriculture		1
ANG (soil)	Z	S13.68787 E17.101756	*Vigna subterranea*	Pristine grassland / bush		2

the major limiting elements in the cultivation of sorghum, the staple crop, a study was carried out to isolate phosphate solubilizing bacteria from the rhizosphere of Seronga floodplain grasses.

Materials and Methods

Time and location of sampling campaigns

A survey for nodulated grain legumes was carried out in Namibia and Angola in the rainy season. Samples were obtained mainly in the Mashare area during 23.03.-24.03.2011. A second campaign in this area was from 25.03.-27.04.2012 and 03.04.2012. Inspection of roots for nodules was also carried out between 30.12.2012-03.01.2013. In Angola, sampling was carried out in the Kuseke area south of Chitembo between 30.03.-01.04.2012. Soils for trapping of rhizobia were also collected in the March –April 2012 field trips on the sampling sites. The coordinates of these sampling sites are given in Table 1, and the locations are shown in Figure 1.

Sampling of plants for PGPR isolation was carried out on selected sites indicated in Table 2 along the Kavango riverine agro-ecology zone in Namibia.

In Botswana, dominant flood plain grasses in Seronga flood plains were selected. The coordinates of the sampling sites are shown in Table 3.

Table 2. Source of bacterial isolates from Namibia with potential for plant growth promotion. Latin name for Mahangu is *Pennisetum glaucum*.

Country	GPS Coordinates	Plant species	Land use form	Isolates
Namibia	17°53`43.80 S 20°14`05.26 E	Mahangu	Subsistance farming	8
Namibia	17°53`49.75 S 20°09`07.07 E	Mahangu	Subsistance farming	3
Namibia	17°55`00.13 S 20°06`16.14 E	Mahangu	Subsistance farming	2
Namibia	17°54`04.40 S 20°14`14.34 E	Mahangu	Subsistance farming	3
Namibia	17°53`43.80 S 20°14`05.26 E	*Sorghum bicolor*	Subsistance farming	2
Namibia	17°55`00.13 S 20°06`16.14 E	*Sorghum bicolor*	Subsistance farming	4
Namibia	17°53`38.49 S 20°09`08.97 E	*Sporobolus sp.*	Pristine	2
Namibia	17°53`33.62 S 20°14`56.13 E	*Sporobolus sp.*	Pristine	3
Namibia	17°52`30.82 S 20°15`21.88 E	*Phragmites australis*	Pristine	1
Namibia	17°53`33.62 S 20°14`56.13 E	*Vetiveria nigritana*	Pristine	1
Namibia	17°53`38.49 S 20°09`08.97 E	*Vetiveria nigritana*	Pristine	1
Namibia	17°53`33.62 S 20°14`56.13 E	Ngwena (Local name)	Pristine	2

Sampling of nodules and isolation of bacterial symbionts

Grain legumes (Table 1) from farmer's fields were inspected for root nodules at their root systems. If nodules were present, several nodules per plant were cut off including some adjacent root tissue. They were stored in 2 ml glass vials with silica gel desiccant at room temperature during the campaign, and at 4°C upon arrival in Bremen. The cultivation of root nodule bacteria was carried out in the following way: desiccated root nodules were rehydrated in 2 ml of sterile water for four hours at room temperature. They were then surface sterilized by immersion in 70% ethanol for 30 seconds followed by a washing step in sterile water and immersion in 5% sodium hypochlorite for two minutes. After six further washing steps, surface sterilized nodules were homogenized with mortar and pestle in 100 to 500 µl (depending on nodules size) of sterile tap water, and a portion was streaked on modified arabinose gluconate medium (van Berkum 1990). The plates were incubated at 28°C for up to 14 days, and pure cultures were obtained by repeated streaking of single bacterial colonies on fresh agar plates.

To confirm the isolates` ability to fix atmospheric nitrogen, a polymerase chain reaction (PCR) based approach targeting the nifH marker gene was carried out. A portion of a bacterial colony was added to 10 µl Lyse and Go PCR Reagent (Thermo Scientific, Rockford, USA), and genomic DNA was released following the manufacturer`s instructions. 2 µL of the lysate served as a template in a PCR consisting of 2.5 U Taq DNA polymerase (Molzym, Bremen, Germany), 5 µl 10X PCR Buffer, 50 µM of each dNTP, and 0.5 µM of each primer FGPH19 and PolR (Poly et al. 2001) in a total volume of 50 µl. DNA was amplified in a Biometra TProfessional thermocycler under the cycling conditions described by Demba-Diallo et al. (2004). The presence or absence of the 429 bp PCR product was determined on a 1.8% agarose gel using PstI-digested DNA of Lambda Phage as a size marker.

Sampling of roots and isolation of PGPRs

Endophytic bacteria were carefully isolated repeatedly from root samples of various plants on selected sites along the Kavango River in Namibia (see Table 2). Pure cultures were obtained and immediately put to long-term storage while further characterization on the isolated bacteria continued. The isolated bacteria were tested for various plant-growth-promoting features including IAA production, phosphate solubilizing potential and bio-control properties.

In Botswana (Table 3), sampling was done by digging out the entire root systems of the selected plants and then

Table 3. Source of phosphate solubilizing isolates to be used as PGPR.

Country	Isolate number	GPS coordinates	Plant species	Land use form
Botswana	S1	18°49`35.48 S 22°25`55.71 E	*Eulesine africana*	Flood plain grassland
Botswana	S2	18°49`35.48 S 22°25`55.71 E	*Imperata cylindrica*	Flood plain grassland
Botswana	S3	18°49`35.48 S 22°25`55.71 E	*Imperata cylindrica*	Flood plain grassland
Botswana	S4	18°48`58.46 S 22°24`53.53 E	*Sesbania seban*	Flood plain grassland
Botswana	S5	18°49`35.48 S 22°25`55.71 E	*Panicum maxinium*	Flood plain grassland
Botswana	S6	18°48`58.46 S 22°24`53.53 E	*Cyperus sp.*	Flood plain grassland
Botswana	S8	18°47`38.4 S 22°24'19.7 E	*Cynodon dactylon*	Cattle grazing Flood plain
Botswana	S9	18°49`35.48 S 22°25`55.71 E	*Urochloa decumbens*	Flood plain grassland
Botswana	S10	18°49`35.48 S 22°25`55.71 E	*Urochloa trichophus*	Flood plain grassland

shaking off the soil to only retain the rhizosphere soil. Each plant was placed in a separate zip lock bag. All the plants were then placed in a cooler box and transferred to the laboratory. Once at the laboratory the roots were excised at the crown and the shoots were sent to the herbarium for identification, while the roots were retained for isolation of phosphate solubilizing bacteria.

Isolation of phosphate solubilizing bacteria

For the isolation of phosphate solubilizing bacteria (PSB) sterile calcium phosphate agar amended with 0.125 g of cyclohexamide/L was used (Nautiyal, 1999). Ten gram of each root sample were placed in a sterile conical flask containing 100 mL of sterile tap water. The mixture was left standing for 15 minutes, with shaking at intervals. A 1 ml aliquot was used to prepare serial dilutions to 10-4. The dilutions were then spread-plated on the solidified calcium phosphate agar and left in an incubator at 25°C. The plates were checked daily for any phosphate solubilizing ability.

After 5 days, halo zones around certain colonies were observed in some of the plates. The colonies with clear halo zones were considered to be PSB and these colonies were picked up using a sterile inoculation loop and streaked onto fresh calcium phosphate agar plates for purification. The purified cultures inoculated onto calcium phosphate agar slants and stored in the refrigerator (4°C).

To determine the phosphate solubilizing ability of the isolates, calcium phosphate agar plates were inoculated with the isolates at the centre using a sterile inoculation loop. The plates were incubated at 25°C for 20 days. These plates were also sealed with parafilm to prevent dehydration and contamination. The zones of clearance (i.e. the halozone) produced by the different isolates and diameter of the colonies were measured at 4 day intervals using a vernier calliper. These measurements were used to calculate the solubilization index (ref) of each isolate.

The solubilizing ability of the isolates was also assessed on other phosphate media i.e., magnesium, aluminium, potassium and iron phosphate.

Results and Discussion

Grain legumes as crops for smallholders in the Northern Kavango region of Namibia and the Chitembo area in Angola

In the Kavango region of Namibia, agriculture is largely dominated by smallholder farms with low crop yields and little developed market chains and food processing. Here as in many African regions, grain legumes can be regarded as "meat for the poor", due to their rich protein content and the relatively low prices of pulses in comparison to meat (Chianu et al. 2011). In our surveys of smallholder farms, cowpea (*Vigna unguiculata*, local name in Kavango makunde) was the main grain legume grown by farmers (Fig. 2A). In Namibia, also Bambara groundnut (*Vigna subterranea*, local name nongomene) is planted (Fig. 3A, B), albeit to a lesser extent. It can be regarded as part of neglected and underutilized species which are often local crops that remain important to poor communities' livelihood but are not exploited to their full potential (Rudebjer et al. 2013). Occasionally, peanuts (*Arachis hypogaea,*

Fig. 2: Intercropping in smallholder farms in the Mashare area in the Kavango region of Namibia. (A) Mahangu and cowpea intercropping. (B) Sampling on a plot for field tests on a traditional smallholder farm.

local name nongongo) were also found. In the Chitembo area, in addition to cowpea, the common bean (*Phaseolus vulgaris*) is utilized as well (Fig. 3F).

Common practice was intercropping of grain legumes with local cereals such as mahangu *(Pennisetum glaucum*), sorghum (*Sorghum bicolor*), or maize (Fig. 2), where legumes were interspersed in an irregular pattern (Fig. 2A). Farmers mostly used local varieties which they propagated themselves and stored grains for the following season. This dryland agriculture system is rainfed, with planting when the rainy season starts, typically November to December. Smallholders did not use chemical fertilizers or manure or any other input. Thus, nitrogen fixation by legume nodule symbiosis would provide an important input of nitrogen into the farming system.

Survey of grain legumes for root nodulation

In the wet season of 2011 and 2012, we undertook a survey of grain legumes and their root nodulation in Namibia and Angola. In Namibia, the North of the Kavango province was targeted, particularly the region around Mashare which belongs to the core sites of the TFO (The Future Okavango) project (Fig. 1A, 1B). The region is characterized by different forms of land use: few pristine woodlands were present; bushveld sites were woody areas used for occasional grazing of cattle; dryland agriculture was the typical form of smallholder farming (see above), and some of these fields had been abandoned (fallows); in small areas near the river, irrigation agriculture with fertilizer inputs was conducted by commercial farmers. In a parallel survey of different land use types and different soils (see Gröngröft et al. 2013), two categories of landscapes and soil types (old flood plains and Kalahari sands) were considered. Soils for trapping (see below) originated from different land use and soil types (Table 1). In Angola, the area for the survey was also one of the core sites of TFO, a rural area dominated by smallholder farms south of Chitembo (Fig. 1, Table 1). Land use types were typically forests and dryland agriculture fields on the summits of hills, horticulture in the wetlands of the valleys, and grassland at the slopes.

Inspection of root systems of pulses in fields of subsistence farmers in the Kavango region often showed a relatively

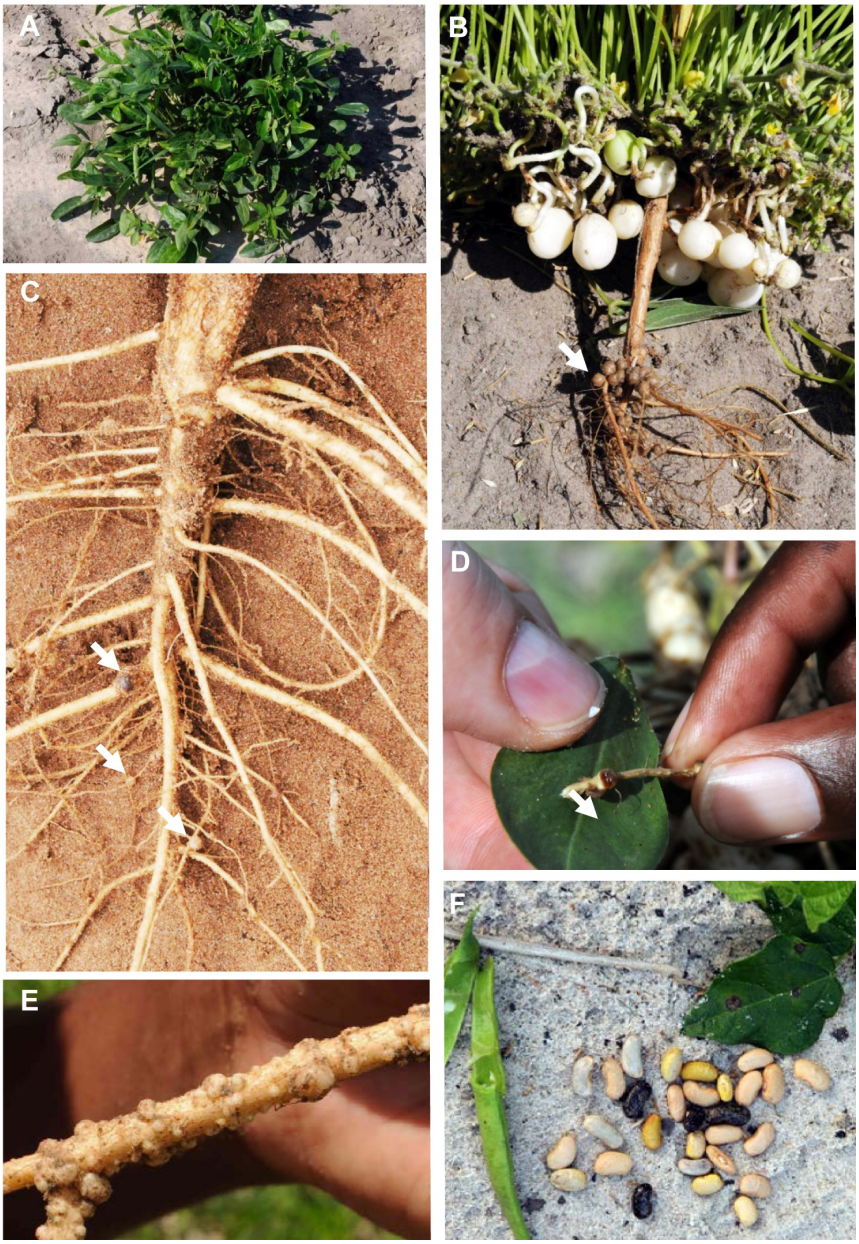

Fig. 3: Examples of nodulated grain legumes detected during the survey in Namibia. (A) Bambara groundnut (*Vigna subterranea*) plant and (B) well-nodulated root system with groundnuts. (C) Cowpea (*Vigna unguiculata*) root system and (D) detached root nodule cut open. (E) Well-nodulated roots of cowpea. Arrows point towards examples of root nodules. (F) Beans (*Phaseolus vulgaris*) cultivated in smallholder farms in Angola.

low degree of nodulation (Fig. 3 C, D), similar observations were made at the Angolan site. Therefore, a survey of traditional legume crops was undertaken for detection of nodulated plants, in order to isolate putatively efficient bacterial symbionts adapted to the soil and climatic conditions. Surveys were carried out in Namibia in the rainy season in December/January or March/April. Generally, nodules were more abundant and less senescent earlier in the season. Most samples originated from the Mashare area in the Kavango region (Fig. 1 B, Table 1). The widest range of grain legumes was found at the MADI (Mashare Agricultural Development Institute), where test fields were under irrigation or under rainfed (dryland) conditions typical for smallholders` farms. Here, cowpea, Bambara groundnut, peanut, and *Lablab purpureus* were cultivated. Several of these plants were nodulated, albeit often poorly. However, from several plants and species, diazotrophs could be isolated

from nodules (Table 1). On some fields of subsistence farmers, nodulated plants appeared to be relatively rare: for example, only from 3 out of 10 inspected individual plants of Bambara groundnut on site H, root nodules could be detected. On the other hand, in a smallholder farmer's field where mainly grain legumes were grown since several years (site M), some root systems were intensely nodulated (Fig. 3 E). Here an enrichment of compatible rhizobia might have occurred over the years.

In total, we could isolate diazotrophs from nodules collected in Southern Africa from several different grain legumes and from different sites: for cowpea, from 9 sites in Namibia and 3 sites in Angola; for Bambara groundnut, from 2 sites in Namibia; for peanut, 5 sites in Namibia; for *Phaseolus vulgaris*, from 4 sites in Angola (Table 1).

In order to increase the diversity of isolates from the underrepresented crop Bambara groundnut, we carried out trapping experiments. Soil was collected from different regions and land use forms (dryland and irrigated agriculture, bushveld in Namibia; fallow, dryland agriculture and pristine grassland in Angola) and transported to Germany with cooling. Surface sterilized grains of Bambara groundnut or occasionally cowpea were transplanted into the soils under aseptic conditions, and the root systems inspected for nodulation of root systems. This approach was also successful for isolation of diazotrophs (Table 1). Initial characterization of isolates indicated that the most diazotrophs, except for those isolated from *Phasaeolus* nodules, belonged to *Bradyrhizobium spp.*.

Survey of cereals and native plants for putatively plant-growth-promoting rhizobacteria and phosphate-solubilizing bacteria

In the Kavango region of Namibia, roots of local cereals like mahangu and sorghum as well as native plants were used for isolation of root-associated bacteria. From 12 different plant samples, 32 isolates were obtained (Table 2).

From roots of grass samples in the Seronga region of Botswana (Table 3), also numerous bacteria were isolated. Many isolates showed different abilities to solubilize phosphate on the different media. Of the many isolates obtained, only nine showed the ability to solubilize phosphate on all the phosphate agar media used. Maximum solubilization was observed on potassium phosphate agar medium. Table 4 shows the solubilization ability of the different isolates on potassium phosphate agar.

Table 4. Diameter of zone of clearance for the different isolates with time (days) on potassium phosphate agar. Values are millimeters. Means followed by the same letter in the same column are not significantly different from each other at 5% according to the Tukey-Kramer test.

Isolate	Grass source	12 d	17 d	19 d	23 d	24 d	27 d
S1	*Eulesine africana*	4b	4.5b	4.6b	5.4b	5.6b	9c
S2	*Imperata cylindrica*	1a	2a	2a	2a	2a	2a
S3	*Imperata cylindrica*	1a	2a	2a	2a	2a	2a
S4	*Sesbania sesban*	1a	2a	2a	2a	2a	2a
S5	*Panicum maximum*	0.6a	2a	2a	2a	2a	2a
S6	*Cyperus sp.*	0.4a	1a	2a	2a	2a	2a
S8	*Cynodon dactylon*	4b	5b	7b	8c	8c	8c
S9	*Urochloa decumbens*	2a	5b	6b	6bc	7c	7bc
S10	*Urochloa trichophus*	2a	4b	5b	5b	6bc	6bc

Botswana soils due to the low rainfall are often slightly alkaline and contain cations which form complexes with phosphorus. Such P deficiencies are very common in most Botswana soils. Phosphorus also forms insoluble complexes with other cations such as aluminium, magnesium, iron and potassium in soil (Thompson & Troeh 2005). It is, therefore, very crucial that any isolate that is intended to be used in the production of a phosphate solubilising biofertilizer should be able to solubilise different phosphate complexes. As such in this study the solubization ability of the isolates was also tested on different agar media i.e., potassium phosphate, magnesium phosphate, iron phosphate, and aluminium phosphate. And only those which showed the ability to solubilize all the phosphates will be selected for further studies.

Conclusions

Our survey has generated a large number of bacteria which may contribute to promote plant growth and soil fertility. Inspection of root systems of grain legumes in smallholder's fields showed large variations in root nodule symbioses, ranging from poorly or rarely to intensely nodulated. Isolation of putatively symbiotic bacteria may help to develop inoculants adapted to the crops and the harsh environmental conditions. Considering that the soil nutrient status in these areas is generally poor and especially low in N content, the role microorganisms in enhancing crop production cannot be ignored or overstated. More work is thus required in this regard to design bioformulations that can be deployed in the areas to boost crop production. Moreover, indigenous grasses in Seronga flood plains flourish in environments where crops show serious P deficiencies, probably because they habour P-solubilising bacteria which they use in acquiring P. In future research, the isolates' ability to solubilise phosphate complexes could be exploited for crop growth.

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