AN ASSESSMENT OF BIOLOGICAL NITROGEN FIXATION ON LEGUMES GROWING ON FARMERS' FIELDS AND IN NATURAL SYSTEMS AND AUTHENTICATION OF ASSOCIATED ROOT NODULE BACTERIA

A Dissertation presented to the Department of Crop Science and Production in partial fulfillment of the requirements of the Degree of Masters of Science (MSc) in Crop Science (Soil Science)

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MASTERS OF SCIENCE (MSc) IN CROP SCIENCE

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December 2016

CERTIFICATION

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Declaration

I hereby declare that this submission is my original work and has not been presented for another Degree in this or any other University.

…….. ……...

AUTHOR'S NAME & SIGNATURE DATE

Dedication

This Dissertation is dedicated to my parents especially my lovely and caring mother Ms. Doricah Bernard who supported my studies, through thick and thin. Her continued support made sure that my studies moved swiftly. To my supervisors, all my colleagues, friends and everyone who played a vital role on my academic life, without your input I would not have managed to reach these far heights.

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Abstract

The low inherent soil fertility, especially nitrogen (N) constrains arable agriculture in Botswana. Nitrogen is usually added to soil through inorganic fertilizer application. In this dissertation, biological N_2 fixation by legumes is explored as an alternative source of N. The objectives of this study were i) to measure levels of N_2 fixation by grain legumes such as cowpea, Bambara groundnut and groundnut in farmers' fields as well as to estimate N_2 fixation by indigenous herbaceous legumes growing in the Okavango Delta; ii) to isolate root nodule bacteria, characterize and authenticate them on their homologous hosts. Flowering plants were sampled from the panhandle part of the Okavango Delta, Tswapong area, Kgalagadi and Gantsi regions. N_2 fixation was measured using the ^{15}N stable isotope natural abundance technique. Root nodule bacteria were isolated, characterized and authenticated under sterile conditions. The $\delta^{15}N$ values of indigenous herbaceous legumes indicated that they fixed N_2 (-1.88 to +1.35 ‰) with the lowest value measured in *Chamaecrista absus* growing in Ngarange (Okavango Delta). The $\delta^{15}N$ values of grain legumes growing on farmers' fields ranged from -1.2 ‰ to +3.3 ‰. For most farms, %Ndfa was above 50% indicating that they largely depended on symbiotic fixation for their N nutrition. With optimal planting density, Bambara groundnuts on farmers' fields could potentially fix about 100 kg N/ha in some parts of Tswapong area and about 60 kg N/ha in areas around the Okavango Delta. Root nodule bacteria exhibited various morphological characteristics. Not all of the authenticated strains fixed N_2, for example, seventeen out of the twenty-six nodulating strains were fixing N_2. Some rhizobial strains such as cowpea strain BUAN316/QAB-Vu48 and *Crotalaria sphaerocarpa* strain BUAN316/XAU-Cs70B induced high shoot and root biomass comparable to 5 mM KNO_3 positive control. Taken together, results from this thesis have shown that herbaceous indigenous legumes and cultivated legumes play an important role in the cycling of N in the soil. It has also been shown that biological N_2 in farmer's field could potentially supply the much needed N for the legumes and the subsequent cereal crops if plant densities are optimized. This desertion has also shown that some of the isolated rhizobial strains have potential for development of elite rhizobial strains as well as Plant Growth Promoting Bacteria (PGPBs) with the potential to increase food security and mitigate climate change.

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List of abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
ADP	Adenosine diphosphate
AOAC	Association of Analytical Communities
ATP	Adenosine Triphosphate
BNF	Biological Nitrogen Fixation
BUAN	Botswana University of Agriculture and Natural Resources
CEC	Cation Exchange Capacity
CIAT	International Center for Tropical Agriculture
IAA	Indole-3-acetic acid
ISPAAD	Integrated Support Programme for Arable Agricultural Development
LKB	Lekobeng
LKD	Lekadiba
GRT	Grootlagte
MPN	Most Probable Number
NGA	Ngarange
N	Nitrogen
NDFA	Nitrogen derived from the atmosphere
QAB	Qabo
YMA	Yeast mannitol agar
XAU	Xauga

XAK Xakao

SSA Sub Saharan Africa

USDA United States Department of Agriculture

1.0 INTRODUCTION

1.1 General introduction

One of the major constraints to increasing arable production in Botswana is the low inherent fertility of soils (Pule-Meulenberg and Batisani, 2003). Nitrogen is the most commonly deficient mineral nutrient in soils, often contributing to reduced plant growth and crop yields. It can be supplied to agricultural crops as fertiliser or fixed through biological nitrogen fixation. Biological N_2 fixation, involving a mutualistic relationship between some members of Rhizobiaceae ("rhizobia") and the Leguminosae, is another source of N for natural and agricultural ecosystems.

So far, researchers have identified five genera within the Rhizobiaceae that have the ability to infect roots and form N_2-fixing nodules in members of the Leguminosae and in the non-legume *Parasponia* (Ulmaceae). These genera of rhizobia include *Rhizobium*, *Bradyrhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Mesorhizobium*, and *Azorhizobium* (Sprent *et al*., 2003). All the five genera of rhizobia with nodulation ability in legumes are α-proteobacteria, but some newly discovered strains include *Methylobacterium*, which nodulates *Lotononis bainesii* in South Africa (Jaftha *et al*., 2002) and *Crotolaria* in Senegal (Sy *et al*., 2001), as well as *Blastobacter denitrificans* and *Devosia* strains, which nodulate the aquatic legumes *Aeschenomene* and *Neptunia* respectively (van Berkum and Eardly, 2002; Rivas *et al*., 2002) in the tropical environments of Africa, Asia and South America.

A while back, a *Burkholderia* strain belonging to the β-proteobacteria was isolated from root nodules of *Aspalathus carnosa* in South Africa with nodulating ability on siratro (Moulin *et al*., 2001). *Ralstonia taiwanensis*, another β-proteobacteria was isolated from nodules of *Mimosa* species, and has been similarly confirmed to nodulate and fix N_2 in the homologous *Mimosa* host

plants (Chen *et al*., 2003). Interestingly, the *Mimosa* is a pantropical genus that has been reported in earlier studies to nodulate with α-proteobacteria-type rhizobia in the Phillipines, Mexico and Brazil (Oyainzu *et al*., 1993; Wang *et al*., 1999), and more recently with strains of *Ralstonia taiwanensis* belonging to β-proteobacteria (Chen *et al*., 2003).

The legume-rhizobial symbiotic interaction leads to formation of nodules which are sites for nitrogen fixation, consequently conversion of gaseous atmospheric N_2 into ammonium through the process termed as the biological nitrogen fixation. The NH_3 formed makes it possible for legumes to survive well under low nitrogen soils and provides residual N to the crops following legume plantation in a crop rotation. It is also worth noting that little quantities of nitrogen can be leaked to the non-legume during the growing season under intercropping (Dakora and Keya, 1997). The benefit of rhizobia to the host plant is determined by the amount of N_2 fixed and released into the host-plant cells.

The accumulated fixed-N in plant organs is easily quantified using ^{15}N isotope technology and/or the N difference method. As shown in Table 1.1, levels of N_2 fixation can vary markedly between species, as well as between sites and countries for the same species (Schulze *et al*., 1991) due to genetic and environmental factors (Dakora and Keya, 1997). For example, in Senegal, *Acacia senegal, A. seyal* and *A tortilis* have been assessed to fix only 5.3, 8.3, and 6.2 kg N/ha respectively (Sprent and Parson, 2000), levels probably enough to support plant growth, but too low to enhance the N economy of the ecosystem. Similar studies (Schulze *et al*., 1991) done in Namibia showed that members of the Mimosaceae exhibited varied $\delta^{15}N$ values, ranging from about 0.15‰ for *A. hereroensis* to a high 7.5‰ for *A. tortilis*. As a result, *A. tortilis* derived only 15% of its N from symbiotic fixation of atmospheric N_2, and *A. hereroensis* 50% (Schulze *et al*., 1991). In Senegal, shoot and leaves of *Crotalaria ochroleua, Crotalaria perrottetii* and *Crotalaria retusa* had a %

Ndfa ranging between 47-53% (Samba *et al*., 2002). Similarly, in Zimbabwe % Ndfa of 61, 67, 79 and 90 were found respectively on *Indigofera erecta, Chamaecrista mimosoides, Indigofera astragalina* and *Crotalaria juncea* (Nezomba *et al*., 2008).

Considering that Namibia (30-400 mm rainfall) and Botswana (150-650 mm rainfall) share similar dry environments, it would be interesting to know the levels of N_2 fixation in herbaceous nodulated legumes of Botswana. However, given the low symbiotic performance of the Mimosaceae in Senegal and Namibia, which have similar soil ecologies with Botswana, it is likely that symbiotic function would be similarly low due to the commonality of the prevailing abiotic factors. Many of the food grain legumes also showed a wide range of symbiotic performance as shown in Table 1.1.

The legume-rhizobial symbiosis plays a significant role in N economy with various legumes species offering variable amounts of nitrogen. However in Botswana besides scanty information regarding rhizobial technology, there are very few researches regarding nitrogen fixation by indigenous legume. This also shows that there is a huge gap which needs to be filled so as to improve the nitrogen status of our soils.

Table 1.1: Amount of N_2 fixed by legumes from various countries

Legume species	Country	N-fixed kg/ha	Reference
Food legumes			

Phaseolus vulgaris	Kenya	17-57	Ssali and Keya (1984)
Vigna unguiculata	Kenya	24-39	Ssali and Keya (1984)
	Ghana	201	Dakora *et al*. (1987)
	Nigeria	122	Eaglesham *et al*. (1981)
	Zimbabwe	73-79	Mapfumo *et al*. (2001)
Arachis hypogea	Ghana	32-134	Dakora *et al*. (1985b)
	Nigeria	11-63	Sanginga *et al*. (2003)
	Kenya	8	Gathumbi *et al*. (2002)
	Zimbabwe	46-50	Chikowo *et al*. (2003)
Glycine max	Nigeria	94	Sanginga *et al*. (2001)
	Zimbabwe	115-127	Ncube *et al*. (2007)
Cajanus cajan	Kenya	142	Guthambi *et al*. (2002)
	Zimbabwe	183	Mapfumo *et al*. (1999)
	Nigeria	86	Sanginga *et al*. (2001)
Vigna subterranea	Ghana	40-62	Dakora *et al*. (1985b)
	Zimbabwe	47-54	Ncube *et al*. (2007)
Herbaceous legumes			
Lablab purpureus	Nigeria	215	Sanginga *et al*. (2001)
	Ivory coast	7 -70	Nezomba *et al*. (2008)
Crotalaria palliada	Zimbabwe	173	Nezomba *et al*. (2008)
Crotalaria ochroleuca	Zimbabwe	26	Nezomba et al. (2008)
Crotalaria juncea	Zimbabwe	58	Nezomba *et al*. (2008)
	Malawi	29 - 142	Nezomba *et al*. (2008)
Eriosema elliticum	Zimbabwe	7	Nezomba *et al*. (2008)
Chamaecrista rotundifolia	Nigeria	144	Sanginga *et al*. (2003)
Chamaecrista mimsoides	Zimbabwe	79	Nezomba *et al*. (2008)
Indigofera astragalina	Zimbabwe	1.5	Nezomba *et al*. (2008)
Indigofera errecta	Zimbabwe	0.5	Nezomba *et al*. (2008)
Indigofera astragalina	Zimbabwe	1.5	Nezomba *et al*. (2008)
Tephrosia villosa	Ivory coast	27-119	Nezomba *et al*. (2008)
Tephrosia radicans	Zimbabwe	1.4	Nezomba *et al*. (2008)
Zornia glabra	Malawi	16	Cadisch *et al*. (1988)
Tree/shrubs legumes			
Sesbania sesban	Senegal	43-102	Ndoye and Dreyfus (1988)
	Kenya	52	Gathumbi *et al*. (2002)
Leucaena leucocephala	Tanzania	110	Hogberg and Kvarnstrom (1982)
	Nigeria	304	Danso *et al*. (1992)
Acacia Senegal	Nigeria	< 20	Sanginga *et al*. (2001)
	Senegal	5.25	Sprent and Parsons (2000)
Acacia seyal	Senegal	8.32	Sprent and Parsons (2000)
Acacia tortilis	Senegal	6.24	Sprent and Parsons (2000)
Acacia albida	Nigeria	< 20	Sanginga *et al*. (2001)
Albizia spp.	Nigeria	60-120	Kadiata *et al*. (1996)

1.1.1 Importance of legumes

Legumes are widely known multi-purpose crops which provide significant source of proteins vital for sustaining nutritional balance hence helping in fighting malnutrion for the resource poor population (Appunu *et al*., 2009). Cowpea being the most common in Africa, has been coined the *poor man's meat* because of its high and inexpensive nutrient contribution to the poor population (Muranaka *et al*., 2016). Various legumes are consumed in different forms; green pods and tender leaves are eaten before the cereals mature hence termed insurance food during the hunger season (Coulibaly *et al*., 2009; Boukar *et al*., 2015). They can also be used as vegetable with its seed used in many African dishes. Green cowpea seeds are sometimes roasted like peanuts then used as a substitute for coffee (Hosseney *et al*., 1996).

N_2 fixation by forage legumes increases the overall N status of the associated grass in pasture. Also, due to their high protein content, herbaceous legumes such as Lablab (*Lab lab pupurea*) and Lucerne (*Medicago sativa*) are used as fodder for livestock while others provides an extra source of proteins to pasture for grazing animals (Giller *et al*., 2001). Several herbaceous legumes also play a vital role in the cropping systems. Their higher moisture and N content which favors rapid N mineralization make them good candidates for green manure (Rutherford *et al*., 2009). For example incorporating 3 months old green manure of *Crotalaria juncea* doubled maize yield (Giller *et al*., 2001). *Crotalaria spp.* can be used as pre-crops for providing green manure while at the same time decreasing the level of detrimental nematodes and increasing the level of beneficial mycorrhizal fungi. Sometimes these herbaceous legumes are used as cover crops where they help in protecting the soil from soil erosion. In Western Africa *Crotalaria spp* are being introduced to the cropping systems mainly for suppressing weeds and improving soils (Sanginga *et al*., 2003).

Legumes are a useful source of phytomedicine. For example, roots for *Acacia mellifera* improves fertility, treating syphilis, malaria and stomach aches (Mahesh *et al*., 2008), *Indigofera arrecta* roots are used for treating stomach aches in Kenya (Kipkore *et al*., 2014) and *Tephrosia sp* are essential in treating leprosy, ulcers, asthma and liver diseases (Dakora *et al*., 1995). They are also essential source of nutriceuticals, are used for religious and beautification purpose, biological control of parasitic pests (Valenzuela *et al*., 2002).

1.2 Justification

The world population is estimated to rise from about 7 ½ billion to as high as 10 billion in 2050. Sub Saharan Africa (SSA) as a rapidly developing region has a population of over 800 million and projected to reach 1.5 billion in 2050 (Gosling *et al*., 2016). This region has the most undernourished population of up to 223 million which is projected to rise by 132 million in 2050. Other than malnutrition, Sub Saharan Africa region is one of the hardest hit by poverty rate. Therefore, increased agricultural production required to meet the food demanded by the rapidly increasing human population leading to intensive farming involving more agrochemical usage (Vanlauwe et *al*., 2014). However excessive chemical fertilizer usage disturbs the ecological balance, pollutes water sources and over usage leads to green-house gas emission (Bhattacharya *et al*., 2000). With intensifying environment-related concerns and alarming climate change effects, sub Saharan Africa smallholder farmer's crop production is faced with challenges worsened by tough resource constraints, erratic rainfall and extreme weather conditions. However, African agriculture has great potential for growth due to its abundant natural resources which need to be explored towards sustainable food security and poverty reduction.

Various alternatives are harnessed to reduce dependence on inorganic N fertilizer for plant nutrients. Soil improvement through biological nitrogen fixation has the potential to significantly improve world food production when optimized. Brazil is an example of a country where advanced rhizobial technology (rhizobial inoculation) successfully replaced fertilizers in about 23 million ha of soybean plantation, saving about 300 kg N/ha which is about 80% of the needed crop nitrogen, a sign of high cost saving in agriculture (Li *et al*., 2016). Bio-fertilizers show wide benefits by reducing financial to environment problems, also a sustainable way of arable production, especially for resource poor farmers who mostly cannot afford the cost of inorganic fertilizers. Bio

fertilizer usage has shown to improve yields and food security in countries such as Zimbabwe where inoculated soybean yield under small scale farming increased from 0.5 – 1.5 to 2.8 – 3.3 t/ha (Giller *et al*., 2011).

In Botswana, farmers rely mostly on inorganic N rich fertilizers which are freely supplied through the ISPAAD program however farmers do not have much knowledge in using these fertilizers leading to overuse (Jefferis *et al*., 2012). This pollutes the environment and escalates the global warming and the program itself is unsustainable. Although rhizobia technology is at infancy, it has the potential to optimize crop production in a sustainable manner. Isolation of indigenous rhizobial strains which are well adapted to the local environmental conditions, are competitive and have higher nitrogen fixing capacity could be a good strategy for the acquisition of elite rhizobial strains which are necessary for optimizing production. These benefits are elevated by abundant adequate knowledge on rhizobial inoculants in terms of their quality and quantification of their population in soil which is a determinant of the need to inoculate. Maximizing elite rhizobial strains usage will lead to improved nitrogen status of soils and at the same time improving legume yield quality and quantity consequently improving food security, especially for the subsistence farmers (Thies *et al*., 1991).

In addition to assessing biological fixed N by indigenous herbaceous legumes from farmers' field and natural systems this study was carried out to characterize and authenticate the isolated root nodule bacteria. Thus finding the locally adapted rhizobial strains which are highly competitive and can be used as inoculant in improving legume production and enrich the soil nitrogen status and agriculture at large, especially for the resource poor farmers. Also, identification of elite rhizobial strains from the indigenous population can be highly beneficial when it is accompanied

by wide dispersal and accessibility to the local community so that they can adopt and use it in improving their production and food security.

1.3 Objectives

The main objective of the study was to assess nitrogen fixed by various herbaceous legumes from different regions of Botswana and to authenticate their associated root nodule bacteria. The specific objectives were:

1. To measure levels of biologically fixed nitrogen using $\delta^{15}N$ natural abundance method from various herbaceous legumes growing on farmer's fields and in natural systems.

2. To isolate, characterize and authenticate associated root nodule bacteria isolated from various herbaceous legumes.

1.4 Hypothesis

- $H1_0$: There are no significant differences in the levels of biologically fixed-N between various herbaceous legumes from different regions of Botswana
- $H1_a$: There are significant differences in the levels of biologically fixed-N between various herbaceous legumes from different regions of Botswana.

2.0 LITERATURE REVIEW

2.1 Status of smallholder agriculture in Sub Saharan Africa

Agriculture is the main industry accounting for a third of gross domestic products and employing about 65% labor force in Sub Saharan Africa (SSA). Smallholder farmers in this region are the principal producers of agricultural output accounting to 80% of all farms responsible for 90% of all the agricultural production (Baiphethi *et al*., 2009). Study by Kusangaya (2014), stated that dependence on subsistence agriculture in Botswana, Zimbabwe, Kenya and Malawi was respectively at 76%, 80%, 85% and 90%. Smallholder farmers are characterized by cultivating small (2 hectares or less) pieces of land and depend mainly on rain farming, normally referred to as "*resource poor*" due to limited access to sufficient labor, affordable input and financial credit (Cooper *et al*., 2008). Studies show that they practice low input/low yield subsistence agriculture with low average yields in comparison to global averages and do not reach commercial markets. Equally Gosling (2016), states that labor productivity and incomes from the Sub Saharan Africa smallholder agriculture is very low (USD 2.00/day) when equated to global averages whereas 60% of their income is spent on food.

2.2 Climate change status in sub Saharan Africa

Rapid agro-chemical usage leads to greenhouse emission which is the principal causer of global warming. In SSA it is anticipated that up to 30% annual precipitation and 50-70 % groundwater decrease rate in situations of 4˚C warming. Greenhouse gas emissions from agriculture accounts for 10-12% of the global anthropogenic emissions, equating approximately 6.1 Gigatons of carbon dioxide equivalent per annum also accounting for 84% and 54% of global N_2O and CH_4 emissions respectively (IPCC, 2014). Research by Stocker (2013) noted that agrochemicals rich in CO_2 led

to increased photosynthetic rate and enhanced dry matter accumulation by plants. Conversely, studies highlighted that increased atmospheric CO_2 negatively affect the nutritional quality of food crops. Plants absorbing the same amount of nutrients in the soil but producing higher biomass leads to dilute or reduced nutrients concentration in the edible plant. Therefore to get the same amount of those nutrients requires consuming more quantities of food which is a challenge intensifying the problem of micronutrient malnutrition in SSA population (Dwivedi *et al*., 2013).

2.3 Major legumes in the world

Reviews by Dakora and Keya (1997) show that Africa farming is characterized by legume production with rare agro-chemical application. Various legumes are grown worldwide which include among others: chickpea (*Cicer arietinum*), common bean (*Phaseolus vulgaris*), cowpea *(Vigna unguiculata*), groundnut (*Arachis hypogaea*), pigeonpea (*Cajanus cajan*), and soybean (*Glycine max*) (Gowda *et al*., 2007). Other minor legumes grown include: faba bean (*Vicia faba*), lentil (*Lens culinaris*), field pea (*Pisum sativum*), bambara groundnut (*Vigna subterranea*), hyacinth bean (*Dolichos lablab*), Kerting's groundnut (*Macrotyloma geocarpum*), lima bean (*Phaseolus lunatus*), yam bean (*Sphenostylis stenocarpa*), mung bean (*Vigna radiata*), black bean (*Vigna mungo*), moth bean (*Vigna aconitifolia*), rice bean (*Vigna umbellata*), and horse gram (*Macrotyloma uniflorum*) (Raemaekers *et al.,* 2001). Other important forage legumes include; alfalfa (*Medicago sativa* L.), clover (*Trifolium L*.) and leucaena (*Leucaena leucocephala)* (Sprent *et al*., 2009).

Majority of farmers in Botswana practice rain fed agriculture which depends on the low and unreliable rainfalls. According to Mayes and Molosiwa (2013), in Botswana arable farming is mainly dominated by Sorghum (*Sorghum bicolor*), Maize (*Zea mays*), Millet (*Pennisetum*

glaucum), Cowpea *(Vigna unguiculata*), Bambara groundnut *(Vigna subterranean)* and groundnut (*Arachis hypogaea*) mainly attributed by their adaptation to these low fertile drought-prone environments. Pulses yields in Botswana are noted to be always low (250-300 kg/ha) while the cereal production is fluctuating at around 18 000 tonnes, nonetheless this is found to be far much less than 10% of the annual requirements of 200 000 tonnes.

2.4 Legume-rhizobial symbiosis

Legumes were generally known for increasing soil nitrogen status since 18th century, through intercropping with other non-leguminous cereal crops. In Nigeria, Sanginga (2001) noted that legumes and cereals are regarded as responsible for improving and depleting soil fertility respectively. However, Deshwal (2013) indicated that the exact mechanisms involved in increasing nitrogen were not known up until late 18th century when Hellriegel and Wilfarth discovered that rhizobia were responsible for increasing nitrogen. In 1679 Malpighi was the first scientist to observe bumps on legumes which were thought to be insect galls, disease infection and or abnormal root growth (Hirsch *et al*., 2001). Rivas (2009) stated that in 1888 the Dutch scientist Martinus Beijerinck firstly isolated and cultured microorganisms from nodules of various legumes and named it *Bacillus radiocicola* but Frank (1889) renamed it, *Rhizobium leguminosarumn*.

2.4.1 Improving the legume-rhizobial symbiosis

Previous researches have shown that different legume species fix varied amounts of nitrogen however, they do not fix up to their potential levels. Rhizobial inoculation has been the best way to improve legumes to biologically fix nitrogen to their capacity. Rhizobial inoculation improves early rhizobia-legume interaction; consequently increasing nodulation at a tender age leading to improved plant growth and yield. Bottomley and Brockwell (1994) stated that inoculation should

be done mainly when; the same or symbiotically related legume in the immediate past history had not been cultivated, there was poor nodulation from previous legume and when a legume follows a non-leguminous crop during crop rotation.

Chemining'wa and Vessey (2006) indicated that through inoculation specific rhizobia are introduced to the legume seeds prior to or during planting hence offering the most effective and compatible strains of bacterial to the grown legume. Research by Woomer (2010) concluded that inoculum was mainly introduced through the following methods namely; slurry, where the inoculum is mixed with water to form slurry then mixed with seeds, adhesive solution and dry application. However, Keyser and Li (1992) reported that legume response to inoculation was an effect of native rhizobial population, nitrogen status of the soil, nitrogen demand and yield potential of the host pant.

2.4.2 Legume inoculation in Africa

Inoculating legumes is not highly practiced in African farming however, fewer population using them are not getting potential results. This is mainly due to using exotic inoculum coupled with lack of knowledge regarding inoculum usage (Bala *et al*., 2011). Poor results were mainly due to out-competitiveness of the native which are also characterized to be inefficient and possibly incompatible to the grown host plant and or low in population to meet yield potential. African soils are also known to be dominated by wide ranges of rhizobia strain; Giller (2001) in Zimbabwe noted diverse rhizobia strains compatible with soybean varieties even in instances with no recent inoculation history. Similarly, Denison (2004) highlighted that these resident rhizobia include both indigenous (native) and naturalized ones from previous inoculation which have been living saprophytically for a while. Rhizobial population of up to 10^2 cells/gram of soil was noted in some

districts of Zimbabwe after 3 years of inoculation (Mpepereki *et al*., 2000). However, Chemining'wa (2006) mentioned that rhizobia spending much time under saprophytic state lose their symbiotic effectiveness.

2.4.3 Hindrance to inoculation success

Native rhizobial populations are characterized functionally for N fixation potential through determination of species, number, and effectiveness. Nevertheless, low Most Probable Number (MPN) counts values for native soil rhizobia can cause major impact as reported by Sessitsch (2012) that less effective strains elicited large responses mostly when the native soil populations are extremely low. According to Naeem (2004) the effectiveness of many inoculum strains used proved no significant difference when compared to the average native strain found in effective populations. High occupancy by strains of less than average effectiveness may slightly suppress nitrogen fixation when there are above 2×10^1 rhizobia per gram of soil. However, it was noted that where a superior inoculum strain known for higher nodulation compared to native rhizobia, there was no response to inoculation when the number of native rhizobia was above 2×10^1/g of soil. Experiment conducted by Turk (1993) concluded that inoculation did not enhance nodule when the number of invasive native rhizobia was $> 1 \times 10^2$ per g of soil. Higher nodulation was attained by addition of excessive rhizobial number in the inoculum, an additional 30 to 40 rhizobia per g of soil available to lima bean and cowpea root systems resulted in 5 to 20 fold increase in nodule number (Turk *et al*., 1993).

2.4.4 Improving inoculum success in Africa

To improve successfulness of inoculants in Africa, exotic inoculants which have failed to give optimum results should be replaced by locally adapted strains. Locally adapted rhizobial strains should be acquired from indigenous plants which are well adapted and striving well under these local conditions. Studies by Nezomba (2015) observed that self-generating nitrogen fixating indigenous herbaceous legumes which are highly adapted to the harsh agro-ecological regions were manipulated to improve productivity of natural fallows contributing to an increased nitrogen economy. In Zimbabwe Mpepereki (1996) concluded that host range and symbiotic effectiveness evaluation of indigenous rhizobial population assisted in identification of effectively adapted rhizobial strains, potential to be a superior inoculant for wide geographical target. Furthermore research by Giller (2011) in Zimbabwe reported that rhizobial screening identified new elite strains performing better than the regularly used inoculants.

Simon (2013) and Berrada (2014) also observed that isolation and characterization of rhizobia identified effective bacterial strains offering economic benefits to the Tanzanian legume farmers hence maximizing agricultural production. Similar studies by Waswa (2014) on identification of elite rhizobial strain for soybeans in Kenya, found that agricultural improvement was attained through identification of native rhizobia characterized with superior symbiotic and competitive abilities. Selection from native rhizhobia was through screening isolates against reference strains for their performance under field conditions until identifying elite strains with greater adaptation and higher competitive ability (Waswa *et al*., 2014).

According to Sharma (2012), prior to inoculum usage it was important to evaluate the competence of the isolated strain, having in depth knowledge about it's; taxonomy, ecology, population,

efficiency and compatibility to given host legume. Supporting studies conducted in Tanzania by Simon (2013) concluded that isolation and testing for the effectiveness of nitrogen fixation from biodiversity ecosystem accompanied by monitoring factors affecting symbiosis. This was vital because native rhizobia have great variation in their nitrogen fixing and competitive ability due to their genetic make-up.

Because rhizobial strains isolated from locally adapted indigenous host legume are equally adapted to the locally harsh conditions. Screening of these strains lead to identification of effective, competitive strain with a broad host range including the field legumes such as cowpea, groundnut and Bambara groundnut which are good candidates to be superior inoculates. Due to their adaptability their inoculants will easily improve legume nodulation consequently improved N_2 fixed and general plant growth.

2.5 Characterization of rhizobial strain

All rhizobia were initially characterized based on shape, general appearance and their interaction with higher organisms (Bevan *et al*., 2011). Based on phenotypic characteristics through Bergey's classification, rhizobia bacteria are categorized as gram negative, aerobic, non-spore forming, motile rod shaped, sizes 1.2-3µm and 0.5 - 0.9 µm length and width respectively (Evans *et al*., 2015). Their movement is made possible by using thread-like structures called flagella while growing through cell division. According to Denison (2011) rhizobial life cycle can be composed of three distinct phases; infective, symbiotic and saprophytic where it lives in the absence of a legume, mainly expressed by the native ones.

According to Freitas (2013), based on growth rate on yeast mannitol agar (YMA), rhizobial genera are categorized by two broad groups; fast and slow growers despite the fact that some include the

intermediate growers as noted from *Mesorhizobium* species. Fast growing *Rhizobium* species from pea, bean, clover, alfalfa and chickpea attain maximum growth within 2 – 5 days and are known for synthesizing acidic products. Conversely slow growing *Bradyrhizobium* from soybean and cowpea species takes 6 - 12 days to show visible growth on YMA and synthesize alkaline products.

Research by Rai (2013) based on the phenotypic patterns of rhizobia illustrated that they are very diverse, showing greater adaptation to abiotic stresses such as saline conditions of about NaCl 1.5%. Their optimal growth is achieved at temperatures of 25 - 30°C and pH 6.8 however, researchers have identified some strains tolerant to temperatures as high as 37°C (Somasegaran and Hoben *et al*., 1994). In South Africa study by Hassen (2014), identified *Ensifer sp* and *Rhizobium sp* which were fast growing, attaining optimal growth at pH 5 while slow growing *Bradyrhizobium sp* were showing great adaptation at pH as high as pH 9.

2.6 Host range and promiscuity

Legumes form a symbiotic relationship with rhizobia however; there is selectivity in the establishment of that mutual relationship. The symbiotic rhizobia-legume interaction which leads to nodule formation consequently nitrogen fixation is mainly controlled by the host specificity and promiscuity (Perret *et al*., 2000). Research by Ampomah (2008) concluded that rhizobia-legume specificity and promiscuity are genetically controlled mechanism possessed by rhizobia and legume making certain rhizobial species or subspecies to only infect and form nodules with specific type of a legume. This is made possible by molecular signal exchange between legume and rhizobium involving both legume symbiotic (sym) genes and rhizobia nodulation (nod) gene (Denarie *et al*., 1993). Perret (2000) observed that a single bacterial strain can nodulate various host legumes due to the existence of several nodD genes in rhizobia strains allowing them to

respond to various types of flavonoids consequently infecting wide range of legumes. Hassan (2012) observed that NodD from different rhizobial species or same strain offers different selective preference for certain flavonoid. On the other hand flavonoid compositions of various legumes differ among various plant species or on cultivars of the same species.

Among the most promiscuous strains of rhizobium is the NGR234 isolated from *lablab purpureus* at New Guinea which has an incredible host range, nodulating about 112 legume genera comprising *Parasponia* a non-legume species. Also from United States Department of Agriculture, *Rhizobium freddi* USDA 257 which was isolated from *Glycine soja* nodulate over 77 legume genera. Supporting studies by Peix (2015) noted that strain CIAT 899 isolated from Centro Internacional de Agricultura Tropical (CIAT) harbors several nodD and nodA copies and have been found to nodulate wide range of legumes including *P.vulgaris, Leucaena leucocephala* and *Macroptilium*. Adhikari (2013) reported that among promiscuous symbiotic interactions, *P. vulgaris* has the ability to be nodulated by diversity of rhizobial species including*; R. leguminosarum bv. phaseoli, R. gallicum (bv. phaseoli and bv. gallicum), R. tropici, R.giardinii (bv. phaseoli and bv. giardinii), Sinhorhizobium meliloti* and *R. etli bv. phaseoli.*

Rhizobial strains with broad host range nodulate wider range of legumes, however due to their competitiveness and persistence in the soil they become inconvenient if they are to be replaced by inoculation. Equally, legumes promiscuity to native rhizobia is beneficial but disadvantageous to inoculation more special if native rhizobia are ineffective. Study by Schuldes (2012) mentioned that rhizobial strain *R. leguminosarum* bv. *trifolii* which nodulate plants of genus *Trifolium* and *S. meloti* experience specific or narrow host range while legume species *Cicereae, Trifoliae* and *Viciae* show restrictive nodulation. Research by Perret (2000), mentioned that legume specificity was a factor of adaptation to specific ecological niche with promiscuous legumes found in tropical

region while Mediterranean legumes found in extreme environmental conditions were high specificity.

2.7 Mechanism of biological nitrogen fixation

Biological nitrogen fixation takes place when the atmospheric nitrogen is enzymatically converted to ammonia. According to Tezcan (2005) the backbone to biological nitrogen fixation is the nitrogenase enzyme which acts as the catalyst to the high energy (ATP) consuming reduction of atmospheric nitrogen to ammonia. Nitrogenase enzyme consists of two metalloproteins; (i) MoFe-proteins with FeMo-cofactor which acts as an active site for substrate reduction whereas the (ii) Fe-protein couples ATP hydrolysis to electron transfer. The inert atmospheric nitrogen molecule composed of two nitrogen atoms linked with a very strong triple bond which requires higher amount of energy to break open it to be reactive, allowing hydrogen atom to be attached to each of the nitrogen atom (equation 1). The fixing rhizobia reduce the inert nitrogen to ammonia with energy from oxidizing carbohydrates obtained from host plant.

$N_2 + 6H^+ + ATP \; 2NH_3 + ADP + Pi$ (Equation 1)

- $N{\equiv}N + 8H^+ + 8e^- + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi$

2.7.1 Modes of rhizobial entry

Nodules are formed after rhizobia in the rhizosphere have entered the plant through various pathways. According to Sprent (2008)**,** two main modes of infection have been described in root nodule symbiosis which are; crack entry or intercellular infection mode and the infection thread mode. Studies show that this direct mode of infection is persisting in about 25 % of legumes (Sprent *et al*., 2006). It is described mainly in woody legumes known to be having hairless roots

or do not produce lateral roots and was also described in *Parasponia*; a non-legume that is nodulated by *Bradyrhizobium* (Boogerd, 1997). According to Chen (2013) crack entry infection mode mainly occur in various subtropical legumes such as *Arachis hypogea* (groundnut).

Crack entry occurs through intercellular penetration where bacteria invade root epidermis then root cortex via natural wounds in the plant caused by epidermal splitting for lateral roots or nodule primordial development and emergence. There is no root infection thread formed and also characterized by infected cells which are not combined with uninfected ones (Goormachtig *et al*., 2004). According to Rasolomampianina (2005) this infection pathway always lead to higher nodule occupancy even by the less effective strains hence associated with some problems as noted in groundnuts which has higher promiscuity level hence affecting effectiveness of quality inoculum strains.

Other than the crack entry infection mode, is the mostly studied and abundant root hair entry and infection thread spreading which is described by about 75% of the legumes. Initial sensing starts with the release of exudates by roots or seed coat (into rhizosphere or spermosphere) including flavonoids and nutrients (such as organic acids and amino acids) which are sensed by the free living rhizobia (Hensen *et al*., 2004). Flavonoids together with NodD protein secreted function as positive inducers of the rhizobial nodulation (nod) genes. Therefore presence of flavonoids at the NodD binding sites activates transcription of the nodulation genes (Cooper and Scherer *et al*., 2012). The bacteria's nod genes then synthesize and release sensed reciprocal signal molecules, the lipo-chito-oligosacharides that bind to specific plant receptor kinases that contain LysM motifs (Hansen *et al*., 2006).

Presence of bacteria in the root cause changes in root metabolism and morphogenesis vital for rhizobial entry into host legume roots, while calcium spiking in root hairs also leads root hairs curling, entrapping the rhizobia inside the pocket. On response to Nod factor the host plant produces a hollow cylindrical infection thread running inside root hair and terminating at nodule premordia formed in the root cortex colonized by invading rhizobia (Gage, 2004). Inside the host root, rhizobia are physiologically converted into bacteriods triggering legume cortical cell division leading to nodule organogenesis. The bacteriods that produces enzyme nitrogenase which catalyze the conversion of atmospheric nitrogen to ammonia are housed in the nodule which supplies with energy and protection from parasitic attack by host tissues.

Bisseling (1990) compared structures of the nodule parenchyma in conjunction with its few and small intracellular spaces form an oxygen diffusion barrier. In combination with the high oxygen consumption rate of the bacteria and the presence of leghemoglobin which also acts as an oxygen buffer. This barrier helps to maintain the low free oxygen concentration in the central tissue suitable for the reaction of an oxygen-sensitive nitrogenase. According to Flynn *et al*. (2015), nodules continue fixing nitrogen for the host plant while still growing till maturity and can be visible to naked eyes after two weeks however, its peak activity is noted at the flowering period thereafter carbohydrates are channeled to plant reproduction and less to the nodules.

The membrane envelope housing bacteriods encloses the oxygen bond protein solution (leghamoglobin) which is responsible for giving active nodule coloration (Burdass *et al*., 2012). Young, healthy effective nodules are often noted by pink, red or brown color, albeit active nodules can be black due to presence of melanin on top of the leghaemoglobin as reported in *Lablab purpureus, Dolichous biflorus,* and *Vigna unguiculata* (Adhikary *et al*., 2015). Conversely growing nodules exposed to sunlight senescent and loose the normal red coloration; breaking down

haemoglobin into legcholeglobin therefore developing a green exterior coloration. Ineffective nodules are sometimes white, greenish to greyish in color throughout the growing season and often smooth textured (Dakora *et al*., 1994).

2.8 Nodule structures

Early nodulation processes are controlled by the NIN protein (nodulation inception gene product) whereby its absence arrests infection thread formation and premodia. The host plant determines morphology and type of nodule formed whereas its effectiveness is bacterial determinant hence varying in genotypic and phenotypic traits (Ferguson *et al*., 2010). According to Subramanian (2013) there are two major types of nodules, they exist as determinate and indeterminate with their differences mainly based on the first internal cell division site, meristematic region maintenance and mature nodule form.

Sprent (2003) observed that indeterminate nodules are mainly noticed on temperate legumes mainly; peas, alfalfa and clover where its initial cell divisions occur in the cortex, endodermis and pericycle. This leads to formation of a nodule primorda and persistent meristem resulting in cylindrical shaped nodules which are amide producers. They are also characterized by the apical meristerm continuously producing and can be infected by rhizobia leading to mature nodule containing heterogeneous bacteriods population. Legumes forming determinate nodules are predominately tropical and subtropical species comprising; soybean and common bean with few noticed on temperate species such as *L. japonicum* (Sprent, 2001). Wopereis (2000) found that mostly the initial cell divisions occur sub-epidermally in the outer cortex leading to spherical shaped nodules, lacking persistent meristem with prominent lenticels and produce ureids. Mature

ones contain relatively homogenous population of the nitrogen fixing bacteriods also characterized by low lifespan (Ferguson *et al*., 2005).

2.9 Major factors controlling biological nitrogen fixation

Sometimes compatible legume-rhizobia symbiosis is formed albeit failing to biologically fix nitrogen to its potential due to a combination of various effects which cause impaired symbiosis establishment. Studies by Giller (2011), reported that infection and nodulation are mostly influenced by the symbiotic partners, genetic makeup and physical factors in the rhizosphere affecting both plant and the rhizobium. Plant developmental stages (such as; growth, receptive physiology and nodulation) and Rhizobium are affected by a variety of environmental factors (such as soil physical, biological and chemical properties). Giller (2001) furthermore concluded that these effects are known to be harsh more special among semi-arid and tropical soils hence considered extreme for these biochemical processes.

2.9.1 Moisture

The legume-rhizobia relationship offer potential results when both partners are given optimum moisture level. Research by Serraj (1999) showed that under soil moisture deficit conditions, biological nitrogen fixation is the mostly affected biochemical processes compared to photosynthesis and transpiration. Under moisture deficit soils there is declined rhizobial population and impaired movement which affects the number of infection threads leading to limited nodulation. Giller (2001) noted nodule decay under moisture deficiency nevertheless, studies by Plied-Blazer *et al.* (1995) mentioned that rhizobial species such as *Rvl,* which is known for nodulating *vitch* and *faba* bean can maintain rhizobial population size under moisture stress however losing its symbiotic efficiency. On the other hand nitrogen fixation is more sensitive to

water logging which prevents root hair, nodulation site development and interferes with oxygen diffusion in the roots leading to plant hypoxia, leading to reduction in respiration and nitrogenase activity (Tobisa *et al*., 2014).

2.9.2 Temperature

Soil temperature is always higher compared to the atmosphere temperature, therefore vital to record soil temperature when dealing with legume-rhizobial symbiosis. Research shows that rhizobial optimum activity is achieved between 27 – 35°C, however studies show that other symbiotic systems can tolerate <25°C and >35°C. Even though few nodules are noticed below 7˚C and above 36˚C optimum growth temperatures for legumes vary among species and their environmental adaptation (Giller *et al*., 2001). In accordance to Micheils *et al*. (1994), critical nitrogen fixation for low land pulses (pea) and high land pulses (groundnuts, cowpea and soybean) is at 30°C and 35°C to 40°C, respectively. Zahran (1999), observed that higher temperatures which are mostly experienced in tropical regions affect Rhizobial growth, development and survival, root hair infection, bacteroid differentiation, nodule structure and nodule functioning.

2.9.3 Salinity

Higher temperatures are found to be mainly associated with increase in soil salt concentration. Salt stress is mostly felt by the symbiosis as compared with free living rhizobia whereas legumes are more sensitive to salinity as compared to rhizobia. Some legumes have higher tolerance while others have greater sensitivity like *soybeans* which are more sensitive compared to *alfalfa*. Research by Munns (2002), observed that soil salinity causes osmotic stress which affects various biochemical plant processes including; photosynthesis, photorespiration and nutrients availability due to osmotic moisture withdrawal from nodules. It also affects vital steps such as nodule

development and metabolism leading to production of very few and small sized nodules consequently hampered nitrogen fixation processes. Similar research by Monica (2013) noted that early rhizobial colonization processes were highly sensitive to salinity which affected peribacteriod membrane structure and limiting bacteriod number. Similar, studies by Jabbar (2012) reported reduced nodule number in pulse crops such as cowpea, Bambara groundnut and groundnut on saline soils even if they are abundant of native rhizobia.

2.9.4 Nutrients availability and deficiencies

Nutrients have great direct and indirect influence on biological nitrogen fixation through affecting plant growth and development where their deficiencies distress major biochemical processes. L'taief *et al*. (2007) reported that phosphorus deficiency is the main limiting element in symbiotic biological nitrogen fixation. Phosphorus is vital in plant growth, pivotal as energy source when ATP is converted to ADP during reduction of N_2 to NH_3. Its deficiency leads to decreased specific-nitrogenase activity in nodules due to low energy status of host plant cells leading to reduced nodulation as a result restricted root growth, photosynthesis and translocation.

According to El-Hamdaoui (2003), application of boron had positive impact on symbiotic biological nitrogen fixation as it is required for nodule development and functioning. Its deficiency inhibits rhizobial-plant molecular signaling, decrease nodule number, alteration of nodule development and inhibited nitrogenase activity (Bellaloui *et al*., 2014). Some studies noted that boron deficient plants produced nodules with a completely changed cell wall structure, infection thread and peribacterial membrane, also having reduced ENOD2 protein in nodules which led to malformed and multifunctioning oxygen diffusion barrier. Under boron limited soils, corresponding research by Muofhe and Dakora (1999) noted that nodule formation was prevented

whereas in some instances nodules formed were low and small in number and size respectively, lacking vascular strand and bacteriods.

According to El-Hamdaoui *et al*. (2003) calcium is essential for the early infection stages mostly 2-3 days after inoculation mainly for enhanced bacterial attachment to root hair, increasing size of the exudates to induce nod-gene activity in rhizobium and for calcium mediated signaling during the infection process. Similarly Melino *et al*. (2012) reported that Mo deficiency other than impacting nodule formation and number, they lead to production of ineffective nodules normally appearing large and normal however with green coloration and appearing senescent. Manganese level in acid soils are high enough to limit nodulation whereas, halved nodulation of *Medicago sativa* was noted through addition of Mn to nutrient solution (Bordeleau *et al*,. 1994).

It has been noted by Giller *et al*. (2001), that aluminum toxicity is related to impaired nodulation because of increasing soil acidity, higher acidity (at pH below 4.5) causes fixation of elements such as phosphorus hence reduced nodulation. Nitrogen fixation stages which are mostly pH sensitive are Rhizobium infection process and attachment. Previous research by Correa *et al*. (2001) noted reduced root growth at pH below 4.8 and root curling only at pH above 5.5 on *Medicago sativa*. A similar study by Taylor *et al*. (1991) concluded that soil acidity limits symbiotic nitrogen fixation through reduced rhizobial survival and persistence causing impaired nodulation.

Higher soil nitrogen inhibits roots infection which consequently impaires nodulation. A study by Muofhe and Dakora (1999) concluded that the effects of nitrates on nodulation are felt regardless of plant age, size or prior nodulation. It was noted that in cowpea and peas ammonium is more

inhibitory in comparison to nitrates where small nitrogen concentrations of 0.02- 0.5 mN impacted nodulation.

2.10 Major benefits from biological nitrogen fixation

With the alarming concerns of greenhouse gas emissions and climate change effects, biologically fixed nitrogen offers vital source of clean nitrogen needed for sustainable agriculture. Research by Unkovich *et al*. (2008) summarized that biological fixed nitrogen was contributing about 50 to 70 million tones to the annual global nitrogen status. Venieraki *et al*. (2011) also reported that symbiotic rhizobia system was estimated to contribute 1.44×10^8 metric tons of nitrogen per year globally where 25% is contributed by cultivated agriculture. In Egypt, Zahran *et al*. (1999) noted that nitrogen contribution of groundnut (*Arachis hypogaea*) to maize growth through intercropping systems at a population density ratio of 4:1was equated to the application of 96 kg of fertilizer N ha^{-1}. Shoko *et al*. (2007) mentioned that farmers can save nitrogen fertilizer estimated at 80 kg nitrogen ha^{-1} from soybeans. Deshwal *et al*. (2013) noted that symbiotically fixed nitrogen can be between 80 and 150 kg N/ha in 90 days with potential to reach up to 340kg. Equally, Giller *et al*. (2011) concluded that soybeans in Zimbabwe fixed 160 – 260 kg N/ha. In Brazil Costa *et al*. (2014) concluded that inoculated pigeon pea exceeded 150 kg N/ha annually.

Studies in Egypt by Moawad and Rahim (2005) on the assessment performance of common beans in Nile Delta soils noted a positive inoculation response in terms of nodule numbers, dry matter and plants biomass accumulation leading to higher nitrogen fixation with average yield increase of about 43% compared to non-inoculated controls. A similar research by Mehrpouyan *et al*. (2011) in Khuzestan province in Iran established that inoculated common bean yield was 35% to

65% higher whereas, soybean yield increased twice when compared with maximum fertilizer treatment and 10 folds when compared to the control plants.

Research by Abaidoo (1989) noted higher shoot dry matter accumulation in inoculated intercropped soybeans compared to the inoculated mono cropped and non-inoculated ones. Higher nitrogen fixation was also recorded in common beans intercropped with maize planted in same hole other than on separate holes. In another cropping study, Allen and Obura (1983) reported that maize following maize plantation yielded 0.5t/ha whereas maize following soybean plantation yielded at least 1.5t/ha. Thies *et al*. (1991) observed that in situations where yield was not increased, inoculation increased protein seed content to a % nitrogen to protein ratio of 1: 6.25. Naeem *et al*. (2004) observed that local isolated strains and exotic strains formed an average of 7 and 3 nodules per plant respectively while maximum dry weight was 4.3 mg/plant and 2.1 mg/plant for local and exotic strains respectively.

Rhizobia studies have noticed added importance in plant growth and development where they act as mediators of nutrient supply due to their modified feeding mechanism aiding in improving nutrient acquisition under low fertility soils. Rhizobia produce and release sidorephores, indole acetic acid (IAA) and organic acids which they use to enhance nutrition consequently improving plant growth. Dakora and Philips (2002) found that siderophores mobilize iron whereas organic acids solubilize phosphorus and manganese. Legume root decomposition leads to accumulation of abundant phenolic nod gene inducer concentration, consequently higher nod factors in the rhizosphere which stimulates plant growth. Complex lipo-chito-oligosacharide Nod factor produced during nodule formation aid in restoring cell division and embryogenesis in cultures, consequently promoting plant growth. Souleimanov (2002) noted that application of nod factor led to increased soybean root biomass and length by 7- 16% and 34 - 44% respectively. Nod factors

secretion indirectly benefits the cropping system through enhancing arbuscular mycorrhizal (AM) fungal symbiosis whereas, McCully (2001) stated that other endophytes found in nodules help in improving plant growth.

Riboflavin produced by rhizobia is easily enzymatically converted into lumichrome, which stimulates CO_2 production hence promoting rhizobial growth and plant biomass accumulation (Phillips *et al*., 1999). Khan *et al*. (2008) concluded that the phenolic compounds produced induced over expression of nod factors in rhizobia also leading to increased photosynthetic rates in various plants due to impact of the compounds released during nodulation. Rhizobial inoculation is also associated to elevating water stress effects in symbiotic legumes with various studies suggesting that rhizobia products; abscisic acid decrease the stomatal conductance therefore sustaining nitrogen fixation during drought periods. Studies by Deshwal (2003), noted that rhizobia act as good bio-control agents in natural agricultural ecosystem due to their suppressive effect in soil pathogens. It was observed that *B. japonicum* caused 75% decrease in sporulation of *Phytophthora magaspema* and also decreases the severity of *phytopthhora* and *fusarium* root rot in beans.

2.11 Measuring biologically fixed nitrogen

Estimating the amount of fixed nitrogen is important as it assist in selecting the most effective rhizobial strain-plant genotype combination which fixes higher N quantities under different conditions. Unkovich (2008) concluded that quantification of fixed N was an important tool in evaluation of indigenous *Rhizobium spp.* to effectively nodulate newly introduced legumes, symbiotic effectiveness of rhizobial inoculants, success of inoculation and evaluating if legume were reaching their potential. Similarly, the rate at which legume biologically fix nitrogen changes

with its physiological growth stages; low at early growth stages when nodules are still establishing and reaching maximum values between early flowering and early seed-filling (Lawrie *et al*., 1997).

Study by Unkovich *et al*. (2008) and Cooper and Scherer *et al*. (2012), concluded that lack of reliable method for measuring biological nitrogen fixation was a major factor affecting research. It is mainly difficult to quantify biological fixation on annual legumes characterized by deep rooted system, which is too laborious and almost impossible to harvest all nodules from such roots whereas very important to analyze the whole plant for quantification. Perennial legumes with their complex rooting system characterized by up-taking soil nitrogen from varied pool makes it difficult to track soil nitrogen uptake and dependence of the nitrogen fixation (Cooper and Scherer *et al*., 2012). According to Boddey *et al*. (2000), estimating quantities of N_2 fixed is usually hampered by wide range of variability in the distribution of certain N_2-fixing agents together with their N_2-fixing intensities as affected by soil, water and nutrient availability together with the competition from other species in the soil.

Generally there is no universal correct technique for measuring N_2 fixation guaranteed to provide the most accurate measure of biological fixed nitrogen for all legumes under various environmental conditions. Liu *et al*. (2011) reported that various methodologies have their own limitations making measuring of exact amount of N_2 fixed becoming a challenge therefore complementary techniques can be used simultaneously to improve quality of the results. Analyzing the nodule in terms of size and color can be vital whereas additional information such as nodule numbers and nodule mass are major indicators of legume productivity. Even though there are various techniques involved, however for quantifying the amount of N_2 fixed by the legume majority of the techniques measure plant dry matter, N concentration (N) and the percentage of N the plant derived from N_2 fixation (%Ndfa). According to Unkovich *et al*. (2000) the choice of the method used to measure

biological nitrogen fixation should be governed by its accuracy, cost, time, and repeatability and is usually measured using the following methods; acetylene reduction assay, xylem sap technique, ^{15}N isotope technique, ^{15} Dilution and Natural ^{15}N abundance.

The $^{15}N/^{14}N$ isotopic analysis method is the mostly applied quantification method used giving satisfactory results. It is based on the principle that there are two stable isotopes of nitrogen in atmosphere (^{14}N and ^{15}N) whereby the prior is lighter and the latter is heavier at an atomic percentage abundant of 99.636 and 0.3663, respectively (Robinson *et al*., 2001). Using this method the main assumptions are that; 1) the $^{15}N/^{14}N$ ratio of the non-nitrogen fixing reference plant is the same as that of the soil and 2) the legume and reference plant explore a soil N pool of identical $^{15}N/^{14}N$ composition. The choice of the reference plant used is one of the major factor affecting results from this method.

Majority of N_2 fixation measurements were done in agricultural environments as compared to natural ecosystems. Various methods have various limitations giving the user to know which method to use when. However, with correct reference plants the $^{15}N/^{14}N$ isotopic analysis method gives reliable results (Unkovich, 2010) therefore chosen to be used in this Dissertation.

3.0 METHODOLOGY

3.1 Sampling

3.1.1 Experimental sites

Regions varying in environmental conditions such as annual rainfall amounts and soil types were selected for this study during the 2014/15 season. Rainfall amounts were collected from the Department of Metrological Services, Botswana. The Okavango region received an annual rainfall between 471-548 mm per annum. The soils from the area are mainly Arenosols (Farrar, 1994). Soils from Okavango Delta are mainly derived from Kalahari Aeolian deposits with a very high sand component. The Tswapong area receives annual rainfall ranging between 362 - 397mm. Soils found in the area are derived from *in situ* weathering of rocks and minerals. Most soils in this area have loamy textures although sandy soils are not uncommon.

Generally all sites (Table 3.1) had slightly acidic soils pH 5.06 – 5.52. Acidic pH negatively affects bioavailability of nutrients to the growing plants whereby vital elements (N, P, K, Ca, Mg, B and Mo) with their optimal bioavailability to plants at pH 6.0 to 7.0, whereas rhizobia's potential activity is at pH 6.8 (Uchida *et al*., 2000; Charman *et al*., 2000). These soils have low available phosphorus (P). P is associated with improving fixation through improved nodule number and weight in different legumes. This is due to the fact that phosphorus acts as plant energy source, increases plant tip and root development, initiates nodule formation and improves efficiency of the symbiosis (Nkaa *et al*., 2014). Soils from all sites have very low cation exchange capacity (CEC) below 6 cmol/kg. CEC measures the capacity of the soil to adsorb and release nutrient to the plants during their growing period. Under field conditions soils with higher CEC can hold onto the applied fertilizer and release those nutrients for the plant.

Table 3.1: Soil pH, phosphorus and CEC from different study sites.

Location	pH ($CaCl_2$)	P (ppm)	CEC (cmol/kg)
Tswapong (Lekobeng)	5.52	7.93	5.07
Okavango (Xakao)	5.06	5.13	1.55
Okavango (Xauga)	5.08	6.15	1.24

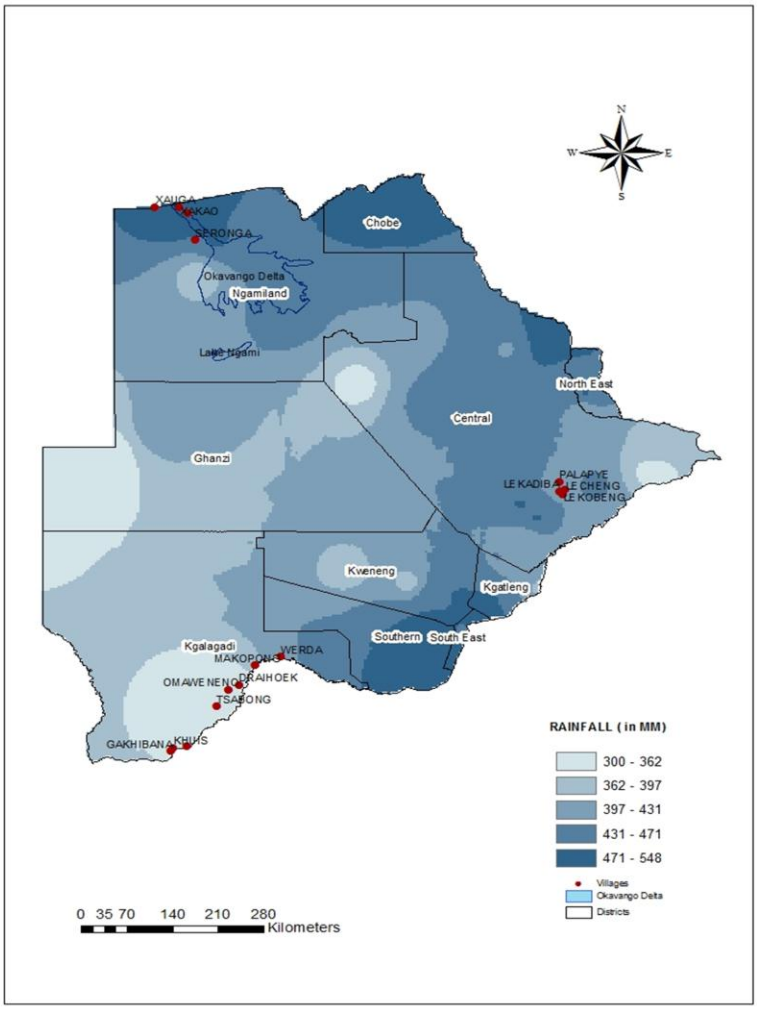

Figure 3.1: Map of Botswana showing various locations where plant samples were collected

3.1.2 Soils and plant sampling

A survey of herbaceous indigenous leguminous plants was undertaken in various study areas (Figure 3.1) determined based on accessibility and abundance of different plant species. At each location, four flowering plants per species were sampled within a 10 m^2 radius area. Collected legume species included legumes in (Table 3.2). Non legume plants (reference plants) with similar growth habit adjacent to the legumes were also sampled as a requirement for the ^{15}N natural

abundance technique of measuring N fixation. Reference plants included; *Terminalia sericea*, *Cassia italica*, *Erlangea musera*, *Ocimum americunum* and *Panicum sp*. For legumes, during sampling, each plant was dug out with intact root and nodules. The top soil within a depth of 0-20cm was sampled at each site.

Table 3.2: Legumes sampled at various locations of the three regions

Okavango	**Tswapong**	**Kgalagadi**
Indigofera flavicans	*Zornia glochidiata*	*Indigofera spp1*
Indigofera tinctora	*Chamaecrista bieinsis*	*Cyamopsis dantata*
Chamaecrista biensis	*Optoptera buchellii*	*Cullen tometosum*
Indigofera astragalina	*Vigna unguiculata subspp*	*Arachis hypogea*
Indigofera daeloides	*Chamaecrista absus*	*Vigna unguiculata*
Tephrosia Purpurea	*Tephrosia Purpurea*	*Vigna subterranea*
Tephrosia lupinifolia	*Rhyncosia totta*	
Chamaecrista absus	*Arachis hypogea*	
Crotalaria astragalina	*Vigna unguiculata*	
Crotalaria sphaerocarpa	*Vigna subterranea*	
Crotalaria pisicarpa		
Vigna unguiculata subspp		
Senna obtusifolia		
Rhyncosia totta		
Zornia glochidiata		
Arachis hypogea		
Vigna unguiculata		
Vigna subterranea		

3.1.3 Sample preparation

On arrival at the BUAN soil science laboratory, plant samples were washed in a stream of water, removing soil off nodules. Nodules were carefully plucked off at about 5mm away from the nodule and kept at -20˚C in zip locked plastic bags before isolating the root nodule bacteria. Shoots and roots were separated and oven dried at 60˚C for 48 hours (Incubator Merk 400L) and weighed to get biomass. Each sample (shoot and root) was pulverized using the 5E-PCM Series Pulverizer (5E-PCM) to 0.05mm mesh.

Soil samples were air dried and passed through a 2 mm sieve before chemical analysis. The top soil of each site was sampled. Soils were analyzed using standard procedures (Tan, 2005) for determining pH, P and CEC. Results of the soil analysis are shown in Table 3.1.

3.2 N_2 fixation parameters

3.2.1 $^{15}N/^{14}N$ isotopic analysis

About 2.0 mg of each pulverized sample was weighed into tin capsule (Elementary Microanalysis LTD, Okehampton, UK) and run on a Thermo Finnigan Delta Plus XP stable light isotope mass spectrometer (Fixon Instrument SPA, Strada Rivolla, Italy) coupled via a Conflo III device to Thermo1112 Flash elemental analysis against two internal reference plant materials namely *Nasturtium sp* and *Acacia sp*. The internal standards had been calibrated against an IAEA standard for N, which is atmospheric air.

3.2.2 %Ndfa and N-fixed

The isotopic composition of ^{15}N was measured as the difference in the number of atoms of ^{15}N to ^{14}N in atmospheric N_2 was calculated in accordance to equation 2 (Junc and Svec, 1958; Mariotti, 1983).

$$\delta^{15}N\ (‰) = \frac{(^{15}N/^{14}N)\ \text{sample}-(^{15}N/^{14}N)\text{standard}}{(^{15}N/^{14}N)\text{standard}} * 1000 \quad \text{(Equation 2)}$$

The proportion of N derived from the atmosphere (%Ndfa) was calculated according to Shearer and Kohl (1986) (Equation 3)

$$\%Ndfa = \left[\frac{(\delta^{15}\text{Nref}) - \delta^{15}\text{Nleg})}{(\delta^{15}\text{Nref} - \text{B value})}\right] \text{x } 100 \quad \text{(Equation 3)}$$

Where; δ^{15}Nref is the mean ^{15}N natural abundance of a non-N_2-fixing reference plants, δ^{15}Nleg is the mean ^{15}N natural abundance of the legume (shoot) and the B value is the ^{15}N natural abundance of legume shoots which were totally dependent on biological N_2 fixation for their N nutrition as shown in Table 3.3.

Table 3.3: B values of varied plant species obtained from literature.

Plant	B value (‰)	Reference
Arachis hypogea	-1.4	Okito *et al*. (2004)
Indigofera spp	-1.5	Okito *et al*. (2004)
Chamaecrista absus	-1.7	de Freitas *et al*. (2012)
Crotalaria spp	-1.1	Gathumbi *et al*. (2002)
Vigna subterranea	-1.4	Nyemba and Dakora (2010)
Vigna unguiculata subsp	-1.7	Okito *et al*. (2004)
Vigna unguiculata	-1.8	Pule-Meulenberg and Dakora (2009)
Tephrosia purpurea	-2.0	Raddad *et al*. (2005)

The amount of N-fixed was calculated using equation 4 as described by (Maskey et al., 2001)

N fixed = ($\frac{\%\text{Ndfa}}{100}$) x Legume shoot N (Equation 4)

3.3 Isolation and characterization of root nodule bacteria

3.3.1 Isolation of rhizobia

Rhizobia from root nodules were aseptically isolated using methods described by Vincent (1970) and Somasegaran and Hoben (1994). Root nodules were defrosted and rehydrated by immersing in sterile water for two hours. They were then surface sterilized by immersing in 98% ethanol for 10 seconds to break the surface tension and remove air bubbles. This was followed by immersing into 3% (v/v) sodium hypochlorite (Jik) for 3 minutes for sterilization then rinsed in changes of distilled water for more than 10 times to remove traces of sodium hypochlorite. Sterilized forceps were used for handling nodules throughout the process were sterilized through dipping in 95% alcohol and flaming.

After surface sterilization individual nodules were crushed over a drop of sterile water using flame-sterilized blunt end forceps in a petri dish then a loopful of slurry was streaked on the Yeast Mannitol Agar (YMA) surface in a dilution manner as described by Vincent (1970). Table 3.4 shows the composition of the YMA. Streaked petri dishes (plates) were sealed with parafilm, labeled accordingly and then incubated at 28°C in an inverted manner monitoring the appearance of colony growth mostly after 3 to 10 days. After colony appearance sub-culturing was done for purification such that there was pure single colony per plate.

Table 3.4: Yeast mannitol agar composition

Ingredients	**Quantity**
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	0.1g
Yeast Extract	0.5g
Mannitol	10g
Bacteriological agar	15g
Actidione (cycloheximide)	20mg
Distilled Water Make up to	1000ml

Note: 0.1N NaOH or 0.1N HCl was used to adjust pH to 6.8

3.3.2 Gram staining

Pure bacterial isolates were confirmed using gram staining in which individual slide was prepared in accordance to (Somasegaran and Hoben, 1994). For each pure isolate, a bacterial smear was streaked and heat fixed on a slide then flooded with Crystal violet for 60 seconds. Smears were then washed under slow pressure flowing water then covered with iodine solution which was drained off before being re-flooded and left for about 60 seconds. Slides were decolorized by 95 % alcohol for at most 60 seconds then washed off with flowing water and blot dried with a paper towel. After drying the smear was counterstained with Safranin for 60 seconds and then washed off with water and air dried, prepared slides were then observed under oil immersion.

3.3.3 Congo red absorption test

Congo red solution was prepared using (Vicent, 1970) procedure then a loopful of bacterial cells were streaked onto Congo red and its absorption observed over several days

3.3.4 Colony shape

Fully grown colonies were examined cross sectional to determine their shape on Yeast mannitol agar (YMA).

3.3.5 Colony texture

Each colony was lifted with a loop to observe how they behaved when moved and or touched.

3.3.5 Colony appearance

Fully grown rhizobial strains were streaked on YMA plates and incubated at 28˚C then monitored to see if the colonies are opaque or translucent

3.4 Authentication of root nodule bacteria as rhizobia

3.4.1 Pot preparation

Sterile environment and aseptic measures were the main priority through-out the experiment. Plastic flower pots were filled with three layers consisting of nutrient free sand sandwiched by the interleaved non-absorbent cotton wool. The pot was firstly lined with the interleaved non-absorbent cotton wool and moistened nutrient-free sand, wrapped by aluminum foil before autoclaving at 121^0C for 45 minutes. After autoclaving seedlings were carefully planted followed by inserting two sterile feeding pipes for delivering nutrient solution then finalized by covering the sand with another layer of the non-absorbent cotton.

Figure. 3.2 The pot experimental set up

3.4.2 Seed treatment

Seed collected for various legumes (wild and field) from different agro-ecological zones of Botswana were sterilized before planting. Those with tough seed coats were scarified through soaking in concentrated sulphuric acid (98% H_2SO_4) for about 2 minutes. Seeds were then surface

sterilized in a similar manner to that of nodules above then submerged in sterile water so as to be fully imbibed before pre-germination in petri dishes and kept in dark places until their radicles emerged.

3.4.3 Inoculum preparation

A loopful of each bacterial culture was transferred into 1.25 mL autoclaved eppendoff tube containing sterilized distilled water which was thoroughly mixed by a Vortex mixer (Finevoterx).

3.4.4 Raising plants

This experiment was conducted in the Botswana University of Agriculture and Natural Resources (BUAN) Department of Crop Science and Production greenhouse located at Sebele Content Farm (24°38'S, 54'N, 994 m above seas level). Pre-germinated seeds were handled by sterile forceps and aseptically grown in the autoclaved pots (Figure 3.2). Each pot had two to three holes with two pre-germinated seeds per hole for insurance purpose in-case one fail to establish well. Refilling was also done quickly after seedling establishment so as to have about 100% plant population. Thinning was carried out such that one plant was left to grow and develop per hole.

Instead of using water, the nitrogen free nutrient solution was used to feed the plants at correct amount and appropriate time. The quarter strength nutrient solution composed of all nutrients required for plant growth under various concentrations as described by (Broughton and Dilworth, 1970) shown in (Table 3.5) was used throughout the experiment until harvesting.

Table 3.5: Nitrogen-free Nutrient Solution

Stock Solution	Element	Form	Mol.Wt	Gram/liter	Molarity of Stock Solution (M)	Vol.(ml) stock solution/ litre
A	Ca	$CaCl_2 \cdot 2H_20$	147.03	294.1	2.0	0.05
B	P	KH_2P0_4	136.09	136.1	1.0	0.05
C	Fe	Fe-Citrate	335.04	6.7	0.02	0.05
	Mg	$MgSO_4 \cdot 7H_20$	246.5	123.3	0.5	0.05
	K	K_2SO_4	174.06	87.0	0.5	
	Mn	$MnSO_4 \cdot H_20$	169.02	0.338	0.002	
D	B	H_3BO_4	61.84	0.247	0.004	0.05
	Zn	$ZnSO_4 \cdot 7H_20$	287.56	0.288	0.001	
	Cu	$CuSO_4 \cdot 5H_20$	249.69	0.100	0.004	
	Co	$CoSO_4 \cdot 7H_20$	281.12	0.056	0.0002	
	Mo	$Na_2MoO_4 \cdot 2H_20$	241.98	0.048	0.0002	

The nutrient stock solutions were prepared separately and mixed at a required appropriate rate during fertilization

Source: Broughton and Dillworth, (1970)

3.5 Treatment and Experimental design

When assessing N_2 fixed, different legume species from Okavango delta and Tswapong agro-ecological zones were sampled. Per location each species was replicated four times. For authentication the number of treatments per species was determined by the number of bacterial strains isolated and each host plant species also included control treatments (one negative or no nitrogen supplement and the other positive or nitrogen supplement of 5 mM KNO_3). The pots were arranged in a Complete Randomized Design and each treatment was replicated three times. Species used for authentication included; *Arachis hypogea*, *Indigofera daeloides*, *Chamaecrista absus*, *Crotalaria sphaerocarpa*, *Vigna subterranea*, *Vigna unguiculata subsp Denkindtiana*, *Vigna unguiculata*, *Tephrosia purpurea* and *Zornia glochidiata*.

3.6 Plant harvesting after authentication

Assessment was done six weeks after inoculation; individual plants were carefully removed from the pot without disturbing root, making sure nodules were intact. Roots were washed by clean tap water then described in terms of their presence of root nodules, number, shape, weight and pigmentation. Whereas nodulation effectiveness was estimated by decapitating plants, separating shoot and roots before oven drying at 60˚C for 48 hours then weighed for dry matter.

3.7 Data collection

Data on δ ^{15}N values, N content, %Ndfa and N_2 fixed was collected. In addition, characteristics of the isolated bacterial isolates were determined. Furthermore after plant growth for authentication of bacterial strains as rhizobia, information on shoot and root biomass, nodulation (nodule number and nodule weight) were recorded.

3.8 Statistical analysis

Data on ^{15}N, % Ndfa, N-fixed, N content, shoot and root weight, nodule number and nodule weight were subjected to analysis of variance (ANOVA) after testing for normality. Where means were statistically different they were separated using the Duncan Multiple Range Test (DMRT) at 5% significant level.

4.0 RESULTS

4.1 Quantification of biologically fixed nitrogen

Table 4.1.1: Shoot biomass and nitrogen fixation characteristics of various wild legumes collected from the Okavango Delta

Agro ecological zone	Location	Sample name	Biomass g/plant	$\delta^{15}N$ (‰)	%Ndfa	N content (mg.plant^{-1})	N-fixed (mg.plant^{-1})
Okavango	Ngarange	*Chamaecrista absus*	7.1±0.8abc	-1.9±0.1g	100.0±1.4a	134.8±16.4abc	134.8±17.0ab
Okavango	Ngarange	*Vigna unguiculata subsp Denkindtiana*	2.6±0.7e	-0.8±0.2d	84.5±3.7de	72.6±15.8cd	68.1±15.7c
Okavango	Ngarange	*Crotolaria sp*	3.7±0.3de	-0.6±0.2d	81.6±3.4de	99.1±6.5bc	80.8±5.4c
Okavango	Ngarange	*Tephrosia sp*	6.2±0.7bcd	-1.5±0.1f	97.1±1.6ab	147.8±19.1ab	143.4±18.6a
Okavango	Ngarange	*Indigofera*	4.1±1.0de	-1.1±0.2e	89.3±2.8bc	87.7±18.6c	78.2±16.0c
Okavango	Seronga	*Crotalaria sphaerocarpa*	4.4±0.9cde	-0.7±0.2d	91.0±2.4bc	97.5±24.0bc	88.0±21.2bc
Okavango	Seronga	*Crotalaria astragalus*	2.5±0.7e	0.2±0.2b	76.6±3.5d	50.1±15.1d	39.5±13.2c
Okavango	Xakao	*Vigna unguiculata subsp Denkindtiana*	2.4±0.6e	1.3±0.5a	56.4±7.8e	81.1±20.5bcd	46.0±12.0c
Okavango	Xakao	*Tephrosia lupinifolia*	5.4±1.2cde	0.6±0.1b	64.1±0.9de	124.9±28.9abc	79.3±17.7c
Okavango	Xakao	*Indigofera daloides*	8.4±1.8a	0.1±0.2c	77.1±2.2d	174.1±29.1a	135.7±25.7ab
Okavango	Xakao	*Indigofera flavicans*	9.9±1.6a	1.4±0.1a	57.5±2.0e	148.8±48.4ab	87.9±30.4bc
		F-Statistics	**5.78*** **	**23.82*** **	**20.87*** **	**2.44***	**3.70****

Note: Means±SE in a column with dissimilar letters are significantly different. *= p< 0.05, ** = p <0.01, *** = p <0.001

Negative $\delta^{15}N$ values means the plant was fixing N_2; the higher the negative $\delta^{15}N$ values = improved N_2 fixation and vice versa

All the plant species were harvested during their flowering stage. There were significant differences in the shoot biomass during that growing stage (Table 4.1.1). *Indigofera flavicans* showed the highest biomass while *Crotalaria astragalus* and *Vigna unguiculata subspp* had the least. There were significant differences in the $\delta^{15}N$ values among the various herbaceous legumes. Plant species from Ngarange exhibited the most depleted values of $\delta^{15}N$ (‰) while plant species from Xakao were comparatively enriched (Table 4.1.1). *Chamaecrista absus* from Ngarange exhibited the most negative $\delta\ ^{15}N$ values (-1.9‰) and consequently displayed a high dependence on symbiotic N_2 fixation while *Indigofera flavicans* from Xakao had the most positive $\delta\ ^{15}N$ value (1.4 ‰) hence it was least dependent on N_2 fixation at 57.5% Ndfa (Table 4.1.1).

With respect to N content, there were significant variations among legumes. *Indigofera daeloides* from Xakao exhibited the highest amount of N per plant while *Crotalaria astragalus* showed the lowest N content. All the sampled legumes from the Okavango Delta depended on symbiotic N fixation with % Ndfa values more than 50% (Table 4.1.1). Because % Ndfa were high, amounts of N fixed (mg/plant) were in some cases very close to the total N content. For example *Tephrosia sp*, the total N content was 147.8 mg/plant while N fixed was 143.4 mg/plant (Table 4.1.1).

Table 4.1.2: Shoot biomass and nitrogen fixation characteristics of various grain legumes sampled from farmers' fields in Tswapong and Okavango regions of Botswana

Agro ecological zones	Crop	Biomass g.plant^{-1}	δ^{15}N (‰)	%Ndfa	N content (mg.plant^{-1})	N-fixed (mg plant^{-1})	Plants/ 0.4ha	N fixed (kg.ha^{-1})	Potential N fixed (kg.ha^{1})
Tswapong	*Vigna Subterranea* L	25.7±2.9ab	-1.2±0.2e	96.4±2.7a	651.6±75.2ab	624.9±66.3a	85	1.3±0.1a	93.7±9.9a
Tswapong	*Arachis hypogea* L	31.3±6.1a	0.5±0.3c	69.3±4.1b	654.7±155.3ab	440.0±84.6b	75	0.8±0.2bc	40.8±7.3c
Okavango	*Arachis hypogea* XN	28.9±1.8a	0.5±0.3c	71.0±4.5b	838.8±62.2a	594.6±55.3ab	78	1.2±0.2ab	59.5±5.5bc
Okavango	*Vigna unguiculata* XN	5.4±0.2c	1.9±0.5b	46.0±6.6c	187.8±6.5c	85.9±12.2cd	82	0.2±0.9c	12.9±1.8d
Okavango	*Vigna Subterranea* XN	7.6±1.3c	1.8±0.3b	50.3±4.4c	211.0±33.5c	110.1±25.9c	78	0.3±2.1c	21.2±5.6d
Okavango	*Arachis hypogea* XM	30.5±4.9a	0.0±0.4d	78.7±5.4b	797.9±130.9a	618.0±96.5a	72	1.1±7.0ab	61.8±7.5b
Okavango	*Vigna Subterranea* XM	15.4±4.7b	3.3±0.4a	28.8±5.8d	431.3±130.0bc	110.6±33.9c	92	0.2±2.6c	16.6±4.0d
	F-statistics	**8.76*** **	**20.60*** **	**21.57*** **	**6.97*** **	**17.90*** **		**19.08*** **	**20.07*** **

Note: Means±SE in a column with dissimilar letters are significantly different. *= p< 0.05, ** = p <0.01, *** = p <0.001

Negative δ^{15}N values means the plant was fixing N_2; the higher the negative δ^{15}N values = improved N_2 fixation and vice versa

Symbiotic performance of the three agricultural grain legumes differed significantly (Table 4.1.2). Values of $\delta^{15}N$ ranged from 0.0 ‰ to 3.3‰. Groundnut growing in farms from both Okavango and Tswapong regions exhibited the lowest $\delta^{15}N$ values. For example at Ngando's farm in Xakao, at Motenya's farm in Xakao and in Lekobeng, $\delta^{15}N$ values of groundnuts were 0.0, 0.5 and 0.5 ‰ respectively. It is noteworthy that Bambara groundnut showed the most enriched $\delta^{15}N$ values irrespective of where they were growing (Table 4.1.2). The $\delta^{15}N$ for cowpea growing in Xakao (Ngando) was similar to that of Bambara groundnut growing in the same farm (Table 4.1.2). All the grain legumes sampled were dependent on symbiotic fixation for their N nutrition. For instance, Bambara groundnut growing in Lekobeng was the most highly dependent on N_2 fixation with a % NDFA of 96.4. Interestingly, of all the sampled grain legumes, the least dependent on N_2 fixation was also Bambara groundnuts in Xakao (Motenya). Dependence of groundnut on N_2 fixation ranged between 69.3 - 78.7%. N-fixed amounts per plant significantly varied among legumes with Bambara groundnut ranging from 110.1 mg/plant in Xakao (Ngando), 110.6 mg/plant in Xakao (Motenya) to 624.9 mg/plant in Lekobeng. The amount of N-fixed by groundnut was consistently higher regardless of location. For example N-fixed amounts were 440.0 mg/plant in Lekobeng, 594.6 mg/plant in Xakao (Ngando) and 618.0 mg/plant in Xakao (Motenya). Surprisingly, cowpea fixed the least amount of N compared to the other two grain legume crops. Based on the planting density on farmers' fields, amounts of N fixed/ha ranged from 0.2 kg N/ha for cowpea in Xakao and Bambara groundnut in Xakao (Motenya) to 1.3 kg N/ha by Bambara groundnut in Lekobeng. Similarly, groundnuts in Xakao (Ngando) and Xakao (Motenya) fixed low N/ha of 1.2 and 1.1 kg N/ha respectively (Table 4.1.2).

4.2 Isolation and characterization of root nodule bacteria

Table 4.2.1: Colony characterization

Characteristics	Percentage of the 56 isolates
Gram staining	
Negative	100.0
Positive	0.00
Congo red	
Pink	
+	26.8
++	33.9
+++	39.3
Colony shape	
Dome	96.4
Flat	3.6
Colony appearance	
Opaque	75.0
Shiny	25.0
Colony texture	
Elastic	23.2
Buttery	76.8

The intensity of the pink color varied, + less intense, ++ middle intensity and +++ high intensity

From various legume species, root nodule bacteria isolated and cultured on YMA showed a wide range of morphological characteristics. In most cases, more than one bacterial strain was isolated

from a single nodule with a maximum of 4 bacterial isolates per nodule. Table 4.2.1 shows that all the root nodule bacteria isolated produced colonies that were gram negative and displayed wide range of characteristics. They produced a range of pink coloration when plated on the Congo red agar with a varying intensity of the pink color where one plus (+) for a lesser intensity while three stars (+++) for the most intense pink (Table 4.2.1). In relation to colony shape, majority (94.4%) of the isolates were dome shaped except of a few flat shaped ones (Table 4.2.1). Of the 56 isolates, 75% of them had an opaque appearance while the rest were shiny. The shiny colonies had an elastic texture while 76.8% of the isolates were buttery.

4.3 Authentication of root nodule bacteria on their homologous host

Table: 4.3.1: Growth and nodulation of *Crotalaria sphaerocarpa* inoculated with root nodule bacteria isolated from its root nodules sample from various ecological zones of Botswana

Strain	Nod	Fix	Shoot DM ($g.plant^{-1}$)	Root DM ($g.plant^{-1}$)	Nodules $plant^{-1}$	Nodule weight ($mg.plant^{-1}$)
BUAN316/LKD-Cs4	+	+	0.10±0.02bc	0.01±0.00cd	4.00±0.88cd	13.30±3.34b
BUAN316/LKD-Cs6	+	+	0.33±0.08abcd	0.01±0.00cd	5.00±0.67cd	16.70±5.78b
BUAN316/NGA-Cs7C	-	-	0.18±0.05bdc	0.01±0.00cd	na	na
BUAN316/QAB -Cs36A	+	-	0.02±0.01d	0.01±0.00cd	2.00±0.33cd	5.00±0.00b
BUAN316/QAB -Cs36B	-	-	0.19±0.04bcd	0.04±0.00ab	na	na
BUAN316/NGA-Cs38	+	+	0.37±0.11abc	0.03±0.01abcd	13.00±3.67b	46.70±12.03b
BUAN316/GRT-Cs52A	+	+	0.36±0.07abc	0.03±0.01abcd	5.00±0.33cd	30.00±5.78b
BUAN316/GRT-Cs52B	-	-	0.15±0.02bcd	0.01±0.00cd	na	na
BUAN316/XAU-Cs68B	+	+	0.30±0.03abcd	0.05±0.01a	26.00±2.91a	650.00±276.08a
BUAN316/XAU-Cs70A	+	+	0.26±0.07bcd	0.02±0.00bcd	6.00±1.15c	43.30±8.83b
BUAN316/XAU-Cs70B	+	+	0.46±0.09ab	0.04±0.01ab	8.00±1.73c	50.00±5.78b
BUAN316-CsC+	-	-	0.63±0.12a	0.03±0.00abc	na	na
BUAN316-CsC-	-	-	0.11±0.02cd	0.02±00bcd	na	na
F statistics			**6.44*** **	**2.22***	**24.05*** **	**4.72****

Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod +or - = positive or negative nodulation, Fix + or - = positive or negative N2 fixation. Nodule weight = fresh nodule weight, DM = Dry weight. na = not applicable due to no nodulation.

Eight out of eleven bacterial strains induced nodules on introduced *C. sphaerocarpa* (Table 4.3.1). There were significant differences in the shoot growth of *Crotalaria sphaerocarpa* plants inoculated with root nodule bacteria isolated from roots of *Crotalaria sphaerocarpa* harvested from different agro ecological zones of Botswana. Apart from nitrate fed plants strain BUAN316/XAU-Cs70B induced the highest shoot growth with a mass of 0.46 g/plant and followed closely by strains BUAN316/NGA-Cs38, BUAN316/GRT-Cs52A, BUAN316/GRT-Cs52B, BUAN316/LKD-Cs6 and BUAN316/XAU-Cs60B. Other strains such as BUAN316/QABCs36A and BUAN316/LKD-Cs4 depressed growth with shoot weights of 0.02 and 0.10 g/plant respectively in comparison to 0.11 g/plant for the negative control.

Bacterial strains that induced significantly higher shoot mass equally induced higher root growth. Root masses were notably higher for BUAN316/XAU-Cs68B, BUAN316/ QAB-Cs36B and BUAN316/XAU-Cs70B (Table 4.3.1). Furthermore, there were significant differences in the ability of the various rhizobial strains to induce N_2-fixing nodules. For instance, strain BUAN316/XAU-Cs68B induced the highest nodule number which weighed significantly more than others nodulating strains while strain BUAN316/HAN-Cs36A produced the least nodules.

Table 4.3.2: Growth and nodulation of *Vigna unguiculata subsp Denkindtiana* (Ngundenyambi) inoculated with root nodule bacteria isolated from its root nodules sample from Xakao in Okavango Delta

Strain	Nod	Fix	Shoot DM ($g.plant^{-1}$)	Root DM ($g.plant^{-1}$)	Nodules $plant^{-1}$	Nodule weight ($mg.plant^{-1}$)
BUAN316/XAK-Ng65A	+	+	0.4±0.04a	0.06±0.01a	58.00±9.40a	170.00±23.12a
BUAN316/XAK-Ng65B	+	+	0.35±0.08a	0.04±0.01ab	66.00±11.57a	103.33±20.30b
BUAN316/XAK-Ng64B	+	+	0.22±0.04ab	0.03±0.01ab	31.00±11.27b	83.33±31.84b
BUAN316/XAK-Ng64A	+	-	0.06±0.03b	0.03±0.01ab	14.00±2.03bc	26.67±3.34bc
BUAN316-NgC+	-	-	0.42±0.07a	0.06±0.01a	na	na
BUAN316-NgC-	-	-	0.03±0.01b	0.01±0.00b	na	na
F statistics			**12.53*** **	**4.18***	**20.16*** **	**13.88*** **

Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod +or - = positive or negative nodulation, Fix + or - = positive or negative fixation. Nodule weight = fresh nodule weight, DM = Dry weight. na = not applicable due to no nodulation.

Three out of the four bacterial strains isolated from Ngundenyambi induced N_2 fixing nodules when its plants were re-infected to satisfy Koch postulates. There were significant differences in shoot growth of Ngundenyambi plants inoculated with root nodule bacteria isolated from root nodules of plants from Xakao. Excluding the N fed positive control, strain BUAN316/XAK-Ng65A and BUAN316/XAK-Ng65B induced significant highest shoot biomass (Table 4.3.2).

BUAN316/XAK-Ng64A induced the least shoot growth of 0.06 g/plant as comparable to 0.03 g/plant of the negative control. Strains inducing significantly higher shoot weight displayed a similar trend in root growth with the highest root weight induced by of strain BUAN316/XAK-Ng65A and BUAN316/XAK-Ng65B. Strains BUAN316/XAK-Ng64B and BUAN316/XAK-Ng64A had similar weight of 0.03 g/plant which was 0.02 g higher that the negative control.

There were significant differences in number of nodules formed with the highest induced by BUAN316/XAK-Ng65B followed by BUAN316/XAK-Ng65A, BUAN316/XAK-Ng64B and BUAN316/XAK-Ng64A respectively at 66, 58, 31 and 14. Strain BUAN316/XAK-Ng65A induced significantly highest nodule weight (170.0 mg/plant) while BUAN316/XAK-Ng64A induced the least nodule weight of 26 mg/plant.

Table 4.3.3: Growth and nodulation of *Vigna unguiculata subsp Denkindtiana* (Monawana) inoculated with root nodule bacteria isolated from its root nodules sample from various ecological zones of Botswana

Strain	Nod	Fix	Shoot DM ($g.plant^{-1}$)	Root DM ($g.plant^{-1}$)	Nodules $plant^{-1}$	Nodule weight ($mg.plant^{-1}$)
BUAN316/NGA-Mo27A	+	+	0.57±0.22ab	0.25±0.10ab	7.00±2.08a	53.33±14.55c
BUAN316/NGA-Mo27B	+	+	0.25±0.05bc	0.09±0.01bc	4.00±0.33ab	96.67±29.09a
BUAN316/NGA-Mo28	-	-	0.04±0.01c	0.02±0.00c	na	na
BUAN316/NGA-Mo29	-	-	0.02±0.00c	0.01±0.00c	na	na
BUAN316/QAB-Mo46	+	-	0.07±0.02c	0.07±0.01c	3.00±1.76b	31.67±11.56b
BUAN316-MoC+	-	-	0.77±0.04a	0.40±0.02a	na	na
BUAN316-MoC-	-	-	0.04±0.01c	0.03±0.00c	na	na
F statistics			**12.77*** **	**14.84*** **	**7.66*** **	**8.95*** **

Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod +or - = positive or negative nodulation, Fix + or - = positive or negative fixation. Nodule weight = fresh nodule weight, DM = Dry weight. na = not applicable due to no nodulation.

Three out of five bacterial isolates were able to re-infect Monawana during authentication (Table 4.3.3). There was significant growth of Monawana plants inoculated with root nodule bacteria isolated from Monawana plants harvested in the wild in different agro ecological zones. Apart from the nitrate fed plants which induced the highest shoot and root mass, BUAN316/NGA-Mo27A induced the next highest shoot growth with a shoot biomass of 0.57 g/plant, followed by

BUAN316/NGA-Mo27B and BUAN316/QAB-Mo46. Strains BUAN316/NGA-Mo28, BUAN316/NGA-Mo29 and BUAN316/QAB-Mo46 were equal to the negative control. A similar trend was observed in root growth, with strain BUAN316/NGA-Mo27A inducing the highest root growth with a biomass of 0.25g/plant followed by BUAN316/NGA-Mo27B and BUAN316/QAB-Mo46 respectively at 0.09 and 0.07g/plant.

Bacterial strains BUAN316/NGA-Mo28 and BUAN316/NGA-Mo29 induced root growth of the same magnitude as the negative control. Nodule number was highest in plants inoculated with strain BUAN316/NGA-Mo27A. However, nodule weight was significantly highest for plants inoculated with BUAN316/NGA-Mo27B and not BUAN316/NGA-Mo27A.

Table 4.3.4: Growth and nodulation of *Chamaecrista absus* inoculated with root nodule bacteria isolated from its root nodules sample from Ngarange

Strain	Nod	Fix	Shoot DM (g.plant^{-1})	Root DM (g.plant^{-1})	Nodules plant^{-1}	Nodule weight (mg.plant^{-1})
BUAN316/NGA-Ca1	-	-	0.06±0.02b	0.02±0.01ab	na	na
BUAN316/NGA-Ca3	-	-	0.06±0.01b	0.03±0.01ab	na	na
BUAN316/NGA-Ca6	+	-	0.07±0.01b	0.03±0.00ab	3.00±0.58b	nd
BUAN316/NGA-Ca8	+	-	0.09±0.01b	0.03±0.01ab	5.00±1.15a	nd
BUAN316-CaC+	-	-	0.16±0.01a	0.06±0.00a	na	na
BUAN316-CaC-	-	-	0.06±0.01b	0.02±0.01b	na	na
F statistics			**8.03****	**3.06***	**9.00****	

Note: Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod +or - = positive or negative nodulation, Fix + or - = positive or negative fixation. Nodule weight = fresh nodule weight, DM = Dry weight. na = not applicable due to no nodulation, nd = not determined due to small size.

Two out of four strains formed root nodules on plant roots. There were no significant difference in the shoot and root masses of *C. absus* plants inoculated with the various root nodule bacteria. Besides the N fed plants, strain BUAN316/NGA-Ca8 induced the highest shoot growth followed by BUAN316/NGA-Ca6 with 0.07g/plant (Table 4.3.4. However, strain BUAN316/NGA-Ca1 and BUAN316/NGA-Ca3 had a shoot mass equal to the negative control at 0.06g/plant. Similar trends were noted in root growth where strains BUAN316/NGA-Ca8, BUAN316/NGA-Ca6 and BUAN316/NGA-Ca3 induced the highest root growth of 0.03g/plant. Different strain induced significant nodule number with BUAN316/NGA-Ca8 which induced the highest shoot and root growth also producing higher nodule number (5) compared to strain BUAN316/NGA-Ca6 with 3 nodules (Table 4.3.4).

Table 4.3.5: Growth and nodulation of *Zornia glochidiata* inoculated with root nodule bacteria isolated from its root nodules sample from Ngarange

Strain	Nod	Fix	Shoot DM ($g.plant^{-1}$)	Root DM ($g.plant^{-1}$)	Nodules $plant^{-1}$	Nodule weight ($mg.plant^{-1}$)
BUAN316/NGA-Zg71	-	-	0.03±0.01a	0.02±0.00a	na	na
BUAN316/NGA-Zg73	-	-	0.01±0.00a	0.01±0.00a	na	na
BUAN316-ZgC+	-	-	0.35±0.04a	0.03±0.00a	na	na
BUAN316-ZgC-	-	-	0.13±0.00a	0.01±0.00a	na	na
F statistics			**0.86ns**	**0.95ns**		

Note: Values shown are means ±SEM. SEM = standard error of means. In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod - = negative nodulation, Fix - = negative fixation. Nodule weight = fresh nodule weight, DM = Dry weight. ns = not significant, na = not applicable due to no nodulation.

None of the two bacterial isolates were able to induced nodulation on the roots of *Zornia glochidiata,* the host from whose root nodules they were isolated from Ngarange (Table 4.3.5). There were no significant differences in the shoot and root masses of inoculated plants (Table 4.3.5).

Table 4.3.6: Growth and nodulation of *Tephrosia purpurea* inoculated with root nodule bacteria isolated from its root nodules sample from various ecological zones of Botswana

Strain	Nod	Fix	Shoot DM ($g.plant^{-1}$)	Root DM ($g.plant^{-1}$)	Nodules $plant^{-1}$	Nodule weight ($mg.plant^{-1}$)
BUAN316/LKD-Tp1	-	-	0.06±0.00cd	0.03±0.00b	na	na
BUAN316/XAU-TpB1	-	-	0.04±0.01cd	0.02±0.00b	na	na
BUAN316/LKD-Tp2A	+	+	0.09±0.00bc	0.05±0.00a	3.00±2.00a	nd
BUAN316/XAU-Tp2B	+	+	0.12±0.01ab	0.05±0.00a	3.00±0.00a	nd
BUAN316-TpC+	-	-	0.16±0.01a	0.06±0.00a	na	na
BUAN316-TpC-	-	-	0.04±0.01d	0.02±0.00b	na	na
F statistics			**18.44*** **	**16.44*** **	**145ns**	

Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod +or - = positive or negative nodulation. Fix + or - = positive or negative fixation. Nodule weight = fresh nodule weight. DM = Dry weight. ns= not significant, na = not applicable due to no nodulation, nd = not determined due to small size.

Half of the bacterial isolates from *Tephrosia purpurea* induced N_2 fixing nodules with a pink interior. There were significant differences in the shoot biomass of *Tephrosia purpurea* inoculated with root nodule bacteria isolated from the root nodules of *Tephrosia purpurea* collected in the natural ecosystem from different ecological zones of Botswana. Strain BUAN316/XAU-Tp2B induced the highest shoot growth, similar to biomass from 5mM NO_3 fed plants. Bacterial strains that induced significantly higher shoot mass equally induced higher root growth such as strain BUAN316/XAU-T2B and BUAN316/XAU-T2A followed by BUAN316/LKD-Tp1 whereas BUAN316/XAU-TpB1 was similar to the negative control (Table 4.3.6).

Table 4.3.7: Growth and nodulation of *Indigofera daeloides* inoculated with root nodule bacteria isolated from its root nodules sample from Nxamasere

Strain	Nod	Fix	Shoot weight (g.plant^{-1})	Root DM (g.plant^{-1})	Nodules plant^{-1}	Nodule weight (mg.plant^{-1})
BUAN316/NXA-Id19	-	-	0.14±0.00b	0.040±0.01ab	na	na
BUAN316/NXA-Id23	-	-	0.14±0.01b	0.037±0.01b	na	na
BUAN316-IdC+	-	-	0.18±0.01a	0.07±0.00a	na	na
BUAN316-IdC-	-	-	0.06±0.01c	0.03±0.00b	na	na
F statistics			**75.42*** **	**5.95** **		

Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod - = negative nodulation, Fix - = negative fixation. Nodule weight = fresh nodule weight, DM = Dry weight. na = not applicable due to no nodulation.

The two bacterial isolates isolated from *Indigofera daleoides* did not induce N_2 forming nodules on the roots of *Indigofera daleoides* sampled from Nxamasere. Interestingly, plants inoculated with the two strains exhibited significantly higher shoot biomass compared to the negative control. Strains inducing higher shoot growth equally induced significant higher root growth with strains BUAN316/NXA-Id19 and BUAN316/NXA-Id23 respectively having the highest mass of 0.04g/plant and 0.037g/plant compared to 0.03g/plant from the negative control (Table 4.3.7).

Table 4.3.8: Growth and nodulation of *Vigna unguiculata* L. (Walp.) inoculated with root nodule bacteria isolated from its root nodules sample from various ecological zones of Botswana

Strain	Nod	Fix	Shoot DM (g.plant^{-1})	Root DM (g.plant^{-1})	Nodules plant^{-1}	Nodule weight (mg.plant^{-1})
BUAN316/LKD-Vu1	-	-	0.15± 0.03b	0.05± 0.01d	na	na
BUAN316/LKB-Vu4	-	-	0.23± 0.01b	0.06± 0.0d	na	na
BUAN316/KOK-Vu8B	+	+	0.26± 0.07b	0.14± 0.02bc	6.00±1.20ab	36.29±20.98a
BUAN316/KOK-Vu12	+	+	0.23±50.05b	0.08± 0.01cd	4.00±0.88b	28.833±2.24a
BUAN316/LKD-Vu45	-	-	0.13± 0.01b	0.06± 0.01d	na	na
BUAN316/QAB-Vu48	+	+	1.17± 0.26a	0.26± 0.03a	7.00±3.39a	43.33±23.36a
BUAN316/LKD-Vu57A	-	-	0.18± 0.02b	0.08± 0.01b	na	na
BUAN316/LKD-Vu57B	-	-	0.20± 0.07b	0.10± 0.02b	na	na
BUAN316/LKB-Vu59	-	-	0.23± 0.05b	0.12± 0.04c	na	na
BUAN316/LKB-Vu60	-	-	0.21± 0.05b	0.10± 0.01cd	na	na
BUAN316/LKD-Vu79	-	-	0.16± 0.04b	0.05± 0.02d	na	na
BUAN316/LKD-Vu90	-	-	0.13± 0.01b	0.07± 0.00dc	na	na
BUAN316-VuC+	-	-	1.18± 0.05a	0.22± 0.00ab	na	na
BUAN316-VuC-	-	-	0.16± 0.02b	0.07± 0.02c	na	na
F statistics			**23.7*** **	**14.49*** **	**8.54*** **	**5.8*** **

Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod +or - = positive or negative nodulation, Fix + or - = positive or negative fixation. Nodule weight = fresh nodule weight, DM = Dry weight. na = not applicable due to no nodulation.

Three out of twelve cowpea isolates were able to induce nodules on the roots of cowpea Black eye variety. There were significant differences in cowpea shoot growth induced by root nodule bacteria isolated from cowpea sampled in farmer's fields under various agro-ecological zones of Botswana. Strain BUAN316/QAB-Vu48 induced the highest shoot growth with a shoot mass of 1.17 g/plant, similar to N fed positive control plants. Plants inoculated with the other two isolates that induced nodules had biomass similar to that of those of non-nodulated plants and the negative control (Table 4.3.8).

Bacterial strains inducing significant higher shoot growth correspondingly induced higher root growth. For example strain, BUAN316/QAB-Vu48 had significant highest root mass of 0.26g/plant similar to that of the N fed plants. Among the nodulating strains, BUAN316/QAB-

Vu48 induced significantly highest number of nodules (7nodules/plant) closely followed by BUAN316/KOK-Vu8B with 6 nodules/plant. Formed nodules displayed significantly different fresh weight which followed the nodule number trend where strain BUAN316/QAB-Vu48 and BUAN316/KOK-Vu12 respectively had the highest and lowest nodule weight (Table 4.3.8).

Table 4.3.9: Growth and nodulation of *Arachis hypogea* inoculated with root nodule bacteria isolated from its root nodules sample from various ecological zones of Botswana

Strain	Nod	Fix	Shoot DM ($g.plant^{-1}$)	Root DM ($g.plant^{-1}$)	Nodules $plant^{-1}$	Nodule weight ($mg.plant^{-1}$)
BUAN316/LKD-Ah1A	+	+	0.29±0.04bc	0.11±0.00ab	41.00±11.23a	nd
BUAN316/LKD-Ah1B	+	-	0.27±0.05bc	0.11±0.03ab	28.00±5.14ab	nd
BUAN316/KOK-Ah5	-	-	0.17±0.07bc	0.08±0.05ab	na	na
BUAN316/LKB-Ah10	+	-	0.25±0.05bc	0.13±0.01ab	20.00±4.00b	nd
BUAN316/KOK-Ah13	-	-	0.12±0.02b	0.03±0.01b	na	na
BUAN316/LKD-Ah18	-	-	0.28±0.05bc	0.09±0.01ab	na	na
BUAN316/GRT-Ah37B	-	-	0.43±0.12abc	0.18±0.06ab	na	na
BUAN316/GRT-Ah37C	-	-	0.54.28±abc	0.16±0.08ab	na	na
BUAN316/GRT-Ah50	-	-	1.02±0.31a	0.19±0.04ab	na	na
BUAN316-AhC+	-	-	0.87.16±ab	0.24±0.03a	na	na
BUAN316-AhC-	-	-	0.33±0.06abc	0.10±0.01ab	na	na
F statistics			**3.94****	**3.15****	**13.89*****	

Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod +or - = positive or negative nodulation, Fix + or - = positive or negative fixation. Nodule weight = fresh nodule weight, DM = Dry weight. na = not applicable due to no nodulation, nd = not determined due to small size.

Three out of nine isolates induced formation on nodules of the roots of groundnut. There were significant differences in groundnuts shoot growth induced by root nodule bacteria isolated from groundnut sampled in farmer's fields from different ecological zones of Botswana (Table 4.3.9). Interestingly, strain BUAN316/GRT-Ah50 induced the highest shoot growth, surprisingly higher than strains that nodulated groundnuts (BUAN316/LKD-Ah1A, BUAN316/LKD-Ah1B,

BUAN316/LKB-Ah10) and even more surprisingly, higher than the plants that received 5 mM NO_3^-. Bacterial strain BUAN316/KOK-Ah5 and BUAN316/KOK-Ah13 slightly depressed the growth of groundnuts plants (Table 4.2.10). The trend for root biomass was similar to that of shoot biomass with strain BUAN316/GRT-Ah50 giving the highest root dry matter. With respect to nodulation, there were no significant differences in nodule number among the strains that induced root nodulation but BUAN316/LKD-Ah1A had the highest number of nodules (41) followed by BUAN316/LKD-Ah1B.

Table 4.3.10: Growth and nodulation of *Vigna Subterranea* inoculated with root nodule bacteria isolated from its root nodules sample from various ecological zones of Botswana

Strain	Nod	Fix	Shoot DM ($g.plant^{-1}$)	Root DM ($g.plant^{-1}$)	Nodules $plant^{-1}$	Nodule weight ($mg.plant^{-1}$)
BUAN316/LKD-Vs6A	+	-	0.25±0.03b	0.10±0.03b	4.00±1.53b	nd
BUAN316/LKD-Vs6B	+	-	0.32±0.07b	0.13±0.02b	5.00±0.33a	nd
BUAN316/LKD-Vs11	-	-	0.3±0.08b	0.11±0.03b	na	na
BUAN316/LKD-Vs20	-	-	0.23±0.01b	0.05±0.00b	na	na
BUAN316-VsC+	-	-	1.25±0.10a	0.25±0.03a	na	na
BUAN316-VsC-	-	-	0.11±0.03b	0.04±0.01b	na	na
F statistics			**45.58*** **	**12.89*** **	**169*** **	

Values shown are means ±SEM. SEM = standard error of means. Star(s) indicate significant difference at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ (***, ** and * respectively). In a column means followed by dissimilar letter(s) are significantly different from each other according to Duncan multiple range test. Nod +or - = positive or negative nodulation, Fix - = negative fixation. Nodule weight = fresh nodule weight. DM = Dry weight. na = not applicable due to no nodulation, nd = not determined due to small size.

Two out of four bacterial strains induced nodules on the roots of *Vigna Subterranea*. There were significant difference between the shoot and root biomass of the *Vigna Subterranea* plant fed with 5 mM KNO_3 and the rest. There were no significant differences in the biomass of the nodulated and non nodulated plants besides the NO_3^- fed plants (Table 4.3.10).

5.0 DISCUSION

The experiment looked at: i) the assessment of the symbiotic performance of herbaceous legumes in nature and in farmers' fields and ii) isolation of root nodule bacteria, their characterization and authentication on homologous hosts. With respect to assessing the N contribution by various herbaceous legumes, first a plant survey was conducted in various agro-ecological zones of Botswana (Table 3.2). A relationship between the numbers of herbaceous leguminous plants known to fix N_2 emerged. Table 3.2 shows that the Okavango Delta which receives higher annual rainfall of up to 600 mm had many plant species compared to the Kgalagadi region which receives less rainfall. This decline in the number of functionally N_2-fixing plants along an aridity gradient was also observed by Pule-Meulenberg and Dakora (2009), who reported that tree and shrub legume species declined with aridity, in that study they reported that the wetter Ngwaketse region had more N_2-fixing tree and shrub legumes compared to Kgalagadi region. They argued that it meant that soil moisture was a constraint to N_2 fixation in Botswana. According to Swap *et al*. (2004), nutrient availability varied inversely with water availability.

A comparison of the symbiotic performance of the herbaceous legumes growing in the panhandle part of the Okavango Delta revealed that all the legumes depended on N_2 fixation for their N nutrition (Table 4.1.1). All species exhibited depleted $\delta^{15}N$ values of less than 5 ‰, indicating that they were fixing N_2 from the atmosphere. This implies that they have a significant role in the nitrogen cycle in that area by adding nitrogen to the ecosystem and used the fixed N for supporting their growth. The variation in N_2 fixation by both wild and field legumes collected from various locations can be attributed to the differences in competitive capability and effectiveness of the indigenous rhizobial population at each location as supported by Martins (2003), that under different soils the rhizobial population differs in species composition and symbiotic effectiveness.

Also, supporting studies by Giller (2001) observed that differences in N fixation were mainly due to variability in soil physical, biological and chemical properties for both rhizobia and plants, environmental conditions, cropping history and the management practices together with adaptability of the symbiotic partners to environmental conditions.

It is not surprising for same legume species from different location to be showing almost similar % Ndfa values which may be a reflection that they are nodulated by similar rhizobial strains with related effectiveness (Table 4.2.1). This is supported by previous literature that nodulation effectiveness was mostly determined by the rhizobial strain rather than the legume species (Ferguson *et al*., 2010). Conversely, variation in N-fixation and its associated characteristics by same legume species (example *Vigna Subterranea* Table 4.2.1) could be indicating the impact of environmental factors on N fixation.

In general, when compared to legumes collected from farmer's fields, wild legumes had higher % Ndfa, for example, *Chamaecrista absus* from Ngarange totally depended on symbiotic fixation for its N nutrition (Table 4.1.1). Higher % Ndfa shows that the legumes derived majority of N from fixing atmospheric nitrogen, indicating that they were growing under N deficient soils compared to field legumes. These results are consistent with (Streeter, 1988, Dakora, 1997), that under low soil N, biological nitrogen fixation is promoted. Equally higher N fixation by wild legumes due to low soil N status could be enhanced by the co-existence of legumes with non-legumes in natural ecosystems. Supporting study by Cramer and Chimphango (2007) mentioned that non legumes are beneficial for up taking and utilizing N in the soil, creating N deficiency in the soil which sequentially boost legumes to biologically fix nitrogen to their optimal level with the consequent improvement of %Ndfa.

Generally field legumes are fixing substantial high amounts of N (mg/plant) however quantities of N (kg N/ha) added by these legumes to the soils as residual N is low to sustain agriculture. Amount of N fixed/ha is mainly determined by the total plant biomass hence increased biomass lead to more fixed N. Ronner *et al*. (2012) reported that environmental factors such as moisture and nutrient deficiencies had a negative impact on the general plant growth which has a direct impact on the amount of N fixed. Other than environmental factors, the difference in plant biomass could be due to different farm cultural practices applied by farmers.

Cowpea was shown to be the least fixing legume (Table 4.1.2). A study by Ronner (2012) reported that long duration indeterminate species were fixing more nitrogen due to their longer growing periods compared to the determinate varieties. Monocropping of legumes by local famers could also be the reason behind low N fixation in grain legumes as supported by Rusinamhodzi *et al*. (2006) that intercropping with cereals like maize or sorghum which has higher N demand causes less N in the soil making legumes to rely more in N_2 fixation.

A major cultural factor contributing to low quantities of fixed N/ha is the legume plant density adopted by the local farmers which was below the recommended density (Table 4.1.2). Due to these low plant densities, farmers miss the opportunity to totally depend on legumes for N requirements for sustaining crop production. The potential N fixed, estimated from the recommended plant density shows that legumes could fix significant levels of nitrogen sufficient to support arable agriculture with *Arachis hypogea* and *Vigna subterranea* respectively potentially fixing 61.8kg N/ha and 93.7 kg N/ha. These amounts of N fixed can sustain sorghum and maize crops under subsistence farming which are fertilized with N rich fertilizer, 2:3:2 (22) at a rate of 200kg/ha. These results support a previous study by Naab *et al*. (2009), that N_2 fixation in Ghana required cowpea plant density optimization instead of manipulating the symbiotic process.

Supporting findings by Kassam (2009) indicated that Africans could sustain sustainable crop production by changing the cropping styles including intensive legume plantation.

To verify the cause of variation in the level of nitrogen fixation by wild herbaceous legumes and field grain legumes, root nodules were collected from the sampled legumes in order to isolate the associated microsymbionts. Fifty seven strains exhibiting varying colony morphological and biochemical characteristics (Table 4.2.1) as well as different nodulation and N_2-fixing abilities (Tables 4.3.1 – 4.3.10) were isolated. Of the 57 bacterial isolates, close to a half (26) nodulated their homologous hosts. Although twenty six strains nodulated their homologous host, only 17 fixed N_2. Without identification of bacteria, it is hypothesized that the other bacterial endophytes may be there as plant growth promoting bacteria (PGPB). For instance, BUAN316/GRT-Ah50 isolated from groundnut, did not nodulate and yet induced the highest biomass production (1.02 g/plant), more than plants that received 5 mM NO_3^- (0.87 g/plant), that is, 15 % more. In some cases, for example, for *C. sphaerocarpa*, eight out of 11 isolates formed nodules (Table 4.3.1), although only six symbiotically fixed N_2, whereas in others, all strains failed to nodulate (Table 4.3.5). It is not uncommon to isolate non-symbiotic endophytes from nodules (Li *et al*., 2008). Their function could be varied, ranging from siderophore production, phosphate solubilization, cellulase activity, xylanase activity, antibiotic resistance and so on (Pule-Meulenberg *et al.*, unpublished data). Excluding the rhizobia, single nodule could harbor various endophytic bacteria (up to four in this study). Concurring with these findings, Hassan (2013) mentioned that multi-rhizobia characterized with different genetically traits can nodulate one host legume leading to superior plant growth, with non-uniform nodules in which bigger ones harbor superior rhizobia. Also, Rambaugh *et al*. (1990) demonstrated that the symbiotic performance of a double strain

inoculant of *Rhizobium leguminosarum* was 2.5 times superior to their sole counterparts in subterranean clover.

Through staining and microscopic examination results reveal that all the root nodule isolates were Gram negative, cementing the fact that they are bacteria Tamas *et al*. (2010). Cultures took different days to show visible colony growth, acid producing fast growing strain took less than five days. Majority of the colonies were slow growers, alkaline produces and taking more than six days to show visible growth. These results are also in agreement with studies by Ndushwa (2013) that bacterial strains were categorized in fast and slow growers based on their growth rate on Yeast Manitol Agar incubated under dark conditions at 28°C. The variation in the intensity of the pink color change of the medium on the Congo red test is corroborated by previous researches which has shown that generally rhizobia absorbs the dye weakly when compared to other bacteria such as *Agrobacterium* which strongly absorbs the dye.

A number of root nodule bacterial strains with the potential for inoculant due to amount of dry matter produced were found. These include strains such as BUAN316/QAB-Vu48 (Table 4.3.8) which nodulated cowpea and BUAN316/XAU-Cs70B (Table 4.3.1) which nodulated *C. sphaerocarpa*. Strains that were not significantly different to the positive control plants shows that they were highly effective taking into consideration the time they took to establish symbiosis after inoculation and the termination period before they could reach their potential fixation stage. This was in agreement with previous literature that legumes reach their potential fixation during early flowering stage, normally 45 days after inoculation however, termination was done earlier. With such performance, if plants were allowed to grow till flowering, majority of strains could have outcompeted the positive control plants.

Except for the controls, plant biomass corresponding to nodulation presence, concurring with previous literature that nodules are a site for biological nitrogen fixation therefore bigger nodules were equated to increased N fixed. Fixed nitrogen in the nodules is then channeled to the upper parts of host plant and utilized for vital enzymatic reaction such as photosynthesis because it is a major constituent of chlorophyll consequently deep green leaf coloration, improved plant growth, enhanced dry matter quality and quantity (Uchida, 2000). These results corroborates the findings by Mfilinge and Ndakiemi (2014) that inoculation by *Rhizobium cicerea* produced significantly higher nodule number, nodule fresh weight, active nodule per plant and general plant growth compared to the negative control plants.

Generally, treatments that failed the Koch's postulate had higher biomass in comparison to the negative control plants which could be a result of the Plant Growth Promoting Rhizobacteria (PGRP). Corroborating findings by Majeed (2015) noted that other than nitrogen fixation rhizobia inoculation offers plants growth promotion phytohormones aiding in improving biological processes such as photosynthesis and solubilizing organic phosphate consequently elevated plant growth. These phytohormones; indolacetic acid (IAA), gibberellins and cytokinins, iron-sequestering siderophores, phosphate-solubilizing enzymes and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Bhattacharjee *et al*., 2008). Similar studies by Glick (2015) found that ACC deaminase was responsible for lowering plant ethylene levels which in turn promoted root development and elongation consequently improving plant nutrient uptake in the rhizosphere leading to increase in total plant biomass. Inoculated plants were seen to be resistant to rust diseases symbolizing that bacterial inoculation suppress pathogen attack to plants, concurring with El-Gali (2012) on the study of rhizobacterial effects in suppressing root rot disease in cowpea,

concluded that bacterial inoculation was a sustainable way of controlling pathogens offering alternative benefit to agrochemicals.

6.0 CONCLUSION

Results from this dissertation have shown that the diversity of indigenous herbaceous legumes decreased with increasing aridity. Herbaceous indigenous legumes play an important role in cycling of N in the soils of Okavango Delta. Even though wild legumes fixed more N compared to field legumes, %Ndfa for most of the farms were above 50% indicating that they largely depended on symbiotic fixation for their N nutrition. If only local farmers could improve legume plant densities, biological N_2 fixation on farmer's field from Tswapong and Okavango Delta could potentially supply the much needed N for the growing legumes and the subsequent cereal crops.

This dissertation has also demonstrated that various legumes are nodulated by more than single root nodule bacteria exhibiting various morphological characteristics. These root nodule bacteria also showed varied N_2 fixation capacity. Not all of the authenticated strains fixed N_2 however, majority of the bacterial strains induced significantly improved growth to its host which was suspected to be due to their Plant Growth Promoting effects. Taken together these results shows that highly fixing indigenous leguminous plants as well as some of the isolated rhizobial strains have potential for development of elite rhizobial strains as well as Plant Growth Promoting Rhizobacteria (PGPRs) potential for use as inoculant for improving N_2 fixation consequently increasing food security and mitigate climate change.

6.1 RECOMMENDATION

Farmers should intensify legume crop production through raising their plant densities in the fields so that they are in good position to benefit more from biological nitrogen fixation which can offer N levels higher enough to sustain agricultural crop production without any need to rely on chemical fertilizers for improving soil fertility.

In addition to improving legume densities, farmers can adopt improved cultural practices including; timely planting, use of improved varieties and nodulating legumes.

Using the $\delta^{15}N$ Analysis future researchers should quantify the amount of N fixed by field legumes grown under optimal population densities.

Farmers should also domesticate the highly fixing legumes species thus including them in their rotational systems and intensively utilizing them as N rich animal feeds.

Future studies should focus on identifying the rhizobial strains that tested positive on authentication.

Researches in future should test the plant growth promotion effects of the isolated root nodule bacteria in the laboratory and on the field.

Future studies must focus on testing the competitive ability of the isolated strains against the native rhizobia under field conditions from various agro ecological zones of Botswana

Effective bacterial strains from wild legumes should also be tested for their host range on wide range of field legumes under field condition.

Researchers should focus in gene transfer from superior strains isolated from the wild legumes into those strains isolated in field legumes.

7.0 REFERENCES

Abaidoo, R. C., and van Kessel, C. (1989). ^{15}N-uptake, N_2-fixation and rhizobial interstrain competition in soybean and bean, intercropped with maize. *Soil Biology and Biochemistry* **21**: 155-159.

Adhikari, D., Itoh, K., and Suyama, K. (2013). Genetic diversity of common bean (*Phaseolus vulgaris* L.) nodulating rhizobia in Nepal. *Plant and soil* **368**: 341-353.

Allen, J. R., and Obura, R. K. (1983). Yield of corn, cowpea, and soybean under different intercropping systems. *Agronomy Journal* **75**: 1005-1009.7

Ampomah, O. Y., Ofori-Ayeh, E., Solheim, B., and Svenning, M. M. (2008). Host range, symbiotic effectiveness and nodulation competitiveness of some indigenous cowpea bradyrhizobia isolates from the transitional savanna zone of Ghana. *African Journal of Biotechnology* **7**: 988-996.

Appunu, C., and Dhar, B. (2006). Symbiotic effectiveness of acid-tolerant Bradyrhizobium strains with soybean in low pH soil. *African Journal of Biotechnology* **5**: 842–845

Appunu, C., N'Zoue, A., Moulin, L., Depret, G., and Laguerre, G. (2009). *Vigna mungo*, *V. radiata* and *V. unguiculata* plants sampled in different agronomical–ecological–climatic regions of India are nodulated by *Bradyrhizobium yuanmingense*. *Systematic and Applied Microbiology* **32**: 460-470.

Appunu, C., Sasirekha, N., Prabavathy, V. R., and Nair, S. (2009). A significant proportion of indigenous rhizobia from India associated with soybean (*Glycine max* L.) distinctly belong to *Bradyrhizobium* and *Ensifer* genera. *Biology and Fertility of Soils* **46**: 57-63.

Arruda, J. S., Lopes, N. F., and Moura, A. B. (2001). Behavior of Bradyrhizobium japonicum strains under different herbicide concentrations. *Planta Daninha* **19**: 111-117.

Baiphethi, M. N., and Jacobs, P. T. (2009). The contribution of subsistence farming to food security in South Africa. *Agrekon* **48**: 459-482.

Bala, A., and Giller, K. E. (2006). Relationships between rhizobial diversity and host legume nodulation and nitrogen fixation in tropical ecosystems. *Nutrient Cycling in Agroecosystems* **76**: 319-330.

Bala, A., Karanja, N., Murwira, M., Lwimbi, L., Abaidoo, R., and Giller, K. (2011). Production and use of Rhizobial inoculants in Africa. *N_2 Africa, Putting Nitrogen Fixation to Work for Smallholder Farmers in Africa.* www.N2Africa.org, 21pp.

Bala, A., Murphy, P. J., Osunde, A. O., and Giller, K. E. (2003). Nodulation of tree legumes and the ecology of their native rhizobial populations in tropical soils. *Applied Soil Ecology* **22**: 211-223.

Bellaloui, N., and Mengistu, A. (2014). Effects of boron nutrition and water stress on nitrogen fixation, seed δ^{15}N and δ^{13}C dynamics, and seed composition in soybean cultivars differing in maturities. *The Scientific World* **407872**: 1-11.

Berrada, H., and Fikri-Benbrahim, K. (2014). Taxonomy of the rhizobia: current perspectives. *British Microbiology Research Journal* **4**: 616-639

Berrada, H., Nouioui, I., Houssaini, M. I., Ghachtouli, N. E. L., Gtari, M., and Benbrahim, K. F. (2012). Phenotypic and genotypic characterizations of rhizobia isolated from root nodules of multiple legume species native of Fez, Morocco. *African Journal of Microbiology Research* **6**: 5314-5324.

Bhattacharjee, R. B., Singh, A., and Mukhopadhyay, S. N. (2008). Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. *Applied Microbiology and Biotechnology* **80**: 199-209.

Boddey, R. M., Peoples, M. B., Palmer, B., and Dart, P. J. (2000). Use of the ^{15}N natural abundance technique to quantify biological nitrogen fixation by woody perennials. *Nutrient Cycling in Agroecosystems* **57**: 235-270.

Boogerd, F. C., and van Rossum, D. (1997). Nodulation of groundnut by Bradyrhizobium: a simple infection process by crack entry. *FEMS Microbiology Reviews* **21**: 5-27.

Bordeleau, L. M., and Prévost, D. (1994). Nodulation and nitrogen fixation in extreme environments. In: *Symbiotic Nitrogen Fixation*, pp. 115-125. Springer Netherlands.

Boukar, O., Fatokun, C. A., Roberts, P. A., Abberton, M., Huynh, B. L., Close, T. J., and Ehlers, J. D. (2015). Cowpea. In: *Grain Legumes*, pp. 219-250. Springer New York.

Brockwell, J., Bottomley, P., and Thies, J. (1995). Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. *Plant and soil* **174**:143-180.143-180.

Broughton, W. J., and Dilworth, M. J. (1970). Methods in legume-rhizobium technology: plant nutrient solutions. *Handbook for rhizobia*, 245-249.

Cadisch, G., Sylvester-Bradley, R., and Nösberger, J. (1989). 15 N-based estimation of nitrogen fixation by eight tropical forage-legumes at two levels of P: K supply. *Field Crops Research* **22**: 181-194.

Chemining'wa, G. N., Muthomi, J. W., and Theuri, S. W. M. (2007). Effect of rhizobia inoculation and starter-N on nodulation, shoot biomass and yield of grain legumes. *Asian Journal of Plant Sciences* **6**: 1113-1118.

Chemining'wa, G. N., and Vessey, J. K. (2006). The abundance and efficacy of *Rhizobium leguminosarum* bv. *viciae* in cultivated soils of the eastern Canadian prairie. *Soil Biology and Biochemistry* **38**: 294-302.

Chen, W. M., James, E. K., Prescott, A. R., Kierans, M., and Sprent, J. I. (2003). Nodulation of Mimosa spp. by the β-proteobacterium *Ralstonia taiwanensis*. *Molecular Plant-Microbe Interactions* **16**: 1051-1061.

Chen, W. X., Yan, G. H., and Li, J. L. (1988). Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *International Journal of Systematic and Evolutionary Microbiology*, **38**: 392-397.

Chikowo, R., Mapfumo, P., Nyamugafata, P., Nyamadzawo, G., and Giller, K. E. (2003). Nitrate-N dynamics following improved fallows and maize root development in a Zimbabwean sandy clay loam. *Agroforestry systems* **59**: 187-195.

Cooper, J. E., and Scherer, H. W. (2012). Nitrogen Fixation. In: *Marschner N Mineral Nutrition of Higher Plants*, 389-408. 3rd Edition. Academic Press, London

Cooper, P. J. M., Dimes, J., Rao, K. P. C., Shapiro, B., Shiferaw, B., and Twomlow, S. (2008). Coping better with current climatic variability in the rain-fed farming systems of sub-Saharan Africa: An essential first step in adapting to future climate change?. *Agriculture, Ecosystems and Environment* **126**: 24-35.

Correa, O. S., Aranda, A., and Barneix, A. J. (2001). Effects of pH on growth and nodulation of two forage legumes. *Journal of Plant Nutrition* **24**: 1367-1375.

Costa, F. M., Schiavo, J. A., Brasil, M. S., Leite, J., Xavier, G. R., and Fernandes Jr, P. I. (2014). Phenotypic and molecular fingerprinting of fast growing rhizobia of field-grown pigeonpea from the eastern edge of the Brazilian Pantanal. *Genetics and Molecular Research* **13**: 469-482.

Coulibaly, O., Alene, A. D., Manyong, V., Sanogo, D., Abdoulaye, T., Chianu, J., and Boukar, O. (2009). Situation and outlook for cowpea and soybean in sub-Ssaharan Africa. *II Project in West and East Africa*. **10**: 5-8.

Cramer, M. D., Chimphango, S. B., Van Cauter, A., Waldram, M. S., and Bond, W. J. (2007). Grass competition induces N_2 fixation in some species of African Acacia. *Journal of Ecology* **95**: 1123-1133.

Dakora, F. D. (1985). Nodulation and nitrogen fixation by groundnut in amended and unamended field soil in Ghana. In *Biological nitrogen fixation in Africa: proc. of the First Conference of the African Association for Biological Nitrogen Fixation (AABNF) held in Nairobi, Kenya, 23 to 27 July 1984/ed. H. Ssali; SO Keya*. Nairobi, Kenya: Nairobi Rhizobium MIRCEN, Department of Soil Science, University of Nairobi.

Dakora, F. D. (1995). A functional relationship between leghaemoglobin and nitrogenase based on novel measurements of the two proteins in legume root nodules. *Annals of Botany* **75**: 49-54.

Dakora, F. D. (1995). Plant flavonoids: biological molecules for useful exploitation. *Functional Plant Biology* **22**: 87-99.

Dakora, F. D. (2003). Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. *New Phytologist* **158**: 39-49.

Dakora, F. D., Aboyinga, R. A., Mahama, Y., and Apaseku, J. (1987). Assessment of N_2 fixation in groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* L. Walp) and their relative N

contribution to a succeeding maize crop in Northern Ghana. *MIRCEN Journal of Applied Microbiology and Biotechnology* **3**: 389-399.

Dakora, F. D., and Keya, S. O. (1997). Contribution of legume nitrogen fixation to sustainable agriculture in Sub-Saharan Africa. *Soil Biology and Biochemistry* **29**: 809-817Dakora, F. D., and Phillips, D. A. (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* **245**: 35-47.

Dakora, F. D., Phillips, D. A., Sande, E., Joseph, C. M., and Zoń, J. (1994). Synthesis, release, and transmission of alfalfa signals to rhizobial symbionts. In: *Symbiotic Nitrogen Fixation*, pp. 69-80). Springer Netherlands.

Danso, S. K. A., Bowen, G. D., and Sanginga, N. (1992). Biological nitrogen fixation in trees and agro-ecosystems. *Plant and Soil*, **141**: 177-196.

de Freitas, A. D. S., de Sampaio, E. V. S. B., da Silva, B. L. R., de Almeida Cortez, J. S., and Menezes, R. S. C. (2012). How much nitrogen is fixed by biological symbiosis in tropical dry forests? 2. Herbs. *Nutrient Cycling in Agroecosystems* **94**: 181-192.

Denarie, J., Debelle, F., Truchet, G., and Prome, J. C. (1993). Rhizobium and legume nodulation: A molecular dialogue. In: *New Horizons in Nitrogen Fixation*, pp. 19-30. Springer Netherlands.

Denison, R. F., and Kiers, E. T. (2011). Life histories of symbiotic rhizobia and mycorrhizal fungi. *Current Biology* **21**: 775-785.

Deshwal, V. K., Pandey, P., Kang, S. C., and Maheshwari, D. K. (2003). Rhizobia as a biological control agent against soil borne plant pathogenic fungi. *Indian Journal of Experimental Biology* **41**: 1160-1164.

Deshwal, V. K., Singh, S. B., Kumar, P., and Chubey, A. (2013). Rhizobia unique plant growth promoting Rhizobacteria: A Review. *International Journal of Life Sciences* **2**: 74-86.

Drew, E. A., and Ballard, R. A. (2010). Improving N_2 fixation from the plant down: Compatibility of *Trifolium subterraneum* L. cultivars with soil rhizobia can influence symbiotic performance. *Plant and Soil* **327**: 261-277.

Drew, E. A., Herridge, D. F., Ballard, R. A., O'Hara, G. W., Deaker, R., Denton, M. D., and Seymour, N. (2012). Inoculating legumes: a practical guide. *Grains Research and Development Corporation*.

Dwivedi, S., Sahrawat, K., Upadhyaya, H., and Oritz., R. (2013). Food, nutrition and agro biodiversity under global climate change. *Advanced Agronomy* **120**:1-128.

Eaglesham, A. R. J., Ayanaba, A., Rao, V. R., and Eskew, D. L. (1981). Improving the nitrogen nutrition of maize by intercropping with cowpea. *Soil Biology and Biochemistry* **13**: 169-171.

Eaglesham, A. R. J., Ayanaba, A., Rao, V. R., and Eskew, D. L. (1982). Mineral N effects on cowpea and soybean crops in a Nigerian soil. *Plant and Soil* **68**: 171-181.

El-Gali, Z. I. (2012). Role of rhizobacteria in suppression of rhizoctonia root-rot disease of bean. *Persian Gulf Crop Protect* **1**: 33-40.

El-Hamdaoui, A., Redondo-Nieto, M., Rivilla, R., Bonilla, I., and Bolanos, L. (2003). Effects of boron and calcium nutrition on the establishment of the *Rhizobium leguminosarum*–pea (*Pisum sativum*) symbiosis and nodule development under salt stress. *Plant, Cell and Environment* **26**: 1003-1011.

Evans, A. T. (2015). Phylogeny and Diversity of Rhizobial Bacteria. *Journal of Phylogenetic and Evolution Biology* **3**: e111.

Farrar, T. J., Nicholson, S. E., and Lare, A. R. (1994). The influence of soil type on the relationships between NDVI, rainfall, and soil moisture in semiarid Botswana. II. NDVI response to soil moisture. *Remote Sensing of Environment* **50**: 121-133.

Ferguson, B. J., Indrasumunar, A., Hayashi, S., Lin, M. H., Lin, Y. H., Reid, D. E., and Gresshoff, P. M. (2010). Molecular analysis of legume nodule development and autoregulation. *Journal of Integrative Plant Biology* **52**: 61-76.

Ferguson, B. J., Ross, J. J., and Reid, J. B. (2005). Nodulation phenotypes of gibberellin and brassinosteroid mutants of pea. *Plant Physiology* **138**: 2396-2405.

Flores, M., Mavingui, P., Perret, X., Broughton, W. J., Romero, D., Hernández, G., and Palacios, R. (2000). Prediction, identification, and artificial selection of DNA rearrangements in Rhizobium: toward a natural genomic design. *Proceedings of the National Academy of Sciences* **97**: 9138-9143.

Freitas, A., Júnior, C., Oliveira, L. A. De, Castro, H. G. De, Cornélio, G. D. L., Rodrigues, G., and Costa, L. (2013). Isolation and phenotypic characterization of rhizobia that nodulate cowpea in the Cerrado in Tocantins State, Brazil. *Journal of Biotechnology and Biodiversity* **4**: 249–259.

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews **68***: 280-300.

Gathumbi, S. M., Cadisch, G., and Giller, K. E. (2002). ^{15}N Natural abundance as a tool for assessing N_2 fixation of herbaceous, shrub and tree legumes in improved fallows. *Soil Biology and Biochemistry* **34**: 1059-1071.

Giller, K. E., Franke, A. C., Abaidoo, R., Baijukya, F., Bala, A., Boahen, S., and Simmons, A. J. (2013). N2Africa: Putting nitrogen fixation to work for smallholder farmers in Africa. *Agro-ecological Intensification of Agricultural Systems in the African Highlands. Routledge, London*, 156-174.

Giller, K. E., Murwira, M. S., Dhliwayo, D. K., Mafongoya, P. L., and Mpepereki, S. (2011). Soyabeans and sustainable agriculture in southern Africa. *International Journal of Agricultural Sustainability* **9**: 50-58.

Giller, K. E., Tittonell, P., Rufino, M. C., Van Wijk, M. T., Zingore, S., Mapfumo, P., and Rowe, E. C. (2011). Communicating complexity: integrated assessment of trade-offs concerning soil

fertility management within African farming systems to support innovation and development. *Agricultural Systems* **104**: 191-203.

Giller, K.E. 2001. *Nitrogen Fixation in Tropical Cropping Systems*. 2nd Edition. CABI Publishing, Wallingford.

Goormachtig, S., Capoen, W., and Holsters, M. **(**2004). Rhizobium infection: lessons from the versatile nodulation behavior of water-tolerant legumes. *Trends in Plant Science* **9**: 518-522.

Gosling, S. N., and Arnell, N. W. (2016). A global assessment of the impact of climate change on water scarcity. *Climatic Change* **134**: 371-385.

Hansen, J., Sato, M., Ruedy, R., Lo, K., Lea, D. W., and Medina-Elizade, M. (2006**).** Global temperature change. *Proceedings of the National Academy of Sciences* **103**: 14288-14293.

Hassen, A. I., Bopape, F. L., and Trytsman, M. (2014). Nodulation Study and Characterization of Rhizobial Microsymbionts of Forage and Pasture Legumes in South Africa. *World Journal of Agricultural Research* **2**: 93-100.

Hirsch, A. M., Lum, M. R., and Downie, J. A. (2001). What makes the rhizobia-legume symbiosis so special? *Plant Physiology* **127**: 1484-1492.

Högberg, P., and Kvarnström, M. (1982). Nitrogen fixation by the woody legume *Leucaena leucocephala* in Tanzania. *Plant and Soil* **66**:21-28.

IPCC (2014). Summary for Policymakers, In: Climate Change 2014, Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jabbar, B. K. A., and Saud, H. M. (2012). Effects of molybdenum on Biological Nitrogen Fixation by combination of *rhizobium* and *azospirillum* in soybean under drip irrigation system. *International Journal of Life Sciences Biotechnology and Pharma Research* **1**: 2250-3137.

Jabbar, B. K. A., and Saud, H. M. (2012). Effects of phosphorus on biological nitrogen fixation in soybean under irrigation using saline water. *Global Journal of Science Frontier Research Agriculture and Biology* **12**: 65-72.

Jaftha, J. B., Strijdom, B. W., and Steyn, P. L. (2002). Characterization of pigmented methylotrophic bacteria which nodulate *Lotononis bainesii*. *Systematic and Applied Microbiology* **25**: 440-449.

Jefferis, K., von Rudloff, L., Kenewendo, B., Sigwele, H., Tlali, B., and Moyo, T. (2012). Status

of Agricultural and Rural Finance in Botswana.

Jourand, P., Giraud, E., Béna, G., Sy, A., Willems, A., Gillis, M., and de Lajudie, P. (2004). *Methylobacterium nodulans sp*. nov., for a group of aerobic, facultatively methylotrophic, legume

root-nodule-forming and nitrogen-fixing bacteria. *International Journal of Systematic and Evolutionary Microbiology* **54**: 2269-2273.

Junk, G. and Svec, H. J. (1958). The absolute abundance of the nitrogen isotopes in the atmosphere and compressed gas from various sources. *Geochimica et cosmochimica acta* **14**: 234–243.

Kadiata, B. D., Mulongoy, K., and Isirimah, N. O. (1996). Time course of biological nitrogen fixation, nitrogen absorption and biomass accumulation in three woody legumes. *Biological Agriculture and Horticulture* **13**: 253-266.

Kassam, A., Friedrich, T., Shaxson, F., and Pretty, J. (2009). The spread of conservation agriculture: justification, sustainability and uptake. *International Journal of Agricultural Sustainability* **7**: 292-320.

Keyser, H. H., and Li, F. (1992). Potential for increasing biological nitrogen fixation in soybean. In: *Biological Nitrogen Fixation for Sustainable Agriculture*, pp. 119-135. Springer. Netherlands.

Keyser, H. H., and Li, F. (1992). Potential for increasing biological nitrogen fixation in soybean. *Plant and Soil* **141**: 119-135.

Khan, M. A., and Akhtar, M. S. (2015). Agricultural Adaptation and Climate Change Policy for Crop Production in Africa. In: *Crop Production and Global Environmental Issues*, pp. 437-541. Springer International Publishing.

Khan, W., Prithiviraj, B., and Smith, D. L. (2008). Nod factor [Nod Bj V (C 18: 1, MeFuc)] and lumichrome enhance photosynthesis and growth of corn and soybean. *Journal of Plant Physiology* **165**: 1342-1351.

Kipkore, W., Wanjohi, B., Rono, H., and Kigen, G. (2014). A study of the medicinal plants used by the Marakwet Community in Kenya. *Journal of Ethnobiology and Ethnomedicine* **10**: 1-15.

Kusangaya, S., Warburton, M. L., Van Garderen, E. A., and Jewitt, G. P. (2014). Impacts of climate change on water resources in southern Africa: A review. Physics and Chemistry of the Earth, Parts A/B/C **67**: 47-54.

L'taief, B., Sifi, B., Zaman-Allah, M., Drevon, J. J., and Lachaâl, M. (2007). Effect of salinity on root-nodule conductance to the oxygen diffusion in the *Cicer arietinum–Mesorhizobium ciceri* symbiosis. *Journal of plant Physiology* **164**: 1028-1036.

Liu, Y., Wu, L., Baddeley, J. A., and Watson, C. A. (2011). Models of biological nitrogen fixation of legumes. A review. *Agronomy for Sustainable Development* **31**: 155-172.

Mahesh, B., and Satish, S. (2008). Antimicrobial activity of some important medicinal plant against plant and human pathogens. *World journal of agricultural sciences* **4**: 839-843.

Mapfumo, E., Chanasyk, D. S., Naeth, M. A., and Baron, V. S. (1999). Soil compaction under grazing of annual and perennial forages. *Canadian Journal of Soil Science* **79**: 191-199.

Mapfumo, P., and Giller, K. E. (2001). Soil fertility management strategies and practices by smallholder farmers in semi-arid areas of Zimbabwe. *International System for Agricultural Science and Technology* **53**: 20-29.

Mapfumo, P., Giller, K. E., Mpepereki, S., and Mafongoya, P. L. (1999). Dinitrogen fixation by pigeonpea of different maturity types on granitic sandy soils in Zimbabwe. *Symbiosis* **27**: 305-318.

Mariotti, A. (1983). Atmospheric nitrogen is a reliable standard for natural ^{15}N abundance measurements. *Nature* **303**: 685–687.

Maskey, S. L., Bhattarai, S., Peoples, M. B., and Herridge, D. F. (2001). On-farm measurements of nitrogen fixation by winter and summer legumes in the Hill and Terai regions of Nepal. *Field Crops Research* **70**: 209-221.

Masson-Boivin, C., Giraud, E., Perret, X., and Batut, J. (2009). Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? *Trends in Microbiology* **17**: 458-466.

Masutha, T. H., Muofhe, M. L., and Dakora, F. D. (1997). Evaluation of N_2 fixation and agroforestry potential in selected tree legumes for sustainable use in South Africa. *Soil Biology and Biochemistry* **29**: 993-998.

Matiru, V. N., and Dakora, F. D. (2004). Potential use of rhizobial bacteria as promoters of plant growth for increased yield in landraces of African cereal crops. *African Journal of Biotechnology* **3**: 1-7.

Mayes, S., Basu, S. M., Molosiwa, O., Redjeki, E. S., Ahmad, N. S., Khan, F., and Stadler, F. (2013). Molecular analysis of Bambara gGroundnut, an underutilised african legume crop as part of the BAMLINK Project–What lessons can we learn. In 2nd International symposium on underutilized species: Crops for the future-beyond food security (pp. 451-458). Kuala Lumpur: Acta Horticulture 979.

McCully, M. E. (2001). Niches for bacterial endophytes in crop plants: a plant biologist's view. *Functional Plant Biology* **28**:983-990.

Melino, V. J., Drew, E. A., Ballard, R. A., Reeve, W. G., Thomson, G., White, R. G., and O'Hara, G. W. (2012). Identifying abnormalities in symbiotic development between *Trifolium spp*. and *Rhizobium leguminosarum* bv. *trifolii* leading to sub-optimal and ineffective nodule phenotypes. *Annals of botany* 206.

Mfilinge, A., Mtei, K., and Ndakidemi, P. (2014). Effect of Rhizobium Inoculation and Supplementation with Phosphorus and Potassium on Growth and Total Leaf Chlorophyll (Chl) Content of Bush Bean Pha*seolus vulgaris*, L. *Agricultural Sciences* **5**: 1413.

Monica, N., Vidican, R., Pop, R., and Rotar, I. (2013). Stress Factors Affecting Symbiosis Activity and Nitrogen Fixation by Rhizobium Cultured in vitro, *ProEnvironment* **6**: 42-45

Moulin, L., ChenZ, W. M., Bénal, G., Dreyfusl, B., and Boivin-Masson, C. (2002). Rhizobia: the family is expanding. *In Nitrogen Fixation: Global Perspectives: Proceedings of the 13th*

International Congress on Nitrogen Fixation, Hamilton, Ontario, Canada, 2-7 July 2001 (p. 61). CABI.

Mpepereki, S., Javaheri, F., Davis, P., and Giller, K. E. (2000). Soyabeans and sustainable agriculture: promiscuous soyabeans in southern Africa. *Field Crops Research* **65**: 137-149.

Mpepereki, S., Wollum II, A. G., and Makonese, F. (1996). Diversity in symbiotic specificity of cowpea rhizobia indigenous to Zimbabwean soils. *Plant and soil* **186**: 167-171.

Mpepereki, S., Zengeni, R., and Giller, K. E. (2006). Manure and soil properties affect survival and persistence of soyabean nodulating rhizobia in smallholder soils of Zimbabwe. *Applied Soil Ecology* **32**: 232-242.

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant, Cell and Environment* **25**: 239-250.

Muofhe, M. L., and Dakora, F. D. (1999). Nitrogen nutrition in nodulated field plants of the shrub tea legume *Aspalathus linearis* assessed using ^{15}N natural abundance. *Plant and Soil* **209**: 181-186.

Muranaka, S., Shono, M., Myoda, T., Takeuchi, J., Franco, J., Nakazawa, Y., and Takagi, H. (2016). Genetic diversity of physical, nutritional and functional properties of cowpea grain and relationships among the traits. *Plant Genetic Resources* **14**: 67-76.

Naeem, F. I., Ashraf, M. M., Malik, K. A., and Hafeez, F. Y. (2004). Competitiveness of introduced Rhizobium strains for nodulation in fodder legumes. *Pakistan Journal of Botany* **36**: 159-166.

Ncube, B., Twomlow, S. J., Van Wijk, M. T., Dimes, J. P., and Giller, K. E. (2007). Productivity and residual benefits of grain legumes to sorghum under semi-arid conditions in southwestern Zimbabwe. *Plant and Soil* **299**: 1-15.

Ndoye, I., and Dreyfus, B. (1988). N_2 fixation by *Sesbania rostrata* and *Sesbania sesban* estimated using ^{15}N and total N difference methods. *Soil Biology and Biochemistry* **20**: 209-213.

Nezomba, H., Mtambanengwe, F., Chikowo, R., and Mapfumo, P. (2015). Sequencing integrated soil fertility management options for sustainable crop intensification by different categories of smallholder farmers in Zimbabwe. *Experimental Agriculture*, **51**: 17-41.

Nezomba, H., Tauro, T. P., Mtambanengwe, F., and Mapfumo, P. (2008). Nitrogen fixation and biomass productivity of indigenous legumes for fertility restoration of abandoned soils in smallholder farming systems. *South African Journal of Plant and Soil* **25**: 161-171.

Nezomba, H., Tauro, T. P., Mtambanengwe, F., and Mapfumo, P. (2010). Indigenous legume fallows (indifallows) as an alternative soil fertility resource in smallholder maize cropping systems. *Field Crops Research* **115**: 149-157.

Nyemba, R. C., and Dakora, F. D. (2010). Evaluating N_2 fixation by food grain legumes in farmers' fields in three agro-ecological zones of Zambia, using ^{15}N natural abundance. *Biology and Fertility of Soils* **46**: 461-470.

Okito, A., Alves, B. R. J., Urquiaga, S., and Boddey, R. M. (2004). Isotopic fractionation during N_2 fixation by four tropical legumes. *Soil Biology and Biochemistry* **36**: 1179-1190.

Oyainzu H, Matsumoto S, Minamisawa K., and Gamou, T. (1993). Distribution of rhizobia in leguminous plants surveyed by phylogenetic identification. *The Journal of General and Applied Microbiology* **39**: 339-354.

Peix, A., Ramírez-Bahena, M. H., Velázquez, E., and Bedmar, E. J. (2015). Bacterial associations with legumes. *Critical Reviews in Plant Sciences* **34**: 17-42.

Plies-Balzer, E., Kong, T., Schubert, S., and Mengel, K. (1995). Effect of water stress on plant growth, nitrogenase activity and nitrogen economy of four different cultivars of *Vicia faba* L. *European Journal of Agronomy* **4**: 167-173.

Pueppke, S. G., and Broughton, W. J. (1999). Rhizobium sp. strain NGR234 and *R. fredii* USDA257 Share Exceptionally Broad, Nested Host Ranges. *Molecular Plant-Microbe Interactions* **12**: 293-318.

Pule-Meulenberg F., and Batisani N.J. (2003). A review of Botswana soil fertility research: Current status and prospects. *UNISWA Journal for Agriculture* **12**: 60-67.

Pule-Meulenberg, F., and Dakora, F. D. (2007). Assessing the biological potential of N_2-fixing Leguminosae in Botswana for increased crop yields and commercial exploitation. *African Journal of Biotechnology* **6**: 325-334.

Pule-Meulenberg, F., and Dakota, F. D. (2009). Assessing the symbiotic dependency of grain and tree legumes on N_2 fixation for their N nutrition in five agro-ecological zones of Botswana. *Symbiosis* **48**: 68-77.

Pule-Meulenberg, F., Belane, A. K., Krasova-Wade, T., and Dakora, F. D. (2010). Symbiotic functioning and bradyrhizobial biodiversity of cowpea (*Vigna unguiculata* L. Walp.) in Africa. *BMC Microbiology* **10**: 89.

Pule-Meulenberg, F., Gyogluu, C., Naab, J., and Dakora, F. D. (2011). Symbiotic N nutrition, bradyrhizobial biodiversity and photosynthetic functioning of six inoculated promiscuous-nodulating soybean genotypes. *Journal of Plant Physiology* **168**: 540-548.

Raemaekers RH (Ed.). 2001. *Crop Production in Tropical Africa*. Directorate General for International Cooperation, Ministry of Foreign Affairs, External Trade and International Cooperation, Brussels, Belgium. 1549 pp

Rai, M. K., Kalia, R. K., Singh, R., Gangola, M. P., and Dhawan, A. K. (2011). Developing stress tolerant plants through in vitro selection—an overview of the recent progress. *Environmental and Experimental Botany* **71**: 89-98.

Rai, M.K., Dash, P.K., Mohapatra, T. and Singh, R. (2012). Phenotypic and Molecular Characterization of Indigenous Rhizobia Nodulating Chickpea in India. *Indian Journal of Experimental Biology* **50**: 340-350.

Rasolomampianina, R., Bailly, X., Fetiarison, R., Rabevohitra, R., Béna, G., Ramaroson, L., and Avarre, J. C. (2005). Nitrogen-fixing nodules from rose wood legume trees (*dalbergia spp*.) endemic to Madagascar host seven different genera belonging to α-and β-proteobacteria. *Molecular Ecology* **14**: 4135-4146.

Rivas, R., Velázquez, E., Willems, A., Vizcaíno, N., Subba-Rao, N. S., Mateos, P. F., and Martínez-Molina, E. (2002). A new species of Devosia that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (Lf) Druce. *Applied and Environmental Microbiology* **68**: 5217-5222.

Robinson, D. (2001). δ ^{15}N as an integrator of the nitrogen. *Trends in Ecology and Evolution* **16**: 153–162.

Rodríguez-Navarro, D. N., Camacho, M., Leidi, E. O., Rivas, R., and Velázquez, E. (2004). Phenotypic and genotypic characterization of rhizobia from diverse geographical origin that nodulate *Pachyrhizus species*. *Systematic and Applied Microbiology* **27**: 737–45.

Rusinamhodzi, L., Murwira, H. K., and Nyamangara, J. (2006). Cotton–cowpea intercropping and its N_2 fixation capacity improves yield of a subsequent maize crop under Zimbabwean rain-fed conditions. *Plant and soil* **287**: 327-336.

Rutherford, J. 2009. *Crotalaria juncea*, a promising green manure crop for the tropics. A Regional Supplement to ECHO Development Notes Issue 3, October 2009

Samba, R. T., Sylla, S. N., Neyra, M., Gueye, M., Dreyfus, B., and Ndoye, I. (2002). Biological nitrogen fixation in *Crotalaria species* estimated using the ^{15}N isotope dilution method. *African Journal of Biotechnology* **1**: 17-22.

Sanginga, N. (2003). Role of biological nitrogen fixation in legume based cropping systems; a case study of West Africa farming systems. *Plant and Soil* **252**: 25-39.

Sanginga, N., Ade Okogun, J., Vanlauwe, B., Diels, J., Carsky, R. J., and Dashiell, K. (2001). Nitrogen contribution of promiscuous soybeans in maize-based cropping systems. *Sustaining Soil Fertility in West Africa* **58**: 157-177.

Scheres, B., Van De Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., and Bisseling, T. (1990). The ENOD12 gene product is involved in the infection process during the pea-Rhizobium interaction. *Cell* **60**: 281-294.

Schulze ED, Gebauer G, Ziegler H, Lange OL (1991). Estimates of nitrogen fixation by trees on an aridity gradient in Namibia. *Oecologia* **88**: 451-455.

Sci, J. C., June, B., Youn, J., Van, K., Lee, J., Kim, W., and Lee, S. (2008). Waterlogging Effects on Nitrogen Accumulation and N_2 Fixation of Super nodulating Soybean Mutants, *Journal of Crop Science and Biotechnology* **11**: 111 – 118.

Serraj, R., Sinclair, T. R., and Purcell, L. C. (1999). Symbiotic N_2 fixation response to drought. *Journal of Experimental Botany* **50:** 143-155.

Sessitsch, A., Hardoim, P., Döring, J., Weilharter, A., Krause, A., Woyke, T., and Hurek, T. (2012). Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Molecular Plant-Microbe Interactions,* **25**: 28-36.

Sharma, S.R., Rao, N.K., Gokhale, T.S. and Ismail, S. (2012). Isolation and Characterization of Salt-Tolerant Rhizobia Native to the Desert Soils of United Arab Emirates. *Emirates Journal of Food and Agriculture* **25**: 102-108.

Shearer, G. and Kohl, D. H. (1986). N_2-fixation in field settings: Estimations based on natural ^{15}N abundance. *Australian Journal of Plant Physiology* **13**, 699–757.

Shoko, M. D., and Tawira, F. (2007). Benefits of soyabeans as a breakcrop in sugarcane production systems in the South Eastern Lowveld of Zimbabwe. *Sugar Journal* **70**: 18-22.

Simon, Z., Mtei, K., Gessesse, A. and Ndakidemi, P.A. (2014). Isolation and Characterization of Nitrogen Fixing Rhizobia from Cultivated and Uncultivated Soils of Northern Tanzania. *American Journal of Plant Sciences* **5**: 4050-4067.

Somasegaran, P. and Hoben, H. J. (1994). Handbook for Rhizobia: Methods in Legume – *Rhizobium technology*. Springer – Verkag, New York, USA.

Souleimanov, A., Prithiviraj, B., and Smith, D. L. (2002). The major Nod factor of *Bradyrhizobium japonicum* promotes early growth of soybean and corn. *Journal of Experimental Botany* **53**: 1929-1934.

Sprent, J. I. (2003). Plant biology: mutual sanctions. *Nature* **422**: 672-674.

Sprent, J. I. (2008). 60Ma of legume nodulation. What's new? What's changing?. *Journal of experimental botany* **59**: 1081-1084.

Sprent, J. I., and Parsons, R. (2000). Nitrogen fixation in legume and non-legume trees. *Field Crops Research* **65**: 183-196.

Sprent, J.I. (2007) Evolving Ideas of Legume Evolution and Diversity: A Taxonomic Perspective on the Occurrence of Nodulation. *New Phytologist* **174**: 11-2.

Ssali S and Keya S.O (1984). Nitrogen level and cultivar effects on nodulation, dinitrogen fixation and yield of grain legumes. Cowpea cultivars. East Africa. *Journal for Agriculture and Forestry* **45**:247-254.

Streeter, J., and Wong, P. P. (1988). Inhibition of legume nodule formation and N_2 fixation by nitrate. *Critical Reviews in Plant Sciences,* **7**: 1-23.

Subramanian S (2013). Distinct Hormone Regulation of Determinate and Indeterminate Nodule Development in Legumes. *Journal for Plant Biochemistry and Physiology* **1**: 2-5.

Swap, R. J., Aranibar, J. N., Dowty, P. R., Gilhooly, W. P., and Macko, S. A. (2004). Natural abundance of ^{13}C and ^{15}N in C3 and C4 vegetation of southern Africa: patterns and implications. *Global Change Biology* **10**:350-358.

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis, M, Boivin-Masson C., and Dreyfus B (2001a). *Methylotrophic Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of Bacteriology* **183**: 214-220.

Tan, K. H. (2005) Soil Sampling, Preparation and Analysis. CRC Press

Taylor, R. W., Williams, M. L., andd Sistani, K. R. (1991). N_2 fixation by soybean-Bradyrhizobium combinations under acidity, low P and high Al stresses. *Plant and Soil* **131**: 293-300.

Tezcan, F. A., Kaiser, J. T., Mustafi, D., Walton, M. Y., Howard, J. B., and Rees, D. C. (2005). Nitrogenase complexes: multiple docking sites for a nucleotide switch protein. *Science* **309**: 1377-1380.

Thies, J. E., Singleton, P. W., and Bohlool, B. B. (1991). Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. *Applied and Environmental Microbiology* **57**: 19-28.

Tobisa, M., Shimojo, M., and Masuda, Y. (2014). Root Distribution and Nitrogen Fixation Activity of Tropical Forage Legume American Jointvetch (*Aeschynomene americana* L.) cv. Glenn under Waterlogging Conditions. *International Journal of Agronomy 2014*

Turk, D., Keyser, H. H., and Singleton, P. W. (1993). Response of tree legumes to rhizobial inoculation in relation to the population density of indigenous rhizobia. *Soil Biology and Biochemistry* **25**: 75-81.

Unkovich, M. J., and Pate, J. S. (2000). An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. *Field Crops Research* **65**: 211-228.

Unkovich, M. J., Baldock, J., and Peoples, M. B. (2010**).** Prospects and problems of simple linear models for estimating symbiotic N_2 fixation by crop and pasture legumes. *Plant and Soil* **329**: 75-89.

Unkovich, M., and Baldock, J. (2008). Measurement of asymbiotic N_2 fixation in Australian agriculture. *Soil Biology and Biochemistry* **40**: 2915-2921.

Valenzuela, H.; Smith, J. (2002). *Cowpea*; University of Hawaii, College of Tropical Agriculture and Human Resources: Manoa, HI, USA, p. 3.

Van Berkum, P., and Eardly, B. D. (2002). The aquatic budding bacterium *Blastobacter denitrificans* is a nitrogen-fixing symbiont of *Aeschynomene indica*. *Applied and Environmental Mmicrobiology* **68**: 1132-1136.

Vanlauwe, B., Wendt, J., Giller, K. E., Corbeels, M., Gerard, B., and Nolte, C. (2014). A fourth principle is required to define conservation agriculture in sub-Saharan Africa: the appropriate use of fertilizer to enhance crop productivity. *Field Crops Research* **155**: 10-13.

Velázquez, E., Peix, A., Zurdo-Piñiro, J. L., Palomo, J. L., Mateos, P. F., Rivas, R., and Martínez-Molina, E. (2005). The coexistence of symbiosis and pathogenicity-determining genes in Rhizobium rhizogenes strains enables them to induce nodules and tumors or hairy roots in plants. *Molecular Plant-Microbe Interactions* **18**: 1325-1332.

Velde, M., Folberth, C., Balkovič, J., Ciais, P., Fritz, S., Janssens, I. A., and Peñuelas, J. (2014). African crop yield reductions due to increasingly unbalanced Nitrogen and Phosphorus consumption. *Global Change Biology* **20**: 1278-1288.

Venieraki, A., Dimou, M., Vezyri, E., Kefalogianni, I., Argyris, N., Liara, G., and Katinakis, P. (2011). Characterization of nitrogen-fixing bacteria isolated from field-grown barley, oat, and wheat. *The Journal of Microbiology* **49**: 525-534.

Vincent, J. M. (1970). The cultivation, isolation and maintenance of rhizobia. *A Manual for the Practical Study of the Root-Nodule Bacteria*, pp. 1-13. Edited by J. M Vicent, Oxford: Blackell scientific.

Wang, E. T., Rogel, M. A., Garcia-de los Santos, A., Martinez-Romero, J., Cevallos, M. A., and Martínez-Romero, E. (1999). *Rhizobium etli* bv. *mimosae*, a novel biovar isolated from *Mimosa affinis*. *International Journal of Systematic and Evolutionary Microbiology* **49**: 1479-1491.

Wang, F., Wang, E. T., Wu, L. J., Sui, X. H., Li Jr, Y., and Chen, W. X. (2011). *Rhizobium vallis sp. nov*., isolated from nodules of three leguminous species. *International Journal of Systematic and Evolutionary Microbiology* **61**: 2582-2588.

Waswa, M., Karanja, N., Woomer, P., and Mwenda, G. (2014). Identifying elite rhizobia for soybean (*Glycine max*) in Kenya. *African Journal of Crop Science* **2**: 60-66.

Woomer, P. L., Singleton, P. W., and Bohlool, B. B. (1988). Reliability of the most-probable-number technique for enumerating rhizobia in tropical soils. *Applied and Environmental Microbiology* **54**: 1494-1497.

Woomer, P., Bennett, J., and Yost, R. (1990). Overcoming the inflexibility of most-probable-number procedures. *Agronomy Journal* **82**: 349-353.

Woomer, P.L., Karanja, N., Kisamuli, S.M., Murwira, M., Bala, A. (2011). Rhizobium collection, testing and the identification of candidate elite strains.

Wopereis, J., Pajuelo, E., Dazzo, F. B., Jiang, Q., Gresshoff, P. M., De Bruijn, F. J., and Szczyglowski, K. (2000). Short root mutant of *Lotus japonicus* with a dramatically altered symbiotic phenotype. *The Plant Journal* **23**: 97-114.

Zahran, H. H. (1999). Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews* **63**: 968-989.

Zengeni, R., and Giller, K. E. (2007). Effectiveness of indigenous soyabean rhizobial isolates to fix nitrogen under field conditions of Zimbabwe. *Symbiosis* **43**: 129–135.