ASSESSMENT OF THE QUALITY AND EFFECTIVENESS OF PLANT GROWTH PROMOTING RHIZOBACTERIA ON SOYBEAN (*Glycine max*) AND COMMON BEAN (*Phaseolus vulgaris*) GROWTH

HEZEKIAH KORIR

A thesis submitted to the Graduate School in partial fulfillment for the requirements of Master of Science Degree in Soil Science of Egerton University

EGERTON UNIVERSITY

August, 2016

DECLARATION AND APPROVAL

Declaration

I declare that this thesis is my original work and has not been shared, presented or submitted wholly or in part for any award in any other Institution.

Signature.....Date.....

Hezekiah Korir

KM13/3342/12

Approval

We confirm that this thesis was prepared under our supervision and has our approval to be for submission to Egerton University.

Signature.....Date.....

Prof. Nancy W. Mungai

Department of Crops, Horticulture and Soils

Egerton University

Signature  Date.....

Dr. Cargele Masso

International Institute of Tropical Agriculture

Nairobi

COPYRIGHT

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to God for enabling me to go through my course of study successfully with good health and state of mind. I greatly appreciate Egerton University, and the department of Crops, Horticulture and Soils, for providing the greenhouse space and laboratory facilities. I am thankful to my supervisors Prof. Nancy Mungai, Dr. Cargele Masso and Dr. Moses Thuita for their efforts, advice, guidance, and patient supervision during the carrying out of this project and preparation of this thesis. I also appreciate the IITA laboratory staff at Duduville, Nairobi for helping with the molecular characterization. I am deeply indebted to the IITA's COMPRO-II Project for funding my studies. I also appreciate my lecturers in the Department of Crops, Horticulture and Soils and Egerton University as a whole for giving me the opportunity to study. My course mates; Alex, Beldina and Jospeth are also appreciated for the encouragement throughout the coursework and during project work. I am also grateful to my parents and family for their moral support during this study.

DEDICATION

This thesis is dedicated to my parents Mr and Mrs. Milgo, my wife Fatuma sharamo and to my daughter Ashley Chepkemoi.

ABSTRACT

The use of plant growth promoting rhizobacteria offers an alternative to the use of chemical fertilizers. The quality and efficacy of inoculants is critical to realize the benefits of inoculation. The objectives of this study were; 1) to test the quality of 3 inoculants (Legumefix, Biofix for soybean and Biofix for common bean) and evaluate the response of soybean and common bean to inoculation; 2) to test the effect of soil fertility on inoculation response of soybean and common bean; 3) to isolate and characterize phosphate solubilizing bacteria from common bean nodules and; 4) to assess the effect of co-inoculation of *rhizobium* and phosphate solubilizing bacteria on growth of common bean in a low phosphorous soil. For objective 1 the inoculants were tested for the purity and number of viable cells. For the efficacy test, seeds were inoculated at the recommended rate at planting. At mid podding, nodule number and weight, shoot and root dry biomass, biologically fixed nitrogen and the symbiotic efficiency of the rhizobia were determined. For objective 2 the legumes were planted and inoculated with the rhizobia inoculants using two soils (Andosol and Nitisol) and data taken as described in objective 1. For objective 3, soils were collected from different parts of Nakuru County and used to trap the nodules and the strains isolated and characterized in the laboratory. For objective 4, common bean were inoculated with *Rhizobium* strains singly or in a combination with two PSB to evaluate their effect on growth. The inoculants showed variable quality in terms of the number of viable cell and the number of microorganisms. Inoculation of common beans with Biofix led to significantly higher number of nodules per plant and nodule weight (117 nodules and 2.09 g respectively) than the control (75 nodules and 0.84 g respectively). Nodulation was higher in high fertility soil in both legumes. Co-inoculation of IITA-PAU987 + *B. megaterium* recorded the highest nodule and shoot dry weight (405.2 mg and 6.84 g respectively) compared to IITA-PAU987 alone (324.8 mg and 5.32 g respectively). CIAT 899 + *B. megaterium* recorded a higher shoot weight (401.2 mg) compared to CIAT 899 alone (337.2 mg) but no significant difference was observed when co-inoculated with *P. polymyxa*. The results on quality testing of inoculants show a need for manufacturing companies to have a quality control system in the production process. These results indicate that co-inoculation of PSB and rhibozia has a synergistic effect on growth of common bean. Testing of the plant growth promoting rhizobacteria under field conditions will further elucidate their effectiveness on grain yield of common bean.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

BNF	Biological Nitrogen Fixation
CAN	Calcium Ammonium Nitrate
CIAT	International Centre for Tropical Agriculture
CRD	Completely Randomized Design
DAP	Diammonium Phosphate
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture organization Statistics
g	grams
ICRISAT	International Centre for Research in Semi-Arid Tropics
ILRI	International Livestock Research Institute
KALRO	Kenya Agricultural and Livestock Research Organization
KEBS	Kenya Bureau of Standards
KEPHIS	Kenya Plant Health Inspectorate Service
Min	Minutes
mm	Millimeter
N_2	Nitrogen gas
NCPB	National Cereals and Produce Board
PCR	Polymerase Chain Reaction
PGPR	Plant Growth promoting Rhizobacteria
PSB	Phosphate Solubilizing Bacteria
Sec	Seconds
SSA	Sub-Saharan Africa
TSBF	Tropical Soil Biology and Fertility

CHAPTER ONE: INTRODUCTION

1.1Background information

Large areas of sub-Saharan Africa (SSA) are affected by various types of degradation, including fertility decline. The fertility decline is to continuous cropping without adequate nutrient replenishment. Consequently, yields are relatively low despite the high potential for improvement. Increasing number of African farmers mention soil fertility decline as a major constraint to farming (De Groote *et al*., 2010; and Tittonel *et al*., 2010) and hence a threat to food security for the growing population.

The nutrient is provided to cropping systems in the form of industrially produced chemical fertilizers. The use of these fertilizers has led to worldwide environmental problems such as greenhouse gas emissions, and eutrophication of water bodies (Mukhtar *et al*., 2013). In addition, these commercial fertilizers are expensive and out of reach of most small-scale farmers in SSA (Chianu *et al*., 2011; Chukukwere *et al*., 2013; Muui *et al*., 2013). Also, African farmers may not be in a position to use fertilizers or to use them optimally, because they do not perceive the benefits, or they cannot afford to buy the fertilizer; or fertilizer may not be physically available (Druilhe & Barreiro-Hurlé, 2012).

To reduce the production cost with mineral fertilizers and provide protection to the environment, increased legume production could be achieved through seed inoculation with beneficial *Rhizobium* bacteria (Hussain *et al*., 2011). Therefore, biofertilizers can help solve the problem of feeding an increasing global population at a time when agriculture is facing various environmental stresses (Bhardwaj *et al*., 2014).

Biological nitrogen fixation (BNF) via legume-*Rhizobium* symbiosis is a potential option which is an environmentally friendly, economically viable and renewable source of nitrogen (N) for resource poor farmers (Bekere & Hailemariam, 2012). Biological nitrogen fixation involving host-specific symbiotic interactions between root nodule bacteria, collectively termed rhizobia, and legumes has received a lot of research attention because of the central role it plays in the maintenance of soil fertility. Estimates of total annual biological nitrogen fixation worldwide range from $130\text{-}180\times10^{6}$ metric tons, with about 50% fixed by rhizobia (Havlin *et al*., 2014). Soybean N fixation is estimated to range between 44 and 103 kg Nha^{-1} (Sanginga *et al*., 2003).

Research on the use of *Rhizobium* inoculants for production of grain legumes shows it is a cheaper and usually more effective agronomic practice for ensuring adequate N nutrition of legumes, compared with the application of N fertilizer (Payne *et al*., 2008). Legume inoculation is the process of introducing commercially prepared sources of rhizobia to promote nitrogen fixation. Use of Rhizobia inoculants may reduce reliance on inorganic N that is expensive to most smallholder farmers in SSA (Cheminingwa *et al*., 2011). Biological nitrogen fixation and grain yields of legumes are normally increased when inoculated with effective and efficient strain of rhizobia.

Because of its current and potential economic importance, the interaction between rhizobia and leguminous plants has been intensively studied and many commercial products have been formulated and are available in the market. Many factors contribute to high quality legume inoculant products. The most important of these include high numbers of efficient rhizobia capable of nodulation and nitrogen fixation and the formulation. When farmers realize that the crops do not respond to inoculation, their confidence in the technology goes down and both the manufacturers and consumers are ultimate losers (Lupwayi *et al*., 2006). Some study results have also shown variable quality in the inoculants produced by some suppliers (Sanginga, 2011). Biofix, for example, a rhizobia inoculant has been shown to increase yield of soybean by 9-10% in DR Congo. This make the investment in inoculation worthwhile for farmers given the small costs associated with inoculation (Sanginga *et al.*, 2011). The success of rhizobia inoculation response in the field is affected by various soil factors such as the cation exchange capacity (CEC), clay content and phosphorous has been widely reported to influence nodulation and subsequent BNF of legumes.

Phosphorus is the most important element in the nutrition of plants, next to N. It plays an important role in virtually all major metabolic processes in plants including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Zaidi *et al*. 2010). Although abundant in soils, in both organic and inorganic forms, its availability is restricted as it occurs mostly in insoluble forms. Inorganic P occurs in soil, mostly in insoluble mineral complexes, some of them appearing after frequent application of chemical fertilizers. Phosphorous stress reduces nitrogen fixation due to decreased nodule formation and reduced nodule sizes and finally affecting the grain quality and quantity (Sadeghipour & Abbasi, 2012).

Phosphate solubilization is an important plant growth promoting activity. A large proportion of soluble inorganic P added to soil is fixed to insoluble form soon after its application, thereby becoming unavailable to plants. Therefore, phosphate solubilizing microorganisms (PSM) have attracted researchers to exploit their potential to utilize phosphate reserves and to enhance crop yields (Khan *et al.,* 2006). Phosphate solubilizers are economical, ecofriendly and have greater agronomic value to compensate the expensive inorganic sources of P fertilizers (Qureshi *et al*., 2012).

Deficiency of phosphorous in soils may limit the full potential of rhizobial inoculation and therefore co-inoculating the soils with phosphate solubilizing bacteria can enhance crop growth (Weisany *et al*., 2013). Taking into account that some plant growth promoting rhizobacteria possess the ability of phosphate solubilization, they could be useful in bean production improvement by increasing P content in the soil and enhancing nodulation and N fixation (Hernendez *et al.,* 2009). The inoculation of plants with selected PSB to increase native population can mobilize P from poorly available sources and therefore improve plant nutrition (Guiñazu *et al*., 2010). The use of PSB inoculants remains limited because of the inconsistent results in different environments, but the potentialities of these resources have not been effectively explored in terms of bacterial species efficient in mobilizing P with crops of economic and social significance.

1.2 Statement of the problem

Replenishing soil fertility in SSA is vital in the pursuit of food security. Majority of Kenyan population live in rural areas and most of them are smallholder farmers. They account for more than 70% of agricultural and food production in Kenya (Salami *et al*., 2010). A major problem facing Kenya's smallholder farmers is declining soil fertility as a result of continuous cropping without sufficient replenishment of soil nutrients. This has led to decreasing food productivity against an increasing population. To reverse this trend and ensure increased yields, there has been investment in soil fertility improvements through soil amendments and other agricultural inputs such as bio-fertilizers, bio-pesticides and inorganic fertilizers. As a result, commercial inoculants are being formulated by manufacturing companies and are made available to farmers mainly through agrochemical outlets across the country. Each of these products is accompanied with a description of its composition and how it improves soil fertility, and its

overall effects on the yields and performance of the crops. However, these products are of inconsistent quality and most of them show variable results in terms of efficacy (Mathu *et al*., 2012) leaving farmers with no substantial alternative to inorganic fertilizers (N'cho *et al*., 2014). The result has been selling and distribution of low/poor quality products to farmers, retailers and wholesalers. When famers realize that the inoculants sold to them are of poor quality, they tend to shy away from adopting the use of the inoculation technology. Poor quality inoculants affect the quality and yield of crops. In addition, low soil phosphorous may limit the full potential of rhizobia inoculation. Phosphatic fertilizers are also expensive to famers and may also have negative impact on the soil and environment. Therefore, there is a need to assess the quality of products and to find alternative effective strains for possible improvement of available commercial products and thus improve soil fertility and crop productivity. The isolation, identification and testing of indigenous phosphate solubilizing bacteria is also important to improve on plant response to inoculation with rhizobia.

1.3 Objectives

1.3.1 General objective

To increase the growth of soybean and common bean through use of quality inoculants and native plant growth promoting rhizobacteria (PGPR)

1.3.2 Specific objectives

1. To test the quality and effect of commercial inoculants on nodulation, plant biomass and biological nitrogen fixation of soybean and on common bean varieties
2. To test the effect of soil fertility status to inoculation response of common bean and soybean.
3. To isolate and characterize native root nodule endogenous plant growth promoting rhizobacteria from common bean in Nakuru county
4. To determine the co-inoculation effect of rhizobia strains and native phosphate solubilising bacteria on common bean growth parameters in a low phosphorous soil

1.4 Hypotheses

H_0;

1. Commercial rhizobial inoculants do not meet the quality standards and do not affect the nodulation, plant biomass and biological nitrogen fixation of common bean and soybean
2. Soil fertility status does not affect the response of common bean and soybean to inoculation
3. The soils do not contain native plant growth promoting rhizobacteria
4. Co-inoculation of rhizobia strains and phosphorous solubilizing bacteria do not enhance growth of common bean in the low phosphorous soil.

1.5 Justification

The increasing prices of chemical fertilizer each year makes it unaffordable to small-scale farmers in sub-Saharan Africa (Yakubu *et al*., 2010). There is therefore need to introduce BNF by legumes as a way of increasing soil fertility. Legumes play an important role by fixing atmospheric N_2 through their symbiotic relationship with *Rhizobium* spp. Rhizobial inoculants are widely accepted as low cost supplements to chemical fertilizers and have no significant harmful effects on soil health and environment (Bagyaraj, 2004). Phosphate solubilizing bacteria provide an environmentally sound and cheap means of improving soil fertility and crop yields. An apparent problem affecting farmers' use of these inoculants is their quality. Poor quality control in the production process negatively affects the viability of the inoculants. Also, most farmers are not aware of the potential of legume inoculation in improving production. Therefore, it is important to assess the quality of these commercial products to cushion farmers against the use of compromised products and to carry out research on the effectiveness of these commercial products. This research aims at assessing the quality of commercial inoculants and tests their effectiveness on common bean and soybean growth as compared to native soil microorganisms. Also the findings and recommendations from this study will be of usefulness to the regulatory bodies such as Kenya Plant Health Inspectorate Service (KEPHIS) since it will help in documenting the quality and efficacy of the products found in the market. The recommendation on effective superior strains will help in improving product formulation by the manufacturers. Subsequently, farmers will have knowledge on the benefit of inoculation and hence can adopt this technology which will in turn improve crop productivity and their living standards. In turn,

there will be improved soil fertility and thereby enhancing food and nutritional security of the small holder farmers.

1.6 Limitation of the study

The evaluation of product efficacy in different soil types using different common bean and soybean varieties was done in a greenhouse environment. The assumption is that the responses of the inoculants in the greenhouse will reflect their performance in the field with similar soil characteristics. Another limitation of this thesis is that nodulation, tissue nitrogen and shoots and root biomass were used to assess the efficacy of the inoculant and bacterial strains. This limits full comparison of the monetary output accrued from inoculation as compared to the chemical fertilizers used by farmers (Di-Ammonium Phosphate and Calcium Ammonium Nitrate) which could have been well explain if yield data was available.

1.7 Thesis layout

This thesis contains five chapters Chapter one describes the general background information of the research topic. Chapter two explores literature review of the different factors contributing to understanding the research topic. Chapter three describes the specific objective one and two, which is laboratory testing of the commercial rhizobial inoculants (biofertilizer) in the laboratory and subsequent efficacy testing in the greenhouse and testing the effect of soil fertility status on inoculation response. Chapter four describes specific objective three i.e. isolation and identification of the plant growth promoting rhizobacteria (PGPR) from root nodules of common bean. It also describes the effect of selected PGPR exhibiting phosphate solubilizing abilities when co-inoculated with indigenous and reference rhizobia strains on common bean and soybean growth in a phosphorous deficient soil. Finally, chapter five highlights the general conclusions drawn from this study and give recommendations on adoption and areas for further research.

References

Bagyaraj, D. (2004). Quality Control and Constraints in Biofertilizer Production and Technology. *Biofertilizers technology* 401-408.

Bekere, W. & Hailemariam, A. (2012). Influences of inoculation methods and phosphorus levels on nitrogen nixation attributes and yield of soybean (*Glycine max* L.). At Haru, Western Ethiopia. *American Journal of Plant Nutrition and Fertilization Technology* 2(2): 45-55.

Bhardwaj, D., Ansari, M. W., Ranjan Kumar Sahoo, R. K. & Tuteja, N. (2014). Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. Review. *Microbial Cell Factories* 13:66.

Chemining'wa, G. N., Theuri, S. W. & Muthomi, J. W. (2011). Abundance of indigenous rhizobia nodulating cowpea and common beans in Central Kenyan Soils. *African Journal of Horticultural Science 5*: 92-97.

Chianu, J. N., Nkonya, E. M., Mairura, F. S. & Akinnifesi, F. K. (2011). Biological nitrogen fixation and socioeconomic factors for legume production in sub-saharan Africa: a review. *Agronomy for Sustainable Development* 31: 139-154.

Chukwukere N.S, Okafor J.A, Iruo, F. (2013). Comparative analysis of fertilizer-use in Imo State: 1990-1999 and 2000-2009**.** *Researcher* 5(9): 94-98.

De Groote, H., Vanlauwe, B., Rutto, E., Odhiambo, G. D., Kanampiu, F. & Khan, Z.R. (2010). Economic analysis of different options in integrated pest and soil fertility management in maize systems of Western Kenya. *Agricultural Economics* 41: 471-482.

Druilhe, Z., & Barreiro-Hurle, J. (2012). *Fertilizer Subsidies in Subsaharan Africa.* ESA Working Paper No. 12-04. FAO, Rome.

Guiñazu, L. B., Andres, J. A., DelPapa, M. F., Pistorio, M. & Rosas, S. B. (2010). Response of alfalfa (*Medicago sativa* L.) to single and mixed inoculation with phosphate-solubilising bacteria and *Sinorhizobium meliloti*. *Biology and Fertility of Soils* 46:185-190.

Havlin, J.L, Tisdale, S.L, Beaton, J.D. & Werner, L.N. (2014). *Soil Fertility and Fertilizers. An introduction to nutrient management*. Eighth Edition. Pearson Education, Inc, Publishing. Prentice Hall.

Hernandez, G., Valdes-Lopez, O., Ramirez, M., Goffard, N., Weiller, G., Aparicio-Fabre, R., FuenteS, S. I., Erban, A., Kopka, J., UdvardI, K. M., & Vance, C. P. (2009). Global changes in the transcript and metabolic profiles during symbiotic nitrogen fixation in phosphorus-stressed common bean plants. *Plant Physiology* 151 (3): 1221-1238.

Hussain, M. I., Akhtar, M. J., Asghar, H. N. & Maqshoof, A. (2011). Growth, nodulation and yield of mash bean (*Vigna mungo* L.) as affected by *Rhizobium* inoculation and soil applied L-tryptophan. *Soil and Environmen*t 30(1): 13-17.

Lupwayi, N. Z., Clayton, G. W. & Rice, W. A. (2006). Rhizobial Inoculants for Legume Crops. *Journal of Crop Improvement* 15(2): 289-321.

Mathu, S., Herrmann, L., Pypers, P., Matiru, V., Mwirichia, R. & Lesueur, D. (2012). Potential of indigenous bradyrhizobia versus commercial inoculants to improve cowpea (*Vigna unguiculata* L. walp.) and green gram (*Vigna radiata* L. wilczek.) yields in Kenya. *Soil Science and Plant Nutrition* 58(6): 750-763.

Mukhtar, N., Hameed, M., Ashraf, M. & Ahmed, R. (2013). Modifications in stomatal structure and function in *Cenchrus ciliaris* L. and *Cynodon dactylon* (L.) pers. in response to cadmium stress. *Pakistan Journal of Botany* 45(2): 351-357.

Muui, C.W., Muasya, R.M. & Kirubi, D.T. (2013). Baseline survey on factors affecting sorghum productionand use in Eastern Kenya. *African Journal of Food, Agriculture, Nutrition and Development* 13(1): 7339-7342.

N'cho, C. O., Yusu, F., A. A., Jefwa, J. M., Odoh, N. C. & Ama-abina, J. T. (2014). Can commercial microbial inoculants increase groundnut phosphorus uptake and yield under low nutrient content soil in Sudan Savanna? Review. *Ivoirian Science and Technology* 23: 199-211.

Payne, F., Murray, P. J. & Cliquet, B. (2008) Root exudates: a pathway for short-term N transfer from clover and ryegrass. *Plant and Soil* 229: 235-243.

Qureshi, M. A. Ahmad Z. A., Akhtar, N. Iqbal, A. Mujeeb, F. and Shakir. M. A. (2012). Role of phosphate solubilizing bacteria (PSB) in enhancing P availability and promoting cotton growth. *The Journal of Animal & Plant Sciences* 22(1): 204-210.

Sadeghipour, O. & Abbasi, S. (2012). Soybean response to drought and inoculation. *World Applied Sciences Journal* 17 (1): 55-60.

Salami, A., Kamara, A. B. & Brixiova, Z. (2010). Smallholder Agriculture in East Africa: Trends, Constraints and Opportunities. Working Papers Series No. 105. African Development Bank, Tunis, Tunisia.

Sanginga, N., Dashiell, K., Diels, J., Vanlauwe, B., Lyasse, O., Carsky, R. J., Tarawali, S., Asafo, A. B., Menkir, A., Schulz, S., Singh, B. B., Chikoye, D., Keatinge, D. & Rodomiro, O. (2003). Sustainable resource management coupled to resilient germplasm to provide new intensive cereal-grain legume-livestock systems in the dry savanna. *Agriculture, Ecosystem and Environment* 100: 305 –314.

Sanginga, N., Baijukya, F. & Franke, L. (2011). Soybean responds to *Rhizobium* inoculation in DR Congo. *N2Africa Podcaster* no. 10, 2-3.

Tittonell, P., Muriuki, A., Shepherd, K. D., Mugendi, D., Kaizzi, K. C., Okeyo, J., Verchot, L., Coe, R, & Vanlauwe, B. (2010). The diversity of rural livelihoods and their influence on soil fertility in agricultural systems of East Africa – A typology of smallholder farms. *Agricultural Systems* 103: 83–97.

Weisany, W., Raei, Y. & Allahverdipoor, K. H. (2013). Role of some of mineral nutrients in biological nitrogen fixation. *Bulletin of Environment, Pharmacology and Life Science* (4): 77-84.

Yakubu, H., Kwari, J. D. & Ngala, A. L. (2010). N_2 fixation by grain legume varieties as affected by rhizobia inoculation in the Sandy Loam Soil of Sudano-Sahelian Zone of North Eastern Nigeria. *Nigerian Journal of Basic and Applied Science* 18(2): 229-236.

Zaidi, A., Ahemad, M., Oves, M., Ahma, E. & Khan, M. S. (2010). Role of phosphate solubilizing bacteria in legume improvement. Microbes for Legume Improvement. Springer, pp 273-292.

CHAPTER TWO: LITERATURE REVIEW

2.1 Economic potential of grain legumes (Soybean and common bean)

Soybean (US) or soya bean (UK) (*Glycine max*) is a species of legume native to East Asia, widely grown for its edible bean which has numerous uses. Soybean is an oil crop that can be grown in Kenya from 0-2200 m altitude and under rainfall regime of 300 to 1200 mm. Kenya is a very small soybean producer, even within the African context. Soybean production in Kenya was 2500 Kg/ha in 2011 (FAO, 2012). An experiment carried out by CIAT-Maseno found a potential yield of up to 3.5 t ha^{-1} for most improved varieties and 2.6 t ha^{-1} for the variety SB 19 (TGx 1740-2F) (CIAT, 2013). Of all grain legumes, soybean has the highest concentration of protein. As a legume, soybean improves soil fertility by fixing atmospheric nitrogen into the soils and this is another strong reason why the adoption of improved varieties of soybean should be promoted (Katungi *et al*., 2009).

High profitability has been demonstrated with improved practices and value addition and industrial demand for soybean products continues to grow. The price of one kilogramme of soybean at Rotterdam port was Ksh. 50.3 in 2012 which recorded the highest price relative to the previous years (World Bank, 2013), and locally farmers are selling a kilogram of soybean at a rate of between Ksh. 37.5 to BIDCO manufacturing company and Ksh. 60 to Kenya Agricultural and Livestock Research Organization (KALRO) (Collombet, 2013).

However, soybean production in Kenya remains low because of knowledge gaps on utilization and processing of soybean, unavailability of quality seed at agro retail outlets, lack of awareness on the right rhizobial inoculants to enhance yields, limited marketing outlets for soybean especially in the rural areas, a narrow range of soy products and consumer awareness on nutritional advantages of soy mainly limited to the educated urban people. Demand for soy products is therefore low as majority of the rural population rarely consume it. To address these gaps, KALRO is undertaking breeding for improved varieties adaptable to the local conditions while the University of Nairobi is working to produce Biofix to inoculate seeds.

Common bean (*Phaseolus vulgaris L)*, also referred to as dry bean, is an annual leguminous plant that belongs to the genus, *Phaseolus,* with pinnately compound trifoliate large leaves. It is grown for its green leaves, green pods, and immature and/or dry seeds. The dry seeds are the ultimate economic part of the bean plant. Common bean is one of the most important

grain legumes in human diets in Africa (Katungi et al., 2009). It provides proteins, complex carbohydrates, and valuable micronutrients for more than 300 million people in the tropics (Katungi *et al.*, 2009). In many areas, common bean is the second most important source of calories after maize. Over 200 million people in SSA depend on the crop as a primary staple (CIAT, 2012). In Kenya common bean is widely grown and consumed particularly by medium and low-income households who are the majority in the country.

Annual consumption can reach 66 kg per person (CIAT, 2012). In terms of acreage, Kenya is the leading producer of common bean with an annual total production of 412, 381 tons in Africa followed by Uganda and then Tanzania (FAO, 2008). Common bean contributes Ksh 13.18 billion annually to the national economy and is a source of dietary protein, especially for the rural and urban poor (ICRISAT, 2013). Common beans consistently get a good price. For example, the price of 90 kg bag of dry beans was Ksh. 8529, Ksh. 7829, and Ksh. 6824 in Eldoret, Kisumu and Nairobi respectively in May 2014 (WFP, 2014).

The production of soybean and common bean is constrained by low soil fertility in many soils of SSA. Farmers therefore need to supplement the soils with inorganic fertilizers especially nitrogen and phosphatic fertilizers to improve on the crop productivity. Gitonga *et al.,* (2010) reported that soybeans grown by most Kenyan farmers receive no inoculants and little or no commercial nitrogen fertilizer. They largely depend on N fixation from natural nodulation by indigenous *Bradyrhizobia* populations.

2.2 Nitrogen and Phosphatic fertilizer use for food crops in Kenya

A huge amount of synthetic fertilizers are used to replenish soil N and P, resulting in high costs and increased environmental pollution (Singh, 2013). Inorganic N fertilizers are frequently used to supply the needed nitrogen to the farming system in both industrialized and non-industrialized nations. Kenyan farmers apply several types of fertilizer on their crops. The most popular type is Di-Ammonium Phosphate (DAP): more than 70 percent of the sample households use at least some DAP. They also use Calcium Ammonium Nitrate (19-33 percent), NPK (25-28 percent), and Urea (8-11 percent). Kenyan farmers apply DAP and Urea on cereal crops such as maize and apply CAN and NPK on cash crops such as tea, coffee, and sugarcane (Yamano & Arai, 2010).

In a review by Morris *et al*. (2007), they found fertilizer use to be unprofitable in many parts of Africa due to high prices and transportation costs. Like many other African countries, almost all fertilizer consumed in Kenya is imported (Ministry of Agriculture, 2012). This makes fertilizer prices particularly susceptible to swings in international commodity prices. Fertilizer prices for the African farmers are often high and food crop prices low. Agriculture in Kenya is predominantly small scale; most of the farmers cannot access fertilizers, amongst other inputs, due to high cost of credit, unavailability of the fertilizer at the right time and most of the farmers are unaware of the type of fertilizer to use at a certain plant growth stage (Bunde *et al*., 2014).

Duflo *et al*. (2010) observed that farmers in Kenya use sub optimal levels of fertilizer and that higher level of fertilizer use, including the official recommendations of the Ministry of Agriculture, are unprofitable for the average farmer. Thus, while fertilizer can be very profitable when used correctly, one reason why farmers may not use fertilizer is that the official recommendations are not adapted to their specific context (Dulfo *et al*., 2010).

Since nitrogen is commonly the most limiting plant nutrient in arable farming in the tropics and also the most expensive element as a mineral fertilizer, biological nitrogen fixation (BNF) holds great promise for smallholder farmers in sub-Saharan Africa. Legumes have been shown to biologically fix atmospheric nitrogen into plant usable. Exploiting this crop attribute may be an alternative to the expensive and unavailable chemical fertilizers especially to small holder farmers.

2.3 Biological nitrogen fixation in grain legumes

With the world's population projected to increase to 8.3 billion by 2025, and the accompanying demand for plant nutrient N, there will continue to be a need to increase the contribution of biological nitrogen fixation to food production (Paul, 2015). Biological nitrogen fixation is a process where atmospheric N_2 is reduced into NH_4^+ by a group of bacteria called rhizobia through symbiotic association with legumes. Legumes have traditionally been used in rotational cropping systems to improve soil fertility. The amount of nitrogen fixed by rhizobia varies with the yield level; the effectiveness of inoculation; the N obtained from the soil, either from decomposing organic matter or from residual N or the environmental conditions (Havlin *et al.,* 2014). The soil factors affecting BNF include excessive soil moisture, drought, soil acidity, P

deficiency, excess mineral N and deficiency of micronutrients such as molybdenum, cobalt and boron (Paul, 2015).

Rhizobia infect the roots of the host plant, which then initiate the development of root nodules where rhizobia fix atmospheric N_2 in exchange for photosynthetic C. Other N_2-fixing bacteria, such as *Frankia* spp., enter into the same type of symbiosis as rhizobia, but only with a small group of non-leguminous plant species. While there is a wide range of organisms and microbial-plant associations that are capable of fixing atmospheric N_2, the symbiotic relationship between rhizobia and legumes is responsible for contributing the largest amounts of fixed nitrogen to agriculture. Grain legumes contribute more than 20 million tons of fixed N to agriculture each year (Herridge *et al*., 2008). For example, in Brazil, rates of nitrogen fixation with soybeans under field conditions can exceed 300 kg of N ha^{-1}each year. Similar rates of nitrogen fixation (260 kg N ha^{-1}) have been recorded for soybean growing under optimal conditions in SSA (N2Africa, 2012). Realization of nitrogen fixation and increased crop production in agriculture is achieved through Rhizobia inoculation.

2.4 Rhizobia inoculation effects on biological nitrogen fixation and yield

Several reports have highlighted low fixation capability of *P. vulgaris* especially if symbiotic association is constrained by various factors including inefficient strains capable of initiating the N-fixation process. This constraint could be eased through seed or soil inoculation with the proper *Rhizobium* bacteria before or at planting to facilitate N-fixation. The goal of inoculation is to introduce a large number of viable host-specific Rhizobia in order to increase infection rates. Inoculation can provide a strain that competes with native bacteria for infection sites on the host, thereby increasing biological nitrogen fixation. Thus, the technology of inoculating legumes is a way to increase the population of the host-specific symbiont in the soil to exploit the natural biological nitrogen fixation potential of the system (Mabruk & Belhadj, 2012). Benefits from BNF can be increased in several ways, including breeding legumes for nitrogen fixation, introducing new legumes into cropping systems or using improved rhizobial strains (Mungai & Karubiu, 2010).

Rhizobial bacteria with billions (10^9) of cells per gram of soil is the minimum a soil should have for effective BNF (Woomer *et al*., 2011). However, Abaidoo *et al*. (2007) showed that *Bradyrhizobia* species populations in many African soils are much lower than this threshold

and, consequently, unlikely to support the magnitude of BNF required for the enhanced growth and performance of soybean. This implies the need for soybean inoculation for the SSA farmers in order to obtain maximum yields as they cannot rely on the responses from the low population of indigenous rhizobia. Inoculation of legumes is especially critical when effective Rhizobia are absent in the soil, or when population densities are low (Catroux *et al*., 2001). In this case, the addition of an inoculant with the host-specific Rhizobia can increase the BNF of the legume.

Mbugua *et al.* (2010) did a research on the beans' ability to fix nitrogen and their argument was that inoculation of seed beans with appropriate Rhizobia strains for enhanced nitrogen fixation provides a more economical and sustainable alternative to the application of nitrogenous fertilizers at Thika, Maragua and Kirinyaga in Kenya. However, a study by Yusuf *et al*. (2012) showed that there was no significant difference in nodulation and shoot dry matter yield between inoculated and uninoculated plants. Instead, the uninoculated plants produced significantly higher pod (20%) and haulm (28%) yields than the average yield of the inoculated plants due to competiveness of the indigenous rhizobia strains in the study soil.

2.5 Competition between native and introduced rhizobia strains

The most studied legume-rhizobia relationship is the one involving soybeans. Studies have shown that soybean has potential for soil fertility improvement through BNF. A study by Muhammad (2010) showed that inoculation generally increased soybean growth and yields above those of the control (uninoculated). Herridge *et al*. (2008) working on 'promiscuous' soybeans reported that the inoculation responses on different soils were large, up to 40-fold increases in nodulation and 87% and 51% increases in shoot dry matter and grain yield, respectively, in sites which were new to soybean. Inoculation responses were recorded at ferralsols sites, both of which were on old soybean land. Inoculation increased total N fixed by as much as 400 % (Herridge *et al.,* 2008). In addition to the good effectiveness of the rhizobial products, research has shown high competitiveness of such strains of up to 100 % nodule occupancy by the inoculated strains over the indigenous strains (Thuita *et al*., 2012). Therefore, commercial products produced elsewhere can be an important source of effective strains for use in areas where soybean is being introduced or where low populations of indigenous rhizobia hinder BNF (Thuita *et al*., 2012). Chemining'wa *et al.* (2011) reported that relative to soil inoculations, inoculation with commercial inoculant improved nodulation for cowpea and shoot

biomass for both cowpea and common bean and their finding suggests that the commercial strains are more efficient in fixing N than indigenous rhizobia.

Mungai & Karubiu (2010) reported that rhizobia isolates from Njoro had comparable symbiotic effectiveness to commercial inoculants and should be tested further using other bean varieties to assess their potential. They carried out a research on the effectiveness of native rhizobia from Njoro area and commercial inoculant (BIOFIX) and compared their effectiveness on the nodulation of common beans. The results from this study confirm that the native rhizobia can be more effective and infective than commercial or introduced rhizobia. Wasike *et al*. (2009) reported nodulation of promiscuous soybean varieties by indigenous rhizobia in Western and Eastern Kenya. There is potential for the use of indigenous strains as inoculants to replace strain USDA 110 currently recommended for the inoculation of soybean production in Kenya (Wasike *et al.,* 2009). Gitonga *et al.*(2010), also reported that seed yield from inoculated soybeans was not significantly higher than that from the uninoculated soybeans, showing a degree of competitiveness among the introduced rhizobia strain and the indigenous rhizobia population. Musandu & Ogendo (2001) also found out that rhizobia inoculation did not influence any of the measured variables in the beans variety under study.

The variable responses to inoculation may be attributed to variation in soil characteristics in terms of population of indigenous rhizobia strains. Some soils have high populations of native rhizobia specific for the intended legume. Native rhizobia may be in sufficient numbers to nodulate both native and introduced legumes. If crop N requirement is provided by the native rhizobia, inoculation may not increase yield. Yahyaabadi, (2008) evaluated the potential of some rhizobia bacteria for nitrogen fixation and other nutrients uptake and concluded that some strains of native bacteria are effective. Sufficient native rhizobia occur when the legume crops are frequently grown on the same fields or with previous legume inoculation. Often, the native rhizobia are low in numbers, are the wrong species or strain for the introduced legume, or are not efficient nitrogen fixers. It is, therefore, recommended to artificially inoculate the seeds with an appropriate strain of rhizobia.

Phosphorous deficiency has been reported to hinder optimum biological nitrogen fixation by legumes. Sufficient phosphorus levels are required to enhance growth of plant organs and promote nodulation and early maturity (Kamara *et al*., 2010). In addition to the P demand of the

host plant, nodules require higher amounts of P and energy than other plant tissues (Mortimer *et al*., 2008). Thus, nodule dry weight is greatly reduced by phosphorus deficiency. The use of microbial inoculants possessing P-solubilizing activities in agricultural soils is considered as an environmental-friendly alternative to further applications of chemical based P fertilizers.

2.6 Solubilization of phosphorous by phosphate solubilizing bacteria

The use of plant growth-promoting rhizobacteria (PGPR), to increase soil fertility and improve growth and yield of crops is a significant alternative to chemical fertilizers in sustainable agriculture (Olivera *et al*., 2014). Beneficial rhizobacteria can improve seed germination, root and shoot growth, nutrient uptake, and plant stress tolerance (Lugtenberg & Kamilova, 2009). Phosphate solubilizing bacteria (PSB) through various mechanisms are able to convert insoluble inorganic and organic soil P into plant available forms (Sharma *et al*., 2013). PSB can be very effective and have potential for enriching the soil P content and enhancing crop yield (Singh, 2013).

Several phosphate solubilizing microorganisms (PSMs) are now recorded to convert the insoluble form of phosphorus to soluble form through acidification, secretion of organic acids or protons (Richardson *et al*., 2009). Ghanem & Abbas (2009) observed an increase in plant height, number of branches, number of pods, grain weight, and eventually higher seed and straw yields in mung bean plants after inoculation of *B. megaterium* in salt-affected soils. The inoculation of plants with selected PSB to increase native population can mobilize P from poorly available sources and therefore improve plant nutrition (Richardson *et al*., 2009). Fernandez *et al*. (2007) reported after field experiments on soybean that PSB significantly increased shoot dry weight, root nodules and their fresh and dry weight and seed yield. Study by Qureshi *et al*. (2012) clearly demonstrated that phosphate solubilising bacteria can play an essential role in growth and yield of cotton and increased more Nitrogen and P content in plants and enhanced available P in soil.

Verma *et al*. (2012) also indicated that seed treatment with two strains of PSB (*Pseudomonas fluoresens* and *Bacillus megaterium*) enhanced the root length of chickpea. Greenhouse and field studies with PGPR strains have demonstrated enhanced nodulation and nitrogen fixation in common bean (Figueiredo *et al.*, 2008). So, PSB has enormous potential in biofertilizer formulations to be exploited in increasing crop yields by increasing fixed P in the soil, as well as by making good use of natural phosphate reserves (Hayat e*t al*., 2012). The

activities of PSB are affected by different soil parameters including soil fertility, temperature, moisture, organic matter, and soil physical properties (Salimpour *et al*., 2010).

2.7 Effect of co-inoculation of rhizobia strains and phosphate solubilizing bacteria

Co-inoculation with N_2-fixing and P- solubilizing microbe has been reported to be more effective approach for providing balanced plant nutrition (Qureshi *et al*., 2011). Co-inoculation studies with rhizobia and plant growth promoting bacteria have shown increased plant nodulation and N_2 fixation (Figueiredo *et al*., 2008). Co-inoculation of nodulating forming rhizobium with PSB results in enhanced legume yield (Qureshi *et al*., 2011; Morel *et al*., 2012). Root and shoot biomass production of soybean also seems to be favorably affected by mix culture of inoculums treatment whether used alone or in combination with phosphorus and responses to Rhizobial inoculation are often observed (Fatima *et al*., 2006). Bai *et al*. (2002) demonstrated that three bacteria isolated from the surface disinfested from soybean nodules when inoculated together with rhizobia increased nodulation and plant weight compared to those receiving just rhizobia. Additionally, combined inoculation of *Rhizobium* and phosphate-solubilizing bacteria (PSB) in lentils has been reported to enhance plant growth (Saini & Khana, 2012). Co-inoculation of phosphate solubilizing bacteria (PSB) *Pseudomonas* sp. and *B. japonicum* (TAL 379) significantly increased nodulation, plant total N, P uptake, seed yield and yield components of soybean over negative control and chemical fertilizers (Argaw, 2012). Anandaraj and Delapierre (2010) reported that inoculation of green gram with the composite inoculants of *Rhizobium* sp., *Pseudomonas fluorescens* and *Bacillus megaterium* were highly beneficial in enhancing the plant growth and yield of green gram besides effecting a reduction in the cost of inorganic fertilizers. Co-inoculation of rhizobia with *Bacillus*, specifically *Bacillus thuringifensis*, *Bacillus megatrium* and *Bacillus cereus* significantly promotes nodulation, plant growth and grain yield of soybean (Mishra e*t al*., 2009).

References

Abaidoo, R.C, Keyser, H.H., Singleton, P.W., Dashiell, K.E, & Sanginga, N. (2007). Population size, distribution and characteristics of indigenous *Bradyrhizobia* spp. that nodulate TGx soybean genotype in Africa. *Applied Soil Ecology* 35(1): 57-67.

Anandaraj, B. & Delapierre, A. L. R. (2010). Studies in influence of bioinoculants (*Pseudomonas fluorescens, Rhizobium* sp., *Bacillus megaterium*) in green gram. *Journal of Bioscience and Technology* 1(2): 95-99.

Argaw, A. (2012). Evaluation of co-inoculation of *Bradyrhizobium japonicum* and phosphate solubilizing *Pseudomonas* spp. Effect on soybean (*Glycine max* L. Merr.) in Assossa Area. *Journal of Agricultural Science and Technology* 14: 213-224.

Bunde, A. O., Kibet, K., Daphen, O. O., Mugo, S. W. & Chomboi. K. C. (2014). Impact of Fertilizer Input Subsidy on Maize Production in Nandi North District, Kenya. *International Journal of Sciences: Basic and Applied Research* 520-540.

Catroux, G., Hartmann, A. & Revellin, C. (2001). Trends in rhizobial inoculant production and use. *Plant and Soil* 230: 21-30.

Chemining'wa, G. N., Theuri, S. W. & Muthomi, J. W. (2011). Abundance of indigenous rhizobia nodulating cowpea and common beans in Central Kenyan Soils. *African Journal of Horticultural Science 5*: 92-97.

CIAT *BEAN PROGRAM*. (2012). Bean. Retrieved From: http:/ciat.cgiar.org/bean-research (Accessed on 28th June 2013).

CIAT (2013). Tropical legume farming in Kenya. A bulletin of the tropical legumes. Retrieved from: http://www.icrisat.org/TropicallegumesII/Bulletn-of-Tropical-Legumes.htm. (Accessed on 15th August 2015)

Collombet, R. N. (2013). Investigating soybean market situation in Western Kenya: constraints and opportunities for smallholder producers. Thesis. Wageningen University.

Duflo, E., Kremer, M. & Robinson, J. (2010). Rates of return to fertilizer: Evidence from field experiments in Kenya. Retrieved from: http://www.povertyactionlab.org/evaluation/rates-return-fertilizer-evidence-field-experiments-kenya (Accessed on 15th October, 2015).

FAO (2008). Production data. Retrieved from: http://faostat.fao.org/faostat. (Accessed on 5th May, 2013)

FAO. (2012). Crops. Retrieved from: http://faostat.fao.org/site/567/default.aspx#ancor. (Accessed on 5th May, 2013).

Fatima, Z., Muhammad, Z., & Fayyaz, C. (2006). Effects of r*hizobium* strains and phosphorus on growth of soy bean (*Glycin Max*) and survival of *rhizobium* and P solubilizing bacteria. *Pakistan Journal of Botany 38* (2): 459-464.

Fernández, L. A., Zalba, P., Gómez, M. A. & Sagardoy, M.A. (2007). Phosphate-solubilization activity of bacterial strains in soil and their effect on soybean growth under greenhouse conditions. *Biology and Fertility of Soils* 43:805-809.

Figueiredo, M. V. B., Martinez, C. R., Burity, H. A. & Chanway, C. P. (2008). Plant growth-promoting rhizobacteria for improving nodulation and nitrogen fixation in the common bean (*Phaseolus vulgaris* L.). *World Journal of Microbiology and Biotechnology* 36: 112-126.

Ghanem, K. H. M. & Abbas, E. E. (2009). Improvement of mung bean growth and productivity in salinity-affected soil after seed inoculation with phosphate-dissolving bacteria. *African Crop Science Conference Proceedings* 9:385-389.

Gitonga, M.N., Gatheri, G.W., Cheruiyot, R., Gitonga, N.M. & Maingi, J.M. (2010). Nodulation and nitrogen fixation in promiscuous and non-promiscuous soybean (*Glycine Max* (L.) Merrill) varieties in Eastern Kenya. *Journal of Tropical Microbiology and Biotechnology*, 6, *3-8.*

Havlin, J.L, Tisdale, S.L, Beaton, J.D. & Werner, L.N. (2014). *Soil Fertility and Fertilizers. An Introduction to Nutrient Management*. Eighth Edition. Pearson Education, Inc, Publishing. Prentice Hall. pp 102-105.

Hayat, R., Ahmed, I. & Sheirdi, R. A. (2012). *An Overview of Plant Growth Promoting Rhizobacteria (PGPR) for Sustainable Agriculture*. In crop production for agricultural improvement. Springer pp 557-579.

Herridge, D. F., Peoples, M. B. & Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil* 311: 1-18.

ICRISAT (2013). Tropical legume farming in Kenya. Retrieved from: http://www.icrisat.org/TropicallegumesII/ Bulletin-of-Tropical-Legumes.htm. (Accessed on 3rd October, 2015).

Kamara, A.Y., Kwari, J., Ekeleme, F., Omoigui, L. & Abaidoo, R. (2010). Effect of phosphorus application and soybean cultivar on grain and dry matter yield of subsequent maize in the tropical savannas of north-eastern Nigeria. *African Journal of Biotechnology* 7(15): 27-43.

Katungi, E., Farrow, A., Chianu, J., Sperling, L. & Beebe, S. (2009). Common Bean in Eastern and Southern Africa: A Situation and Outlook Analysis. Retrieved from http://www.icrisat.org/what-we-do/impi/projects/tl2-publications/regional-situation-outlook-reports/rso-common-bean-esa.pdf (Accessed on 28th April 2013).

Mabrouk, Y. & Belhadj, O. (2012). Enhancing the biological nitrogen fixation of leguminous crops grown under stressed environments. *African Journal of Biotechnology* 11(48): 10809-10815.

Mbugua, G.W., Wachiuri, S.M., Karoga, J. & Kimamira, J. (2010). Effects of commercial rhizobia strain inoculants and triple superphosphate fertilizer on yield of new dry bean lines in central Kenya. *Proceedings of the 12th KARI Biennial Scientific conference*, 2010, pp 370-379.

Ministry of Agriculture. (2012). Economic review of agriculture: 2012. Nairobi, Kenya.

Mishra P. K., Mishra, S., Selvakumar, G., Bisht J. K., Kundu, S., Gupta, H. S. (2009). Co-inoculation of *Bacillus thuringeinsis*-KR1 with *Rhizobium leguminosarum* enhances plant growth and nodulation of pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.). *World Journal of Microbiology and Biotechnology* 25:753-761.

Morel, M. A., Braña, V. & Castro-Sowinski, S. (2012). Legume crops, importance and use of bacterial inoculation to increase production. *Crop Plant* 12: 218-240.

Morris, M., Kelly, V., Kopicki, R. J., & Byerlee, Derek. (2007). *Fertilizer Use in African Agriculture: Lessons Learned and Good Practice Guidelines*. Washington DC: World Bank

Mortimer P. E., Pérez-Fernández, M. A. & Valentine A. J. (2008). The role of arbuscular mycorrhizal colonization in the carbon and nutrient economy of the tripartite symbiosis with nodulated *Phaseolus vulgaris. Soil Biology and Biochemistry* 40:1019–1027.

Muhammad, A. (2010). Response of a promiscuous soybean cultivar to rhizobial inoculation and phosphorus in Nigeria's Southern Guinea Savanna Alfisol Nigerian. *Journal of Basic and Applied Science* 18(1): 79-82.

Mungai, N. W. & Karubiu, N. M. (2010). Effectiveness of rhizobia isolates from njoro soils (Kenya) and commercial inoculants in nodulation of common beans (*Phaseolus vulgaris*). *Journal of Agriculture, Science and Technology* 12(1): 47-59.

Musandu, A. A. O. & Ogendo, O. J. (2001). Response of common bean to rhizobia inoculation and fertilizers. *The Journal of Food Technology in Africa* 6(4): 121-125.

N2ARICA (2012). Quantifying the impact of the N2Africa project on biological nitrogen fixation. Retrieved from: http://www.n2africa.org/sites/ (Accessed on 17th June 2013).

Olivera, S., Delić, D., Kuzmanović, D., Protić, N., Rasulić, N. & Knežević-vukčević, J. (2014). Growth and nutrient uptake in oat and barley plants as affected by Rhizobacteria. *Romanian Biotechnological Letters* 19(3): 72-77.

Paul, E.A. (2015). *Soil Microbiology, Ecology, and Biochemistry*, 4th Edition. Academic Press, Boston.

Qureshi, M. A., Shakir, M. A., Iqbal, A., Akhtar, N. & Khan, A. (2011). Co-inoculation of phosphate solubilizing bacteria and rhizobia for improving growth and yield of mungbean (*Vigna radiata* L.). *The Journal of Animal & Plant Sciences* 21(3): 491-497.

Qureshi, M. A. Ahmad Z. A., Akhtar, N. Iqbal, A. Mujeeb, F. and Shakir. M. A. (2012). Role of phosphate solubilizing bacteria (PSB) in enhancing P availability and promoting cotton growth. *The Journal of Animal & Plant Sciences* 22(1): 204-210.

Richardson, A. E., Barea, J. M., McNeill A. M. & Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil* 321:305–339.

Saini, P. & Khana, V. (2012). Evaluation of native rhizobacteria as promoters of plant growth for increased yields in lentils (*Lens culinaris*). *Recent research in science and technology* 4(4): 5-9.

Salimpour, S., Khavazi, K., Nadian, H., Basharati, H. & Miransari, M. (2010). Enhancing phosphorous availability to canola (*Brassica napus* L.) using P solubilizing and sulfur oxidizing bacteria. *Australian Journal of Crop Science* 4(5): 330-334.

Sharma S.B., Sayyed, R.Z., Trivedi, M.H. & Gobit, T.A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorous deficiency in agricultural soils. *SpringerPlus* 2: 587-601.

Singh J. S. (2013). Plant growth promoting rhizobacteria. Potential microbes for sustainable agriculture. *Resonance* 275-281.

Thuita, M., Pypers, P., Herrmann, L., Okalebo, J.R., Othieno, C., Muema, E. & Lesueur, D. (2012). Commercial rhizobial inoculants significantly enhance growth and nitrogen fixation of a promiscuous soybean variety in Kenyan soils. *Biology and Fertility of Soils* 48: 87–96.

Wasike, V., Vanlauwe, B., Mungai, N., Wachira, F., Mumera, L., Sanginga, N., Herrmann, L., Mburu, H, Malala, P., Wasilwa, L. & Lesueur, D. (2009). The Competitiveness of indigenous *Bradyrhizobium Spp* and *Bradyrhizobium Elkanii* strains on promiscuous and specific soybean varieties in Kenyan soils. *Plant and soil* 322:25-35.

World Bank, D.P.G., 2013. Commodity price forecast. World Bank.

Woomer, P., Bala, A. & Abaido, R. (2011). Rhizobia *Strain Isolation And Characterization Protocol.* Retrieved From N2Africa:www.N2Africa.org/. (Accessed on 3rd July 2013).

WFP (2014). Kenya Market watch. Bulletin 2014 Issue.

Yahyaabadi, M. (2008). Evaluation of nitrogen fixation potential and nutrients uptaking in some common bean symbiosis bacteria. *Proceeding of 10th Iranian Congress of Crop Production and Plant Breeding. 19-21 August*, 2008. *Karaj*. *Iran*.p:75.

Yamano, T. & Arai, A. (2010). Fertilizer policies, price, and application in East Africa GRIPS Discussion Paper 10-24.

Yusuf, A. A., Jemo, M. and Abaidoo, R. C. (2012). Effects of rhizobium inoculation and phosphorus sources on the productivity of groundnut in Samaru, northern Guinea savanna of Nigeria. *In*: M.K.A. Adeboye, A.J. Odofin, A.O. Osunde, A. Bala and S.O. Ojeniyi (Eds). *Soil resources management, gobal climate change and food security*. *Proceedings of the 35th Annual Conference of the Soil Science Society of Nigeria*. p. 191 - 198.

CHAPTER THREE: EFFICACY ASSESSMENT OF COMMERCIAL RHIZOBIA INOCULANTS ON COMMON BEAN AND SOYBEAN GROWTH

3.1 Abstract

The use of inoculants offers an alternative to the use of chemical fertilizers. However, the quality and efficacy of these inoculants is critical to realize the benefit of inoculation. This study tested the quality of 3 inoculants (Legumefix - soybean, Biofix-soybean and Biofix – common bean) available in the Kenyan market and evaluated the response of soybean and common bean to legume inoculants. Seeds were inoculated at the recommended rate at planting. Plants were grown under greenhouse conditions in a completely randomized design. Non-inoculated seeds (control) were also planted and plants grown under the same conditions. At mid podding, nodule number and fresh weight per plant, above ground biomass and root dry biomass were determined. Biologically fixed nitrogen was determined using the nitrogen difference method and the symbiotic efficiency of the rhizobia also determined. The quality assessment results showed variable quality of the inoculants in terms of the number of viable cell count and the number of microorganisms in the product. Nodule number and fresh weight per plant were higher with inoculant application for soybean and common bean relative to the controls. Biological nitrogen fixation was significantly improved as a result of inoculation. High fertility soil resulted in higher nodulation in both legumes. In common bean, AFR 708 variety responded better to inoculation than the GLP 2 and Lyamungu-85 variety while in soybean the early maturing SB19 variety resulted in higher nodulation and growth. The results on quality testing of the inoculants suggest that the inoculants in the market are varied in terms of quality and therefore need for manufacturing companies to have a quality control system in the production process. The response to inoculation also depends on the variety being grown and the soil fertility status. Further work under field conditions is recommended to confirm these findings.

Keywords: Biological nitrogen fixation, Biofix, common bean, inoculant, soybean, Legumefix, Rhizobia.

3.2 Introduction

A major problem facing Kenya's smallholder farmers is declining soil fertility as a result of continuous cropping without sufficient replenishment of soil nutrients. This has led to decreasing food productivity against an increasing population. Nitrogen is the most commonly deficient crop nutrient. The demand for nitrogen in a deficient soil is normally achieved by the use of chemical fertilizers. However, the high cost of mineral nitrogen fertilizers and their un-availability at the required time are the two major constraints responsible for low fertilizer nitrogen inputs (Yakubu *et al*., 2010). Legume improves the soil fertility through its ability to fix atmospheric nitrogen in association with rhizobia. This emphasizes the importance of developing alternative means by use of beneficial bacteria that are sustainable, environmentally friendly and affordable. As such, biofertilizers containing rhizobia are an essential component of an integrated soil fertility management strategy (Uribe *et al*., 2012).

Biological nitrogen fixation and grain yields of legumes are normally increased when inoculated with effective and efficient strains of *Rhizobium* (Zarei *et al*., 2012). *Rhizobium* inoculation in legumes stimulates growth and increases yields and is an alternative source to the expensive commercial nitrogen fertilizers (Bambara and Ndakikemi, 2009; Morad *et al*., 2013). Lesueur *et al.* (2012) reported that the utilization of effective good-quality rhizobial inoculants by farmers has a real potential to improve legume yields in unfertile soils requiring high applications of mineral fertilizers. They tested effective soybean commercial inoculants in different locations in Kenya and found out that application of the rhizobial inoculants significantly increased soybean yields in about 75% of the farms evaluated.

Masso *et al*. (2014) reported a lack of enough background information to substantiate the potential benefits of biofertilizers for resource-poor small scale farmers in Sub-Saharan Africa and therefore the need for continued evaluation (Jefwa e*t al*., 2014). Also, most of the biofertilizers fail to meet the quality standards acclaimed by the manufacturers (Mathu *et al*., 2012; COMPRO-II, 2013a). Otieno *et al*. (2009) conducted a study to investigate the response of grain legume to inoculation. Their results indicated an increasing trend of number of nodules and nodular weight, seed yield and growth parameters with seed inoculation. Studies were conducted by Giri & Joshi (2010) through application of rhizobium as a biofertilizer on nodule formation and growth of chickpea and to evaluate the efficiency of seed inoculation for nitrogen

fixation. They reported a 10.83% and 14.06% increase in total shoot and root length respectively and 9.0% more germination as compared with control.

However, inoculation of legumes with rhizobial inoculants does not show positive response to nodulation and crop growth at all instances because of a variety of biotic or abiotic factors that affect nodulation of plants (Aung *et al*., 2013). For example, Mungai & Karubiu (2010) reported unresponsiveness of common bean to inoculation with commercial rhizobial inoculant (Biofix) in terms of shoot and root dry matter and biological nitrogen fixation. Legume inoculants can sometimes fail because of poor quality, poor survival during storage and death on the legume seed after inoculation. Some indigenous isolates have been found to be as good or superior in nitrogen fixation effectiveness to commercial inoculant strains under greenhouse conditions (Mungai & Karubiu, 2010). Mweetwa *et al*. (2014) also noted a lack of commercial rhizobia inoculation responses in cowpea, soybean and groundnuts.

The uncertain performance of *Rhizobium* inoculants may explain the limited farmer adoption despite their potential to reduce the mineral fertilizer requirement, and reduce cost of production. Smallholders may benefit from good quality products that are correctly applied to the appropriate crop under appropriate soil and crop management (Jefwa *et al*., 2014). There is therefore need to validate the quality and the efficacy of these products under different soil conditions to add knowledge on profitable technologies for adoption to the farmers. The objectives of this study were twofold:

i. To test the quality of three commercial inoculants (Biofix-soybean, Biofix- common bean and Legumefix-Soybean) through cultural and biochemical characterization
ii. To test the effectiveness of Biofix and Legumefix inoculants on common bean and soybean growth in two different soil types.

3.3 Materials and Methods

Three commercial products were tested for their quality in the laboratory and efficacy in greenhouse using two different soil types.

3.3.1 Commercial Rhizobia inoculants

Commercial inoculants Biofix-Soybean, Biofix-Common bean from MEA Limited and Legumefix- Soybean from United Kingdom were purchased. Biofix is the most available and commonly used inoculant by farmers in Kenya and although legumefix is not produced locally,

some early studies have shown Legumefix- soybean performing better than Biofix- soybean in other part of the country (COMPRO II, 2013b). Three samples of Biofix from three different batches each were purchased for the characterization purpose for both the Biofix-Soybean and Biofix-Common bean. The Biofix packets purchased were 50 g pack sizes and the different batches were used to study the consistency of quality during production of the inoculant. For the Legumefix, one pack was used for the laboratory quality test to confirm the earlier findings by Atieno *et al*. (2012).

3.3.2 Culturing on media and plate counting

The procedures in this work on isolation, characterization, testing for effectiveness of bacteria was done following the guidelines from Somasegaran & Hoben (1994) and Woomer *et al.* (2011a). The rhizobia in the inoculants were cultured on Yeast Extract Mannitol Agar (YEMA). YEMA is a slightly selective Mannitol-based medium that favours the growth of rhizobia (Mannitol ($10gL^{-1}$), K_2HPO_4 ($0.5\ gL^{-1}$), $MgSO_4.7\ H_2O$ ($0.2\ gL^{-1}$), Nacl ($0.1\ gL^{-1}$), Yeast Extract ($1.0\ gL^{-1}$), Agar ($15\ gL^{-1}$) and pH adjusted to 6.8 and the YEMA cultures were placed in an incubator at 28°C.

The 19 samples (nine of Biofix-common bean, nine of Biofix-soybean and one of Legumefix) were serially diluted by weighing 1 g of the inoculant into 9 ml of sterile physiological saline (9 g sodium chloride per litter of sterile water) up to 10^{-8} and 0.1 ml of each dilution was poured and evenly spread on the culture media. The plates were incubated at the optimum temperature of 28°C. Different bacterial colonies that grew on the same plate were separated and re-cultured by streaking in new YEMA plates and incubated at the optimal temperature. The isolated colonies were purified three times on YEMA media.

Different types of colonies formed by the bacteria present in the product were counted separately using a counter and the initial concentration of each calculated. Only the plates with about 30 to 300 isolated colonies were selected. The counting was done on pure plate culture after restreaking. The concentration of the bacteria in the initial suspension was calculated as follows (Olsen *et al.*, 1996):

$M = n*V/C$

M: concentration of the initial suspension (number of cells per ml)

n: number of isolated colonies counted on the selected plate(s)

C: dilution (sum of dilutions / number of selected plates)

V: spread volume (0.1 ml * number of selected plates)

3.3.3 Determining gram stain reactions of various bacteria

Thin smears of the various bacterial colonies were made and fixed by heat. Then the smears were stained with crystal violet for 30 seconds, washed lightly with water and flooded with iodine solution. After 30 seconds, the iodine solution was drained and the smear decolorized with 95% ethanol for 10 seconds. Then it was counter stained with safranin 30 seconds. Finally, it was washed with water, air dried and the preparation observed under an oil immersion. The Gram stain procedure separates bacteria into two groups: Gram-positive and Gram-negative organisms. Gram-positive organisms retain the crystal violet stain after treating with iodine and washing with alcohol, and appear dark violet after staining. Gram-negative organisms lose the violet stain after treating with iodine and washing with alcohol but retain the red coloration of the counter-stain, safranin.

3.4 Effectiveness test of commercial products in the greenhouse

3.4.1 Plant material and soil

The test crops used were two soybean varieties; Nyala and TGx1740-2F (SB19) and three common beans varieties; AFR 708 (Chelalang), Lyamungu-85(Tasha) and GLP 2 (Rosecoco). Nyala is an early maturing soybean variety with an average on-farm yield of 700 $kgha^{-1}$with large grain size. It can be intercropped with other crops and nodulates with specific strains of rhizobia, while SB19 is a medium maturing variety with promiscuous nodulation and has high grain and biomass yield with an average yield of 900 $kgha^{-1}$ (ICRISAT, 2013). AFR 708 and Lyamungu-85 varieties are newly released common bean varieties by Egerton University with special attributes of being high yielding, pest and disease resistant. AFR 708 is reported to yield more than the local checks (Njoka *et al*., 2009), that is, GLP 2 and Mwezi Moja (GLP 1004). GLP 2 is a high yielding variety suitable for medium altitudes while Mwezi moja is a medium yielder and suitable in dry areas (KALRO, 2008). Maize (H614D) was included as a reference crop for biological nitrogen fixation (BNF) estimation. Nitisol (Chuka- S00°20.472' E037°41.691') and Andosol (Njoro- S00°23.723' E037°35.043') soil were collected from the 0-20 cm top layer, air-dried, sieved to pass 2 mm and thoroughly homogenized. Moisture content (MC) at field capacity (FC) was determined to standardize water addition in the pot trials as

described by Somasegaran & Hoben, (1994). The Andosol FC water content was determined to be 483 ml while that of the Nitisol was found to be 520 ml.

Soil samples (20 cm depth) were collected at the beginning of the study from the two sites for characterization of initial soil chemical properties. The soils were air-dried, prepared and analysed using standard procedures as described by Okalebo *et al*. (2002). Soil pH was determined using a glass electrode pH meter at 1:2.5 soil/water ratio. Available P was extracted using the Mehlich-3 and determined using the ammonium vanadate method and amount determined using a spectrophotometer. Organic carbon was determined by Walkley and Black sulfuric acid– dichromate digestion followed by back titration with ferrous ammonium sulfate whereas nitrogen was determined using the Kjeldahl method.

The common bean experiment consisted of three treatment; T1= Biofix, T2= Di-ammonium phosphate (DAP) and T3= Control (No fertilizer, no inoculation), while the soybean consisted of four treatments; Biofix, Legumefix, DAP and Control (No fertilizer, no inoculation). Detailed treatment structure is shown in Table 3.1 and 3.2. The experiment was laid out in a completely randomized design (CRD) with three replicates in the greenhouse and the pots were rotated regularly on the benches to minimize the effect of shading.

Table 3.1: Treatment structure for testing commercial products in common bean

Factor	**treatment**	**Description of treatment**
Variety	3	AFR 708, Lyamungu-85 and Rosecocco
Soil type	2	Vitric Andosol and Rhodic Nitisol
Inoculation	3	Biofix, 1 negative(no inoculation, no Nitrogen) + 1 positive control (no inoculation, + nitrogen source)
Reps	3	
No. of pots	3×2×3×3=**54**	

Table 3.2: Treatment structure for testing commercial products in soybean

Factor	treatments	Description of treatments
Variety	2	Nyala and SB 19
Soil type	2	Vitric Andosol and Rhodic Nitisol
Inoculation	4	Biofix, Legumefix, 1 negative (no inoculation, no nitrogen) + 1 positive control (No inoculation, + Nitrogen source)
Reps	3	
No. of pots	2×2×4×3=**48**	

Maize for BNF estimation (2 soils × 1 variety × 3 replicates) = 6 pots

Total number of pots 48 + 6 = 54 pots

3.4.2 Soil preparation and product application

Pot volume used was 5.3 L (15.0 cm inner diameter and 30 cm length), and contained different heights of soil for Njoro and Chuka soil respectively. Each pot was filled with 4 kg of soil. The soils have different bulk densities and accounted for the different heights of soil-filled tubes and this was done by putting the same amount of soil (4 kg) of the two soils but packing them to different heights to ensure a similar bulk density. PVC tubes were closed at the bottom using a nylon mesh, and placed on plastic plates.

Four grams of agricultural lime was applied to the pots containing the Nitisol one week before planting which is equivalent to 2t ha^{-1} lime required to raise the pH of such soil (Kisinyo *et al*., 2014) in order to increase the efficiency of inoculation. Biofix and Legumefix were applied based on manufacturer's recommendations of 50 g of the inoculant to 15 kg of soybean and 10 kg for common bean with large sized seeds. For the greenhouse experiment, 100 g of soybean was coated with 0.3 g of the inoculant while 0.5 g of the inoculant was used to coat 100 g of the common bean. Immediately after coating, the seeds were spread on paper and allowed to dry in a shady place. For the positive control, common beans received 12.2 kg Nha^{-1} (DAP at 67.5 kg ha^{-1}) to simulate farmers' practices (Mungai & Karibiu, 2010) with each pot receiving 0.135 g while soybean received 22.5 kg N ha^{-1} (DAP at 125 kg ha^{-1}) recommended rate (KALRO, 2006) at 0.25g per pot.

3.4.3 Planting, nutrient addition, thinning and water addition

Seeds were surface-sterilized by soaking in 3.5% NaOCl solution for 5 minutes and then thoroughly washed with distilled water. Three healthy seeds of uniform size were then planted per pot, and thinned to one plant per pot of comparable height and vigour at 7 d after planting. Uninoculated pots were planted before those with inoculated soybeans to avoid contamination during planting. Basal nutrients were applied as minus-N solutions (750 mg K, 270 mg Ca, 165 mg Mg, 60 mg S, 36 mg Mn, 1.5 mg Zn, 0.6 mg Cu, 0.9 mg B, 0.15 mg Mo and 0.15 mg Co pot^{-1}) (Somasegaran & Hoben, 1994). Stock solutions were diluted in 20 L of distilled water and 10 ml added to plants every two days. The pots were watered regularly to maintain the soil at field capacity. The watering was done by taking three representative pots from each of the two soils and weighing to determine the amount of water (average of the three) to bring the soil water content back to FC.

3.5 Data collection

3.5.1 Determination of nodule number and nodule weight

At mid podding, plants were carefully uprooted from the pots and placed on sieves to avoid loss of nodules during cleaning. The soil was then gently washed off the roots under a stream of running tap water. The nodules were then carefully removed from the roots, counted and weighed.

3.5.2 Determination of biomass yield, biologically fixed nitrogen and symbiotic efficiency

The above ground and root biomass were determined at mid-podding after drying to a constant weight at 65 ºC. Total nitrogen fixed was determined using the Nitrogen Difference Method as described by Unkovich *et al*. (2008). In this method, legumes in all the treatments were grown under greenhouse conditions alongside the non N fixing reference crop, maize (Abdul-Latif, 2013). At mid podding, percent tissue N was determined in all plants using the Kjeldahl method (Bremner & Mulvaney, 1982). The percent nitrogen fixed was then determined by calculating the difference between the N in the legumes and that in the reference crop. The symbiotic efficiency of the inoculation treatments was obtained by comparing the nitrogen concentration in the inoculant treatment with the nitrogen content in the mineral N application treatment.

3.6 Data Analyses

Data were analysed using SAS Statistical Package Version 9.3 (SAS 2010). To determine the effects due to inoculation, analysis of variance at 95% confidence limit was done and means separated using the least significance difference (LSD) test.

Statistical model:

$\mathbf{Y_{ijkl} = \mu + \alpha_i + \beta_j + \tau_k + R_l + \alpha\beta_{ij} + \alpha\tau_{ik} + \alpha\,\beta\tau_{ijk} + \Sigma_{ijkl}}$

μ = overall mean

α_i = Effect due to the i^{th} variety

β_j = effect due to the j^{th} soil type

τ_k = Effect due to the k^{th} treatment

R_l = Effect due to the l^{th} replicate

$\alpha\beta_{ij}$ = Effect due to the i^{th} variety in the j^{th} soil

$\alpha\tau_{ik}$ = Effect due to the i^{th} variety in the k^{th} treatment

$\alpha\beta\tau_{ijk}$ = Effect due to the i^{th} variety with the k^{th} treatment in the j^{th} soil

Σ_{ijkl} = Random experimental error

Symbiotic efficiency was calculated using the following formula (Beck *et al*., 1993)**;**

S.E. (%) = A/B ∗100

Where, S.E. = symbiotic effectiveness, A = the amount of nitrogen in the plant inoculated, B = the amount of nitrogen in the nitrogen applied control (DAP).

3.7 Results

3.7.1 Plate culturing and colony counting

Legumefix showed one type of colony forming unit: rod shaped colonies and gram negative. For Biofix (Soybean), all the three batches cultured showed one type of colony morphology when plated on YEMA and YEMA/CR: opaque, rod shaped and gram negative. Biofix for common beans presented a mixture of bacterial strains with some of the colonies absorbing the Congo Red while other colonies were opaque which is characteristic of Rhizobia strains (Figure 3.1). The results showed a variation in the colony forming unit (CFU)/g in the products from batch to batch (Table 3.3).

Table 3.3: Laboratory quality control of commercial plant growth promoting rhizobacteria

Product	Batch No	Package	Plate counting (Colony forming unit) per g of product) x 10^9	Number of organisms
Biofix – Soybean	22071302S	1	9.8	1
		2	11	1
		3	8.8	1
	14011402S	1	8.3	1
		2	7.1	1
		3	9.3	1
	01081302S	1	7.5	1
		2	8.4	1
		3	7.8	1
Biofx-Common bean	08081302B	1	5.1	3
		2	6.9	3
		3	7.2	3
	14011402B	1	6.2	3
		2	6.8	3
		3	5.8	3
	27011402B	1	8.0	3
		2	7.8	3
		3	6.9	3

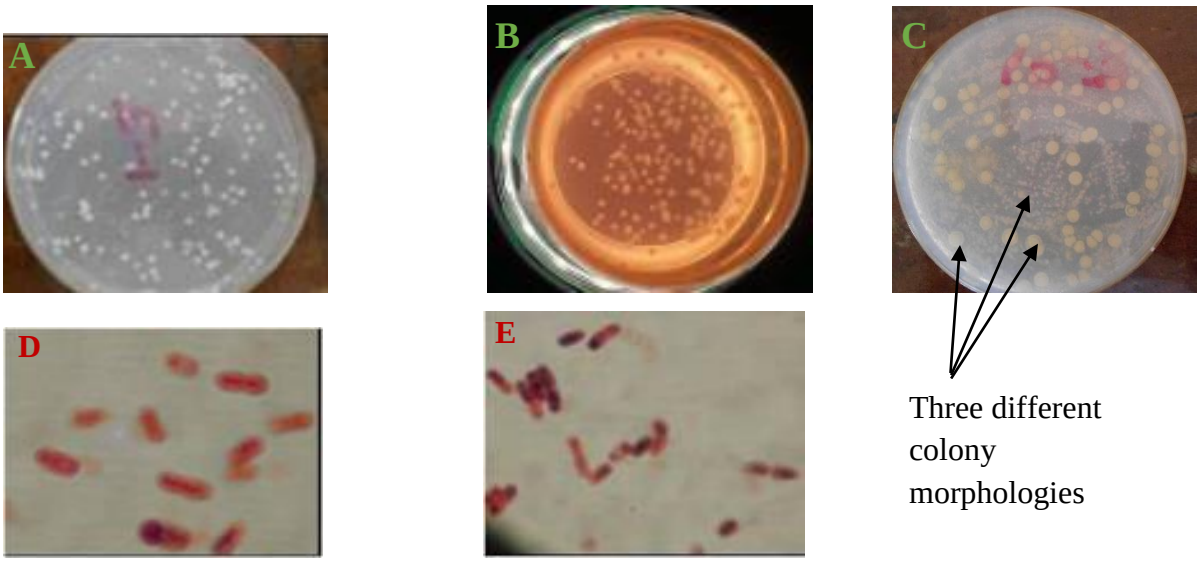

Figure 3.1: Plate cultures of the products - A: Legumefix (10^{-5}), B: Biofix for soybean (10^{-6}) - C: Biofix for common bean (10^{-3}) showing three different colony morphologies, D: Legumefix and E: gram stain reaction for Biofix-soybean.

3.6.2 Chemical properties of the study soils

The study soils varied in terms of pH with the soils from Chuka having a pH of 5.01 (moderately acidic) and Njoro soil having a pH value of 6.32 (slightly acidic). The Chuka soil had low level of extractable P while the Njoro soils had sufficient amount of P. The total nitrogen and organic carbon were moderate for the two soils (Okalebo *et al*., 2002; Mungai *et al*., 2009) (Table 3.4). Chuka soil is characterized as a Rhodic Nitisol, while the Njoro soil is characterized as Vitric Andosol (Jaetzold, 2005).

Table 3.4: Chemical properties of the study soils

Soil	Depth (cm)	pH (H_2O)	Extractable P (mg kg^{-1})	Organic Carbon (%)	Total Nitrogen (%)
Nitisol	0-20	5.01	14.0	2.46	0.16
Andosol	0-20	6.32	43.6	3.62	0.21

3.6.3 Effect of rhizobial inoculation on number of nodules of common bean

The nodule number per plant of common bean was significantly affected by the soil type and the inoculation. There was no significant effect due to the varieties (Appendix I).

However there was significant interaction among the soil, treatment and the varieties (Appendix I). In terms of soil type, Andosol recorded a significantly higher nodule number (112) than the Nitisol (20) (Figure 3.2). Inoculation of the common bean varieties with Biofix also led to a significant increase in the number of nodules (117 nodules) compared to the uninoculated control (75 nodules) and the di-ammonium phosphate application (6 nodules).

In the soil by variety by inoculation interaction, Biofix had a significant effect on nodulation of common bean varieties in the Andosol with AFR 708 and GLP 2 variety having significantly higher nodule numbers than Lyamungu-85. Also, in Andosol inoculation of Lyamungu-85 variety with Biofix did not increase the nodule number compared to the control. However, in Nitisol, inoculation of Lyamungu-85 variety with Biofix led to significantly higher nodule numbers than the control (Figure 3.2).

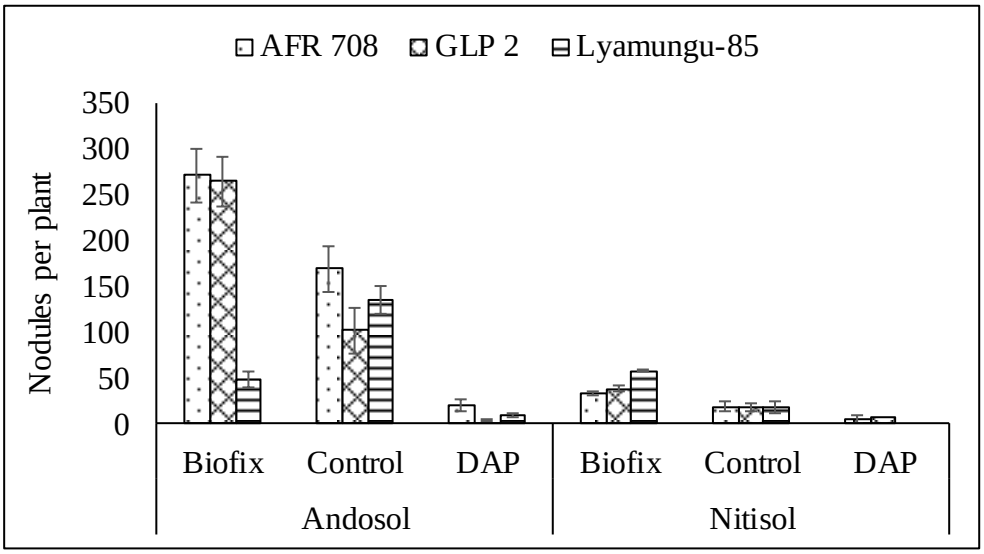

Figure 3.2: Interactive effect of soil*variety* inoculation on the number of nodules per plant in common bean. Error bars represent standard error of the means; DAP- Di-Ammonium Phosphate

3.6.4 Effect of rhizobial inoculation on nodule fresh weight of common bean

The nodule weight of common bean was significantly affected by the soil, variety and inoculation. Also significant interactions effects of soil*inoculation, variety*inoculation and soil*variety*inoculation were observed (Appendix I). Andosols recorded a higher nodule weight (1.46 g) than the Nitisol (0.54 g per plant) in beans. Inoculation of common beans with Biofix

led to significantly higher nodule weight (2.09 g) than the uninoculated control (0.84 g). Di-ammonium phosphate application recorded the least nodule weight. The varieties also responded differently with AFR 708 recording the highest nodule weight (1.29 g) that was significantly different from the Lyamungu-85 variety (0.58 g), but not from GLP 2 (1.14 g). In the Andosol, GLP 2 variety recorded the highest nodule fresh weight and was significantly different from the Lyamungu-85 variety (Figure 3). In the Nitisol however, AFR 708 variety recorded the highest nodule fresh weight following inoculation compared to GLP 2 and Lyamungu-85 varieties (Figure 3.3).

In the three-way interaction, Biofix had a significant effect on the nodule weight of the common bean varieties in Andosols with AFR 708 and GLP 2 variety having significantly higher nodule weight compared to the Lyamungu-85 variety. Also, in Andosols inoculation of Lyamungu-85 variety with Biofix did not increase the nodule weight compared to the control. However, in the Nitisol, inoculation of Lyamungu-85 variety with Biofix led to significantly higher nodule weight over the control (Figure 3.3).

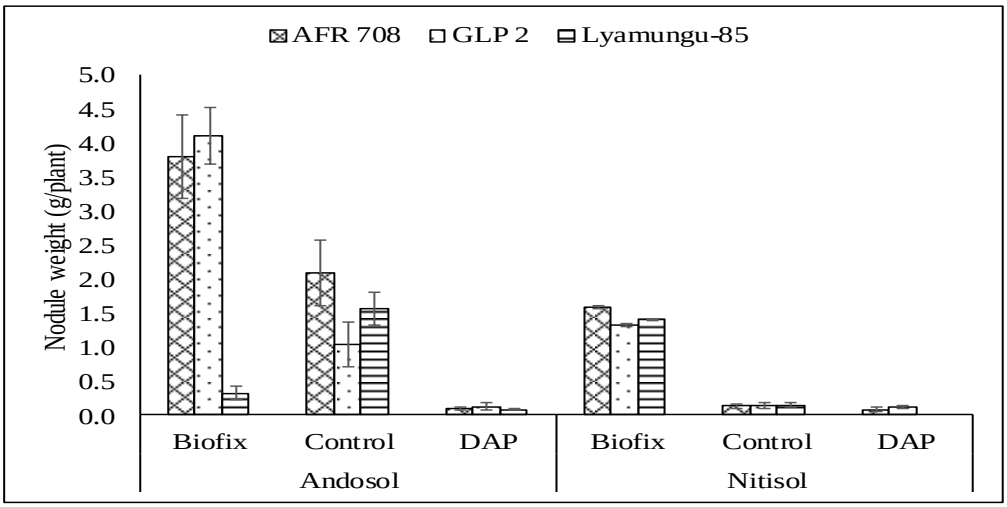

Figure 3.3: Interactive effect of soil, variety and inoculation on nodule weight of common bean. Error bars represent standard error of the means; DAP- Di-Ammonium Phosphate

3.6.5 Effect of rhizobial inoculation on shoot dry weights of common bean

For the common bean, there was significant effect due to the soil type, inoculation, soil*variety and soil*inoculation interaction. However, there was no significant difference among the varieties, variety*inoculation interaction and soil*variety*inoculation interactions (Appendix I). The Andosol recorded a significantly higher shoot dry weight (13.37 g) than the

Nitisol (8.33 g). In terms of the inoculation effect, DAP application recorded the highest shoot weight (14.08 g) that was significantly higher than the Biofix inoculation and the uninoculated control. Additionally, inoculation with Biofix (11.94 g) significantly increased the shoot dry biomass compared to the uninoculated control (6.53 g).

In the soil by variety interaction, the three common bean varieties had a higher shoot dry weight in the Andosol compared to the Nitisol. In the Andosol, AFR 708 variety performed better than Lyamungu-85 variety but no significant difference was noted between AFR 708 and GLP 2 variety. However, in the Nitisol, GLP 2 and Lyamungu-85 varieties performed better than AFR 708 variety (Figure 3.4 A). In terms of the soil by treatment interaction, Biofix inoculation led to a significantly higher shoot dry weight in the Andosol compared to the Nitisol. The increase in shoot dry weight due to Biofix inoculation over the control was more pronounced in the Nitisol (330.1%) compared to the Andosol (19.6%). Di-Ammonium Phosphate application recorded the highest shoot dry weight in both soils types (Figure 3.4 B).

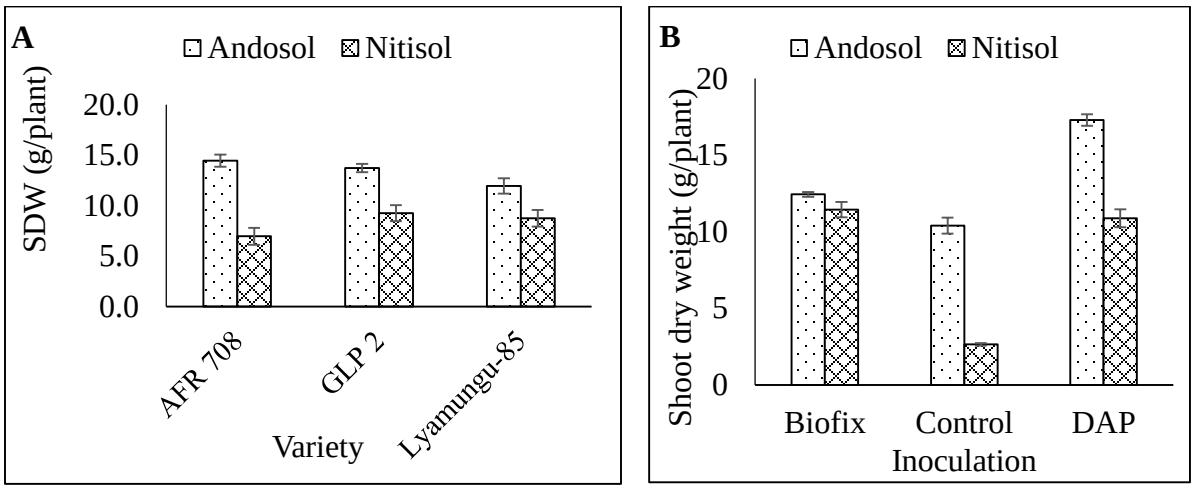

Figure 3.4: Interactive effect of soil*variety (A) and soil*inoculation (B) on the shoot dry weight (SDW) of common bean. Error bars represent standard error of the means; DAP- Di-Ammonium Phosphate

3.6.6 Effect of rhizobial inoculation on root dry weights of common bean

For the common bean, the root dry weight was significantly affected by the soil type and the treatment. However, there was no significant effect due to the variety and the interactions of the variety and the inoculation (Appendix I). In the Andosol, DAP application recorded a significantly higher root dry weight (7.6 g) than the Biofix inoculation and the control. However,

the inoculation of the bean with Biofix led to a significantly higher root dry weight (5.6 g) than the control (4.0 g) (Figure 3.5). In the Nitisol Biofix inoculation of common bean with Biofix significantly increased the root dry weight compared to the uninoculated control. However there was no significant difference between the Biofix inoculation and the DAP application (Figure 3.5).

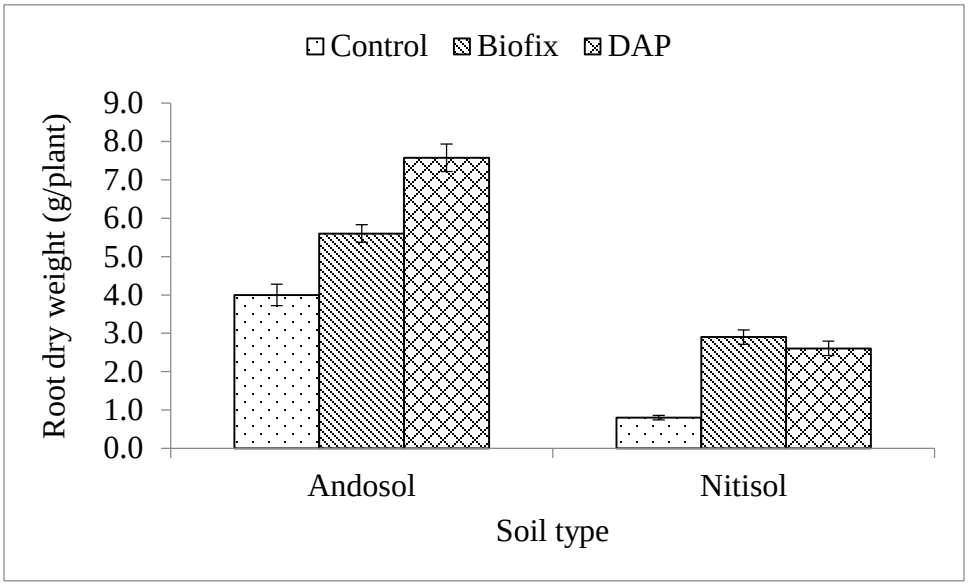

Figure 3.5: Effect of soil type and inoculation on the root dry weight of common bean. Error bars represent standard error of the means.

3.6.7 The effect of inoculation on plant tissue N, biological nitrogen fixation and symbiotic effectiveness of common bean

The plant tissue N content and the BNF were significantly affected by inoculation. The variety, soil and the interactions were however not significant (Appendix I). Inoculation of common bean with Biofix significantly increased the tissue nitrogen compared to the control. DAP application recorded the highest plant tissue nitrogen. In terms of BNF, Biofix inoculation significantly fixed more nitrogen compared to the uninoculated control. However, the symbiotic efficiency of Biofix inoculation was less than 100% (Table 3.5).

Table 3.5: Effect of inoculation on plant nitrogen content, biological nitrogen fixation (BNF) and symbiotic efficiency (SE) of common bean

Treatment	Plant Nitrogen (mg/g)	BNF (kg/ha)	Symbiotic Efficiency (%)
Biofix	36.9b	29.3a	79
Control	27.8c	17.9b	-
DAP	46.5a	-	-
LSD α = 0.05	7.1	7.1	-

Means followed by the same letter within a column are not significantly different at α = 0.05

3.6.8 Effect of rhizobial inoculation on number of nodules of soybean

The number of nodules of soybean were significantly affected by the variety, treatment and variety*inoculation interaction. The soil type had no significant effect on the nodulation of soybean (Appendix II). The SB19 variety recorded a significantly higher number of nodules per plant (20 nodules) than the Nyala variety. Legumefix recorded significantly higher nodules (29 nodules) than Biofix (22 nodules), the uninoculated control (16 nodules) and the DAP application. Biofix also recorded significantly higher nodules than the uninoculated control. In the variety by treatment interaction SB 19 variety recorded significantly higher nodule numbers than the Nyala variety when inoculated with Legumefix and Biofix (Figure 3.6).

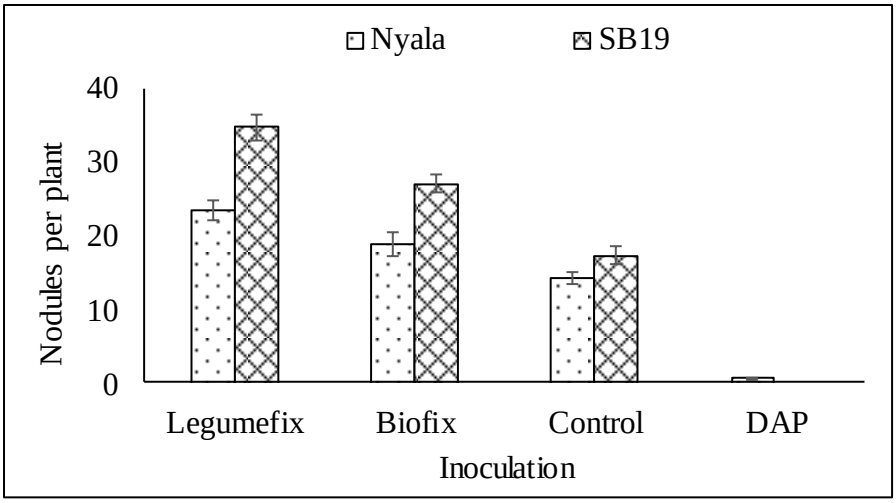

Figure 3.6: Interactive effect of variety*inoculation on the number of nodules plant of soybean. Error bars represent standard error of the means; DAP- Di-Ammonium Phosphate

3.6.9 Effect of rhizobial inoculation on nodule weight of soybean

The nodule weight of soybean was significantly affected by the soil and inoculation but the variety did not have a significant effect on the nodule weight (Appendix II). Soybean recorded a significantly higher nodule weight (122.1 mg) in Andosols compared to Nitisols (62.4 mg). Legumefix inoculation recorded the highest nodule weight (189.1 mg) compared to the uninoculated control (77.9 mg) and the DAP application (2.08 mg). There was significant difference in nodule weight for soybean inoculated with Legumefix and Biofix in both the soil tpes Application of DAP suppressed the nodule fresh weight in both soils and varieties tested in both the Andosol and the nitisol (Figure 3.7).

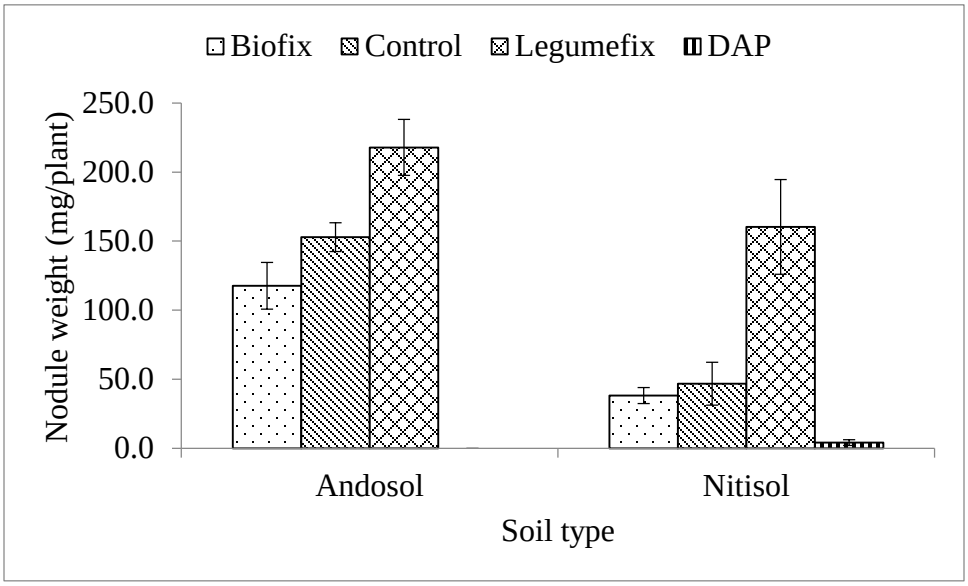

Figure 3.7: Effect of soil type and inoculation on the nodule fresh weight of soybean. Mean followed by the same letter for each section are not significantly different at α=0.05. DAP- Di-Ammonium Phosphate

3.6.10 Effect of rhizobial inoculation on shoot dry weights of soybean

In soybean, there was a significant difference in shoot dry weight between the soil type, variety and among the inoculation. There was also a significant soil*variety interaction on the shoot dry weight (Appendix II). In the Andosol, inoculation with Legumefix recorded a significantly higher shoot dry weight than the control. However, there was no significant difference between the Biofx application and the control (Figure 3.8A). In the Nitisol, inoculation of the soybean with Legumefix and Biofix led to significantly higher shoot dry

weight than the control (Figure 3.8A). In terms of variety, SB19 recorded a significantly higher shoot dry weight (7.52 g) than the Nyala variety (4.34). In the soil*variety interaction, SB 19 variety recorded significantly higher shoot dry weight in both the Andosol and the Nitisol compared to the Nyala variety in both the soil types (Figure 3.8B).

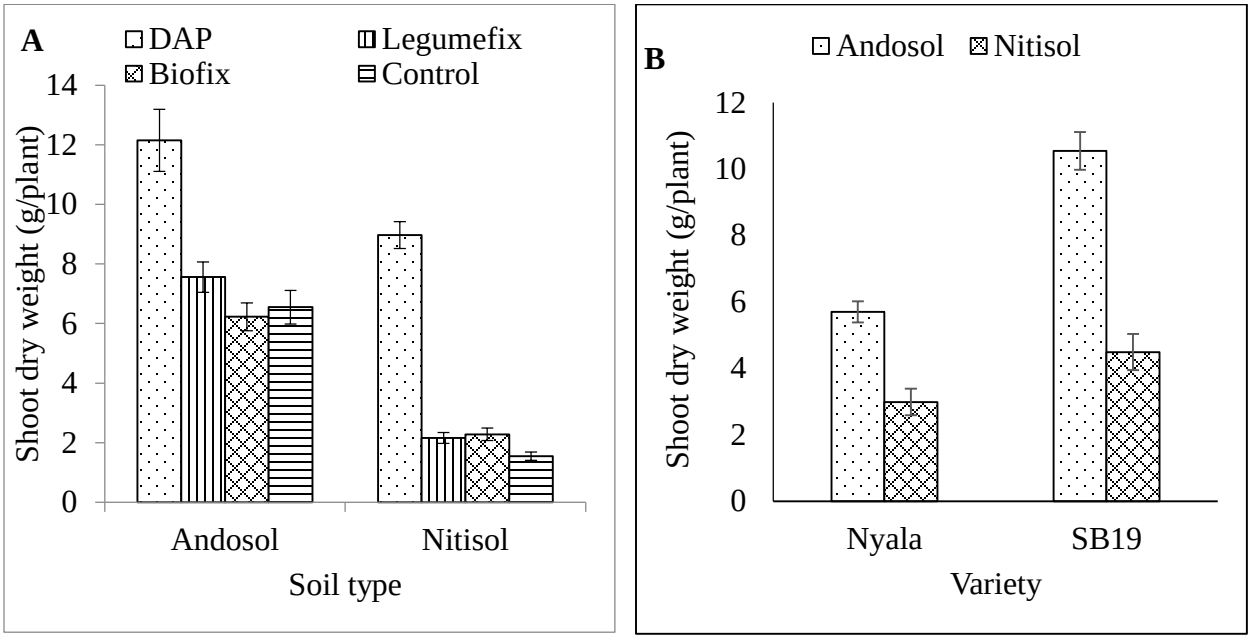

Figure 3.8: Effect of soil*inoculation (A) and the soil*variety interaction (B) on the shoot dry weight of soybean. Mean followed by the same letter are not significantly different at α=0.05. Error bars represent standard error of the means

3.6.11 Effect of rhizobial inoculation on root dry weights of soybean

The root dry weight of soybean was significantly affected by the soil type, variety, treatment and soil*variety interaction (Appendix II). Similarly, in soybean; the Andosol recorded a significantly higher root dry weight (3.25 g) compared to the Nitisol (0.89 g). The SB19 variety recorded a significantly higher root dry weight than the Nyala variety. Inoculation of soybean with Legumefix and Biofix did not increase the root dry weight compared to the uninoculated control (Figure 3.9A). The highest root dry weight was recorded due to the addition of DAP across the varieties and soils. In the soil*variety interaction, SB 19 variety recorded significantly higher root dry weight in both the soils compared to the Nyala variety (Figure 3.9B).

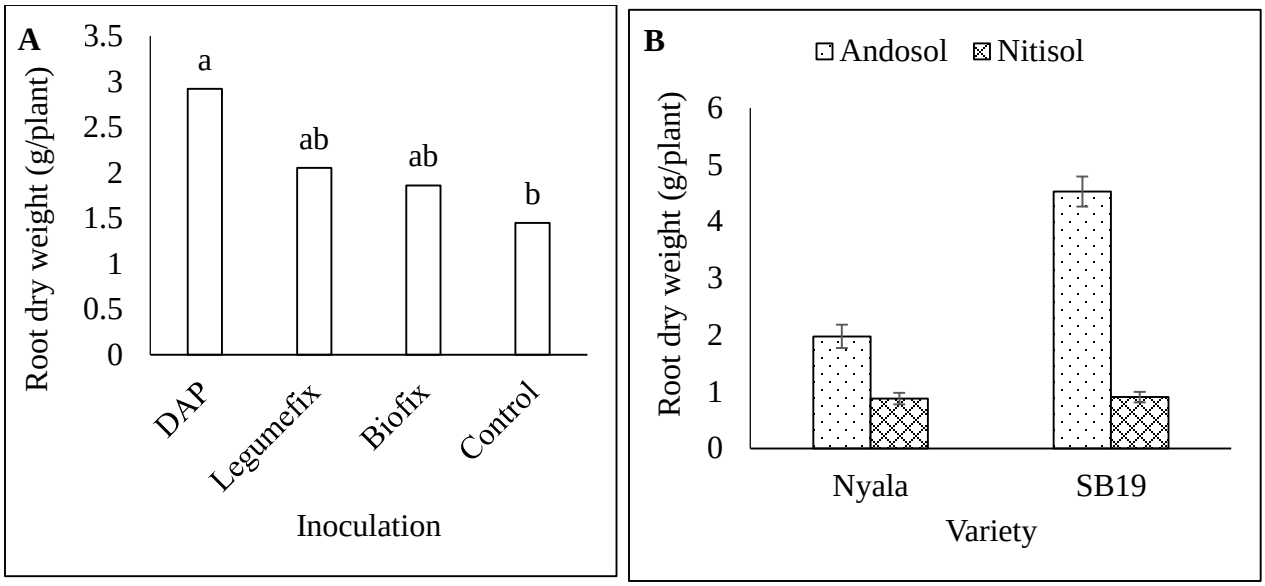

Figure 3.9: Effect of inoculation (A) and the soil*variety interaction (B) on the root dry weight of soybean. Mean followed by the same letter are not significantly different at α=0.05. Error bars represent standard error of the means

3.6.12 Effect of inoculation on plant tissue N, biological nitrogen fixation and symbiotic effectiveness of soybean

For the soybean, the plant tissue N and the BNF were significantly affected by the treatment. The variety, soil and the interactions were however not significant (Appendix II). Inoculation increased the tissue nitrogen compared to the control. Legumefix inoculation recorded a significantly higher nitrogen level compared to the uninoculated control. However, no significant difference was noted when compared to the Mineral N application and Biofix inoculation. Additionally, inoculation of soybean with Legumefix fixed a significantly higher nitrogen level compared to the uninoculated control and recorded a symbiotic efficiency of 105.6% that was higher than 100% compared to Biofix inoculation that recorded a symbiotic efficiency of 88.1% (Table 3.6).

Table 3.6: Effect of inoculation on plant nitrogen content, Biological nitrogen fixation (BNF) and symbiotic efficiency (SE) of soybean

Treatment	Plant Nitrogen (mg/g)	BNF (kg/ha)	Symbiotic Efficiency (%)
Biofix	46ab	40.5ab	88
Legumefix	55a	51.9a	105
Control	38b	31.1b	-
Mineral N	52ab	-	-
LSD $\alpha = 0.05$	14	14	-

Means followed by the same letter within a column are not significantly different at $\alpha = 0.05$

3.7 Discussion

3.7.1 Quality of inoculants

The three products showed variation in their quality especially Biofix for common bean which had more than one type of microorganism. The company producing the inoculants tested in this study did not give information on the exact composition of the products and thus the strains were not obviously 'contaminants' (Herrmann *et al*., 2010). Mathu e*t al*. (2012) reported more than one strain of microorganism in the product tested (Biofix for cowpea). Also, Biofix 704 and 2447 for green gram revealed that the product did not contain *Bradyrhizobia* strains. In addition, Biofix inoculants have been shown to carry additional contaminating microbes, which may interfere with the survival of rhizobia and subsequent root nodulation (Balume, 2013). The presence of multiple strains, some of which may not be rhizobia raises concern about the quality (Mathu *et al*., 2012). Study by Hassen *et al*. (2014) on the viability and shelf life of commercial inoculants also indicated that the inoculant products available on the market are sub-standard soybean inoculants products and this may have led to nodulation failure. The study by Hassen *et al*. (2014) also showed that a wide variety of soybean inoculants with strains of *Bradyrhizobium* other than the recommended WB74 strain are being manufactured and/or imported by different companies to be sold to the South African farmers. The two products analyzed in this study showed higher values than the minimum number (CFU/g) that is required for inoculants in most parts of the world of 10^9 microorganisms per gram of a product (Woomer *et al*., 2011b). This

may be an indication of good quality inoculants with high cell numbers (Atieno *et al*., 2012; KEBS, 2015).

3.7.2 Inoculation effect on nodulation of common bean and soybean

In the present investigation rhizobium inoculation of common beans and soybean significantly increased the nodule number per plant compared to the uninoculated control. This may be due to the presence of adequate rhizobia strains in the root rhizosphere which initiated the formation of the nodules. The result of this study was in accordance with earlier finding by Tahir *et al*. (2009) who reported increase in soybean nodule numbers and nodule dry weight from 73 to 125 and 1.36 g to 1.53 g respectively by inoculation alone.

There was a variation in nodulation of soybean following inoculation with Legumefix and Biofix with Legumefix recording higher nodulation than Biofix inoculation. Results of nodulation among the inoculants indicate that the strains in Legumefix could be of higher quality in terms of efficiency and effectiveness than in Biofix. A study by Aliyu *et al*. (2013) showed that some of the rhizobia strains used in the trials did not show significant difference from control while others recorded even lower nodulation. The Common bean and soybean had nodules even without inoculation in Andosol. This indicates that the soil contains native rhizobia that nodulated the legumes (Chemining'wa *et al*., 2004). Nodulation observed in control plots indicates that native common bean and soybean rhizobia in the soils are compatible with varieties tested.

This study found that N fertilizer application significantly reduced the number of nodules and nodule fresh weight per plant. This indicates the preference of host plant to utilize available N added to the soil which requires less energy than fixing N from the atmosphere. Muhammad (2010) and Van der Bom (2012) also reported that application of nitrogenous fertilizer resulted in reduction of nodules number and rate of nitrogen fixation in soybean. Inhibitory effect of added nitrogen fertilizer to nodulation had been reported by Otieno *et al*. (2009), Bekunda *et al*. (2010) and Thuita *et al.* (2012).

3.7.3 Effect of inoculation on shoots and root dry weights of common bean and soybean

It has also been reported that nodule number, dry weight and soybean shoot yield increased when seeds inoculated with Rhizobium (Javaid & Mahmood, 2010; Musyoki e*t al*., 2011). Otieno *et al.* (2009) also reported similar results that *rhizobial* inoculation significantly increases nodule number and dry weight in studied legume species compared to application of

farmyard manure and N-fertilizer. Lamptey *et al*. (2014) reported that soybean seeds inoculated with commercial Rhizobium inoculants (Legumefix) established better grew more vegetatively, produced higher shoot biomass and nodulated more vigorously.

The results from this study also indicated that the rhizobial inoculants did not significantly increase the shoot and root dry weight compared to the control. This is explained by the fact that enhanced growth of the legumes is mainly due to nitrogen fixed by rhizobia (Mweetwa *et al*., 2014). Another factor that may have resulted in no inoculation response is the quality of inoculants used. Similarly, other authors have reported inability of inoculation with Biofix and other rhizobial inoculant to result in significant increase in shoot dry biomass in common bean, soybean, cowpea and greengram over the control (Mungai & Karubiu, 2010; Mathu *et al*., 2012 and Gitonga *et al*., 2010).

3.7.4 Effect of soil fertility status on inoculation

The present study also revealed different inoculation effect depending on the type of soil with the Nitisol being more responsive to inoculation than the Andosol. Success of rhizobia inoculation is highly site specific and depends on a number of interactions including environmental, soil and biological factors (Argaw, 2012). Soil factors that influence plant and rhizobial growth include acidity, temperature, moisture, fertility influence infection and nodulation of legumes (Cooper & Schere, 2012). This may be as a result of high N level in the Andosol compared to the Nitisol and this may have hindered the legumes in responding to rhizobia inoculation as much as the Nitisol. When the soil N levels are high the legume will use the readily available N rather than fix nitrogen through the BNF process. Soil pH has been widely reported to influence nodulation because it can induce deficiency in some essential nutrients (Giller, 2001) such as P. Aliyu *et al*. (2013) reported a difference in inoculation effect of soybean in an Ultisol and Inceptisol. This study shows that soils varying in fertility status will respond differently to rhizobial inoculation. There is therefore need to supplement the soil with other nutrients such as P and starter nitrogen and also need of liming acidic soils to reduce deficiency of other plant nutrients.

3.7.5 Effect of common bean and soybean varieties on the response to inoculation

The common bean and soybean varieties tested showed a variation in their response to inoculation. This may be attributed to the genetic makeup of the varieties. Cultivar variation affects levels of nitrogen fixation in many legume crop species, and in some crops particular

combinations of strain and cultivar have been shown to be especially efficient at fixing nitrogen. There are varying reports on the interaction between variety and strain in soybean (Solomon *et al*., 2012; Mhango, 2015). SB19 responded well to inoculation compared to Nyala variety and this may be due to the SB19 being a promiscuous variety and Nyala being a specific nodulating soybean variety and SB19 could have been nodulated by other rhizobium species in the soil. Thuita *et al*. (2012) and Atieno *et al*. (2012) working with the two varieties in soils from Central and Coast province of Kenya also reported similar results with SB19 responding to inoculation more than Nyala variety.

In the common beans varieties uninoculated Lyamungu-85 variety was able to form more nodules than those that had been inoculated with Biofix in the Andosol while in GLP 2 and AFR 708 variety the inoculated had more nodules than uninoculated in the same soil. These results suggested that different varieties of beans had preference for certain rhizobia and indigenous rhizobia strain were better than inoculant strains for the Lyamungu-85 variety in the Andosol. This is in line with what was reported by Gicharu *et al*. (2013) working on three climbing bean cultivars who showed variation in nodulation of the cultivars in response to inoculation with rhizobial strains.

3.7.6 Effect of inoculation on biological nitrogen fixation and symbiotic efficiency of the inoculants

Plant tissue analysis from this study also revealed that inoculation of common bean and Soybean with Biofix did not significantly increase shoot nitrogen over the control. Previous studies assessing the response of several legumes to inoculation with Biofix revealed that there was generally no significant increase in the amount of total nitrogen accumulated with inoculation (Mungai & Karubiu, 2010; Khafa, 2013 and Mweetwa *et al*., 2014) on inoculation of bean genotypes. Others studies also found indigenous Rhizobium strains to be highly effective symbiotic N fixers than introduced commercial inoculants (Evans, 2005; Yadav *et al*., 2011).

In soybean, variation in tissue N and biologically fixed N was observed between the Legumefix (soybean) and Biofix (soybean) inoculation. The symbiotic efficiency of Legumefix inoculant was greater than that of Biofix inoculation. This indicates the presence of strains with a high potential in the Legumefix compared to the Biofix showing that some inoculant strains are of higher quality than others. Rhizobia strains of different origin vary in their symbiotic

efficiency (Zaman-Allah, 2007). Additionally, the biologically fixed N was significantly higher than the control though the response depended on the legume (COMPRO II, 2013c). The approach of using effective or superior exotic rhizobia strains as inoculants has failed in various environments due to various reasons including the use of ineffective and non-competitive rhizobia strains as inoculants (Slattery *et al*., 2004). The total nitrogen accumulated by legumes has previously been shown to reduce with Biofix inoculation. For example, a 4.13% reduction was observed in groundnuts (COMPRO II, 2013c).

3.8 Conclusions

The products tested contained adequate number of viable microorganisms. However, for Biofix (Common bean) there is need to specify the different microorganisms found in the product since it has positive effect on plant and can coexist with the rhizobia strain.

Rhizobia inoculation of common bean seeds resulted in enhanced nodule numbers and weight in genotypes tested across the soil types except for Lyamungu-85 variety in Andosols. This indicates importance of testing genotypes in various locations to improve selection of genotypes for the required trait they have been tested for. In this study, Legumefix performed better than Biofix in enhancing soybean nodulation.

For the greenhouse evaluation, the results of the study indicated that the products generally had positive influence on the measured parameters compared to control and thus inoculation would be beneficial in improving productivity of common bean and soybean as assessed using nodulation and shoot and root biomass. Additionally, field testing is required to determine whether improvement to growth would result in yield increase.

References

Abdul-Latif, A. (2013). Contribution of Rhizobium and phosphorus fertilizer to biological nitrogen fixation and grain yield of soybean in the Tolon District. MSc. Thesis. Retrieved from: http://www.n2africa.org/sites/n2africa.org/files/images/ABDUL-LATIF%20ABDUL-AZIZ%20MSc%20Thesis.pdf (Accessed on 27th October, 2015).

Aliyu, I. A, Yusuf, A. A. & Abaidoo, R. C. (2013). Response of grain legumes to rhizobial inoculation in two savanna soils of Nigeria. *African Journal of Microbiology Research* 7(15): 1332-1342.

Argaw A. (2012). Evaluation of symbiotic effectiveness and size of resident *R. Leguminosarum* var. viciae nodulating lentil (*Lens culinaris* Medic.) in some Ethiopian soils. *Archives of Agronomy and Soil Science* 3: 1-17.

Atieno, M., Hermann, L., Okalebo, R. & Lesueur, D. (2012). Efficiency of different formulations of *Bradyrhizobium japonicum* and effect of co-inoculation of *Bacillus subtilis* with two different strains of *Bradyrhizobium japonicum. World Journal of Microbiology and Biotechnology* 28:2541–2550.

Aung, T. T., Buranabanyat, B., Piromyou, P., Longtonglang, P, Tittabutr P., Boonkerd, N. & Teaumroong, N. (2013). Enhanced soybean biomass by co-inoculation of *Bradyrhizobium japonicum* and plant growth promoting rhizobacteria and its effects on microbial community structures. *African Journal of Microbiology Research* 7(29): 3858-3873.

Balume K. M. I. (2013). Assessment of quality control of inoculants used on bean and soybean in eastern and central Africa (Master's thesis). University of Nairobi, Nairobi, Kenya.

Bambara, S. & Ndakidemi, P. A. (2010). Effects of *Rhizobium* inoculation, lime and molybdenum on nitrogen fixation of nodulated *Phaseolus vulgaris* L. *African Journal of Microbiology Research* 4: 682-696.

Beck, D., Materoni, L., & Afandi, F. (1993). Practical *Rhizobium* sp. legume technology manual. International Center for Agricultural Research in Dry Areas, 1-54.

Bekunda, M., Sanginga, N. & Woomer, P. L. (2010). Restoring soil fertility in Sub-Saharan Africa. *Advances in Agronomy* 108: 183-236.

Bremner, J. M. & Mulvaney, C. S. (1982). *Nitrogen total*. In: Methods of soil analysis (A. L. Page et al., ed). Agronomy monograph 9, part 2, 2nd ed. American Sociaty of Agronomy, Madison, W. I. pp. 595-624.

Chemining'wa, G. N., Muthomi, J. W. & Obudho, E. O. (2004). Effects of rhizobia inoculation and urea application on dry matter accumulation and green gram legume at Katumani and Kabete sites of Kenya. *Legume Research Network Project Newsletter* 11: 13-17.

COMPRO II (2013a). The building blocks for biofertiliser and biopesticides policymaking in Africa. Policy Brief, 2.Retrieved from http://www.iita.org/projects.html

COMPRO II (2013b). Sequencing results for Rhizobia inoculants. Retrieved from http://www.compro2.org/outputs2.html

COMPRO II (2013c). Testing Rhizobia products on cowpea (RVU), common bean (RP), greengram (RVR) and groundnut (RA). Retrieved from http://www.compro2.org/outputs2.html

Cooper J. E. & Schere H. W. (2012). *Nitrogen fixation*. Plant-soil relationship, part II. p. 389-408.

Evans, J. (2005). An evaluation of potential Rhizobium inoculant strains used for pulse production in acidic soils of south-east Australia. *Australian Journal of Experimental Agriculture* 45: 257–268.

Gicharu, G. K., Gitonga, N. M., Boga, H., Cheruiyot R. C. & Maingi, J. M. (2013). Effect of inoculating selected climbing bean cultivars with different rhizobia strains on nitrogen fixation. *International Journal of Microbiology Research* 1(2): 25-31.

Giller K. E. (2001). Nitrogen fixation in tropical cropping systems. CABI Publishing, Wallingford, UK.

Giri, N. & Joshi, N. C. (2010). Growth and yield response of chick pea (*Cicer arietinum*) to seed inoculation with *Rhizobium* sp. *Nature and science* 8(9): 232-236.

Gitonga, M.N., Gatheri, G.W., Cheruiyot, R., Gitonga, N.M. & Maingi, J.M. (2010). Nodulation and nitrogen fixation in promiscuous and non-promiscuous soybean (*Glycine Max* (L.) Merrill) varieties in Eastern Kenya. *Journal of Tropical Microbiology and Biotechnology* 6: *3-8.*

Hassen, A. I., Bopape, F. L., Rong, I. H., & Seane, G. (2014). Nodulation efficiency of *Bradyrhizobium japonicum* strain WB74 on soybean (Glycine max L. Merrill) is affected by several limiting factors. *African Journal of Microbiology Research* 8(20): 2069-2076.

Herrmann L., Atieno M., Okalebo J. R. and Lesueur, D. (2010). Molecular identification of the strains contained in commercial products for improving agriculture in Africa. *Poster presentation in the International Society for Microbial Ecology (ISME) conference* 13, Seattle, USA, August 2010.

ICRISAT (2013). Tropical legumes. Retrieved from: http://www.icrisat.org/TropicallegumesII/ Bulletin-of-Tropical-Legumes.html (Accessed on 18th October, 2015).

Jaetzold, R., Schmidt, H., Hornetz, B. & Shisanya, C. (2005) *Farm Management Handbook of Kenya*. Ministry of Agriculture, Kenya, in Cooperation with the German Agency for Technical Cooperation (GTZ), Nairobi.

Javaid, A. & Mahmood, N. (2010). Growth, nodulation, and yield response of soybean to biofertilizers and organic manures. *Plant Journal of Botany* 42 (2): 863-871.

Jefwa, J.M., Pypers, P., Jemo, M., Thuita, M., Mutegi, E., Laditi, M. A., Faye, A., Kavoo, A., Munyahali, W., Herrmann, L., Atieno, M., Yusuf, A., Ibrahim, A., Ndug'u-Marigoi, K. W., Asrat, A., Muletta, D., Ncho, C., Kamaa, M. & Lesueur, D. (2014). Do commercial biological and chemical products increase crops yields and economic returns under small holder farmer conditions? In Challenges and opportunities for agricultural intensification of the humid highland systems of Sub Saharan Africa. Eds Vanlauwe, B., Asten, P. V. and Blomme, G. Springer, pp 81-96.

KALRO. (2006). Better soybean yields through good agricultural practices. Retrieved from: http://africasoilhealth.cabi.org/wpcms/wp-content/uploads/2014/05/259-KARI-soybean-leaflet.pdf (Accessed on 17th April, 2015).

KALRO. (2008). Better bean varieties. Retrieved from: http://www.kalro.org/fileadmin/publications/brochuresI/Beanvariety.pdf (Accessed on 17th April, 2015).

KEBS. (2015). Biofertilizer specification. Draft Kenya standard 2015. Second edition. Retrieved from: http://waterfund.go.ke/safisan/Downloads/Bio%20Fertilizer%20Guidelines(2).pdf (Accessed on 25th July, 2016).

Khafa, B. P. (2013). Response of common bean genotypes to inoculation with Rhizobia and effects of P and N on biological nitrogen fixation. Msc. Thesis. Sokoine university of Agriculture, Morogoro, Tanzania.

Kisinyo, P. O, Othieno, C. O., Gudu, S. O. , Okalebo, J. R., OpalA, P. A., Ng'etich, W. K., Nyambati, R. O., Ouma, E. O., Agalo, J. J., Kebeney, S. J., Too, E. J., Kisinyo, J. A., & Opile, W. R. (2014). Immediate and residual effects of lime and phosphorus fertilizer on soil acidity and maize production in Western Kenya. *Experimental Agriculture* 50 (1): 128–143

Lamptey, S., Ahiabor, B. D. K., Yeboah, S. & Osei, D. (2014). Effect of rhizobium inoculants and reproductive growth stages on shoot biomass and yield of soybean (*Glycine max* (L.) Merril). *Journal of Agricultural Science* 6(5): 44-54.

Lesueur, D., Atieno, M., Mathu, S. & Herrmann, L. (2012). Importance of Rhizobia in Agriculture: Potential of the Commercial Inoculants and Native Strains for Improving Legume Yields in Different Land-Use Systems. *Geophysical Research Abstracts* 14: 632.

Masso, C., Jefwa, J.M., Jemo, M., Thuita, M., Tarus, D. & Vanlauwe, B. (2014). Impact of inadequate regulatory framework on the adoption of biofertilizers (E.g PGPR) technologies. A case of Sub-Saharan Africa. In: *Recent advances in biofertilizers and biofungicides (PGPR) for sustainable agriculture*. Sayyed, R., Sudini, H., Kumar, K.V.K, Armanda, A. & Gopalkrishnan Eds. Cambridge Scholars Publishing pp 259-268.

Mathu, S., Herrmann, L., Pypers, P., Matiru, V., Mwirichia, R. & Lesueur, D. (2012): Potential of indigenous bradyrhizobia versus commercial inoculants to improve cowpea (*Vigna unguiculata* L. walp.) and green gram (*Vigna radiata* L. wilczek.) yields in Kenya. *Soil Science and Plant Nutrition* 58(6): 750-763.

Mhango, J. (2015). Efficacy and competitiveness of indigenous bradyrhizobia strains on soybean (*Glycine max (L.) Merr*.) productivity in Malawi. Msc. Thesis. Egerton University, Kenya. Retrieved from: http://www.n2africa.org/sites/n2africa.org/files/images/Joseph%20Mhango%20Thesis%202015%20MSc%20Soil%20Science.pdf. (Accessed on 29th September, 2015).

Morad, M., Sara, S., Alireza, E., Reza, C.M. & Mohammad, D. (2013). Effects of seed inoculation by rhizobium strains on yield and yield components in common bean cultivars (*Phaseolus vulgaris* L.). *International Journal of Biosciences (IJB*) 3: 134-141.

Mungai, N. W., Bationo, A., & Waswa, B. (2009). Soil properties as influence by soil fertility management in small scale maize farms in Njoro, Kenya. *Journal of Agronomy* 8(4): 131-136.

Mungai, N. W. & Karubiu, N. M. (2010). Effectiveness of rhizobia isolates from Njoro soils (Kenya) and commercial inoculants in nodulation of common beans (*Phaseolus Vulgaris*). *Journal of Agriculture, Science and Technology* 12(1): 47-59.

Musyoki, M., Majengo, C., Mutegi, E., Thonar, C., Faye, A., Okalebo, R., Thuita, M., Lesueur, D. & Pypers, P. (2011). Increased soybean production through rhizobial inoculation under smallholder conditions in distinct agro-ecological zones of Kenya. *Nitrogen & global change key findings – Future Challenges Conference, Edinburgh;* April, 11-15, 2011.

Mweetwa, A. M., Mulenga, M., Mulilo, X., Ngulube, M., Banda, J.S.C, Kapulu, N. & Ng'andu, S.H. (2014). Response of cowpea, soyabeans and groundnuts to non-indigenous legume inoculants. *Sustainable Agricultural Research* 3(1): 84-95.

Njoka E. M**.,** Kimurto P. K**.,** Towett, B. K. Ogumu, M. O., Ngolo, A., Macharia, J. K. & Gatongi, I**.** (2009). Development and Release of 3 bean varieties *(AFR 708, Lyamungu-85 and Ciankui*) for Commercial Production in high ad medium altitudes of Kenya. *KEPHIS Reports, 2009 and Kenya Gazette Vol 9 (3), 2009.*

Okalebo, J. R., Gathua, K. W. & Woomer, P. L. (2002). *Laboratory Methods of Soil and Plant Analysis*: A Working Manual, KARI, SSSEA, TSBF, SACRED Africa, Moi University. Second Edition, Nairobi, Kenya. pp. 128.

Olsen P. E., Sande, E. S., & Keyser, H. H. (1996). The enumeration and identification of rhizobial bacteria in legume inoculant quality control. NifTal center, USA. pp 29-45.

Otieno, P. E., Muthomi, J. W., Chemining'wa, G. N. & Nderitu, J. H. (2009). Effect of *rhizobia* inoculation, farmyard manure and nitrogen fertilizer on growth, nodulation and yield of selected food grain legumes. *African Crop Science Conference Proceedings* 8. 305-312.

SAS software version 9.3 (2010). SAS Institute Inc., Cary, NC, USA. Licensed to Institute of Tropical Agriculture.

Slattery, J., Pearce, D. & Slattery, W. (2004). Effects of resident rhizobial communities and soil type on the effective nodulation of pulse legumes. *Soil Biology and Biochemistry*, 36: 1339-1346.

Solomon, T., Pant, L. and Angaw, T. (2012). Effects of inoculation by *Bradyrhizobium japonicum* strains on nodulation, nitrogen fixation, and yield of soybean (*Glycine max* L.Merill) varieties on nitisols of Bako,Western Ethiopia. *International Scholarly Research Network Agronomy* 2012:1-8.

Somasegaran, P. & Hoben, H.J. (1994). *Handbook for Rhizobia*. Springer Laboratory pp 511.

Tahir, M. M., Abbasi, M. K., Rahim, N., Khaliq, A. & Kazmi, M. H. (2009). Effect of *Rhizobium* inoculation and NP fertilization on growth, yield and nodulation of soybean (*Glycine max* L.) in the sub-humid hilly region of Rawalakot, Azad, Jammu and Kashmir Pakistan. *African Journal of Biotechnology* 8(22): 6191-6200.

Thuita, M., Pypers, P., Herrmann, L., Okalebo, R., Othieno, C., Muema, E. & Lesueur, D. (2012). Commercial rhizobial inoculants significantly enhance growth and nitrogen fixation of a promiscuous soybean variety in Kenyan soils. *Biology and Fertility Soils* 48:87–96.

Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, R., Giller, K., Alves B. & Chalk, P. (2008). Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monograph No. pp. 136. 258.

Uribe, D., Sa´nchez-Nieves, J. & Vanegas, J. (2012). Role of microbial biofertilizers in the development of a sustainable agriculture in the tropics. In soil biology and agriculture in the tropics. Dion, P. eds. Pp 240-250. Springer.

Van der Bom, F. (2012). Response of *Phaseolus vulgaris* L. and *Pisum sativum* L. to N and P fertilizers and inoculation with *Rhizobium* in Kenya. MSc. Thesis, Wageningen University.

Woomer, P., Bala, A. & Abaido, R. (2011a). *Rhizobia strain isolation and characterization protocol.* Retrieved From N2Africa:www.N2Africa.org/. (Accessed on 3rd July 2013).

Woomer, P.L., Karanja, N., Kisamuli, S.M., Murwira, M. & Bala, A. (2011b). A revised manual for rhizobium methods and standard protocols available on the project website. Retrieved from: www.N2Africa.org (Accessed on 18th April, 2015).

Yadav, J., Verma, J. P., Rajak,V. K., Tiwari, K. N. (2011). Selection of effective indigenous Rhizobium strain for seed inoculation of chickpea (*Cicer aritenium* L.) production. *Bacteriology* 1:24–30.

Yakubu, H., Kwari, J. D. & Ngala, A. L. (2010). N_2 fixation by grain legume varieties as affected by rhizobia inoculation in the sandy loam soil of Sudano-Sahelian Zone of North Eastern Nigeria. *Nigerian Journal of Basic and Applied Science* 18(2): 229-236.

Zaman-Allah, M., Sifi, B., L'Taief, B., El-Aouni, M. H. & Drevon, J. J. (2007). Rhizobial inoculation and P utilization response in common bean (*Phaseolus vulgaris*) under greenhouse and field conditions. *Experimental Agriculture* 43: 67-77.

Zarei, I., Sohrabi, Y., Heidari G.R., Jalilian, A. & Mohammadi, K. (2012). Effects of biofertilizers on grain yield and protein content of two soybean (*Glycine max* L.) cultivars. *African Journal of Biotechnology* 11(27): 7028-7037.

CHAPTER FOUR: IDENTIFICATION OF NODULE ENDOPHYTIC PHOSPHATE SOLUBILIZING BACTERIA AND CO-INOCULATION OF RHIZOBIA STRAINS AND PHOSPHATE SOLUBILIZING BACTERIA FOR COMMON BEAN PRODUCTION IN LOW PHOSPHORUS SOIL

4.1 Abstract

Nitrogen (N) fixation by legume-r*hizobium* symbiosis is important to agricultural productivity and is therefore of great economic interest. Growing evidence indicates that other soil beneficial bacteria can positively affect symbiotic performance of rhizobia. The effect of co-inoculation of *rhizobium* and phosphate solubilizing bacteria (PSB), on nodulation and growth of common bean (*Phaseolus vulgaris* L.) was investigated using a low phosphorous soil under greenhouse conditions. PSB strains *Paenibacillus polymyxa* and *Bacillus megaterium* were isolated from common bean nodules from Nakuru County in central Rift Valley in Kenya. The PSB strains, two rhizobia strains (IITA-PAU987 and IITA-PAU983) and one reference rhizobia strain (CIAT 899) were used in this study. Two common bean varieties were inoculated with *Rhizobium* strains singly or in a combination with PSB to evaluate the effect on nodulation and growth parameters. The experiment was laid out as a completely randomized design with three replicates. Co-inoculation of IITA-PAU987 + *B. megaterium* recorded the highest nodule weight (405.2 mg) compared to IITA-PAU987 alone (324.8 mg), while CIAT 899 + *B. megaterium* (401.2 mg) compared to CIAT 899 alone (337.2 mg). CIAT 899 + *B. megaterium* recorded a significantly higher shoot dry weight (7.23 g) compared to CIAT 899 alone (5.80 g). However, there was no significant difference between CIAT 899 + *P. polymyxa* and CIAT 899 alone. Combination of IITA-PAU987 and *B. megaterium* led to significantly higher shoot dry weight (6.84 g) compared to IITA-PAU987 alone (5.32 g) but no significant difference was observed when co-inoculated with *P. polymyxa*. IITA-PAU983 in combination with *P. polymyxa* led to significantly higher shoot dry weight (7.15 g) compared to IITA-PAU983 alone (5.14 g). There was a significant contrast between the co-inoculation with rhizobia strains and PSB compared to single rhizobia inoculation on the root dry weight. These results indicate that co-inoculation of PSB and rhibozia has a synergistic effect on the growth and they can be used in improving biofertilizers formulation for common bean production. Testing of the plant growth promoting

rhizobacteria under field conditions will further elucidate their effectiveness on grain yield of common bean.

Keywords: *Phaseolus vulgaris* L., plant growth promoting rhizobacteria, phosphate solubilizing bacteria, rhizobia

4.2 Introduction

Nitrogen (N) and phosphorus (P) are the most limiting nutrients for plant growth. Phosphorus is generally deficient in most of the soils due to its ready fixation (Collavino *et al*., 2010). Inadequate P restricts root growth, photosynthesis, translocation of sugars, and other such functions, which directly or indirectly influence nitrogen fixation by legume plants (Olivera *et al*., 2004).

The replenishment of N and P nutrients is mostly done through application of inorganic fertilizers to the soil. However, prices of nitrogen and phosphatic fertilizers have increased in many third world countries. Therefore, it is very challenging for farmers to supplement N and P fertilizers in the soil to avoid the nutrient deficiencies. Given the negative environmental impacts of chemical fertilizers and increasing costs, utilization of phosphate solubilizing bacteria (PSB) is advantageous for sustainable agricultural practices. Thus, one area of increasing interest is the use of microorganisms with the ability to solubilize mineral and organic P (Shiri-Janagard *et al*., 2012). The association between PSB and plant roots plays a key role in P nutrition in many agroecosystems, particularly in P-deficient soils (Goldstein, 2007; Jorquera *et al*., 2008). The use of microbial inoculants possessing P-solubilizing activities in agricultural soils is considered as an environmental-friendly alternative to further applications of chemical based P fertilizers.

Symbiotic and non-symbiotic bacteria have been isolated from the root nodules of a wide range of legumes (Zakhia *et al*. 2006; Kan *et al.* 2007; Li *et al*. 2008 and Pandya *et al*., 2015). Bacillus is attractive as a potential inoculant in agriculture, as it produces very hardy spores that can survive for prolonged periods in soil and in storage containers (Araújo, 2008). The inoculation of plants with selected PSB to increase native population can mobilize P from poorly available sources and therefore improve plant nutrition (Guiñazu *et al*., 2010). Increased growth and P uptake have been reported for *Paenibacillus polymyxa* and *B. megaterium* in tomato (EI-Yazeid & Abou-Aly, 2011).

Growth and yield of legumes have been shown to increase with inoculation with rhizobia. This can be further enhanced by inoculating them with co-cultures of rhizobia and the PSB especially in P deficient soil conditions. Co-inoculation with P-solubilizing bacteria and *Rhizobium* stimulated plant growth more than their separate inoculations (Walpola & Yoon, 2013). Bai-YuMing *et al*. (2003) reported that co-inoculation of Bacillus strains in soybean plants with *Bradyrhizobium japonicum* provided the largest increases in nodule number, nodule weight, shoot weight, root weight, total biomass, total nitrogen and grain yield. Results by Tariq *et al.* (2012) showed that non-rhizobial plant growth promoting bacteria improve nodulation and grain yield of the legumes upon co-inoculation with crop specific rhizobia. Remans *et al*. (2007) also reported an increased ability of *Rhizobium* isolates nodulation on bean plants as the result of phosphate solubilizing bacteria co-application. Silva *et al*. (2007), found specific nodulation stimulus and increase in root dry matter in *Vigna unguiculata* co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus polymyxa* (Loutit (L) and *Bacillus sp.* (LBF-410). Similarly, endophytic plant-growth promoting bacteria and nitrogen-fixing *Rhizobium* species have been found to work in synergy to promote nitrogen fixation efficiency in lentils (Veena & Poonam, 2011).

The PSB which increases the efficiency of the *Rhizobium* species in one legume does not necessarily do the same in other legumes. For example, the strain *Bacillus sp*. CECT 450 increased nodulation in common bean when co-inoculated with *Rhizobium tropici* CIAT 899, but it reduced nodulation in soybean when co-inoculated with USDA 110 strain (Camacho *et al*., 2001). Similarly, in a study by Elkoca *et al*. (2012) it was noted that except for OSU-142 + M-3, inoculation with dual and triple mixtures with *Rhizobium*, OSU-142, and M-3 had no significant effect on common bean yield compared with single inoculations of these bacteria. The variable responses to co-inoculation underscores the need to find appropriate rhizobia strain and phosphate solubilizing bacteria which will enhance growth of common bean and soybean under low phosphorus levels.

In Kenya, much emphasis in bio-fertilization of legumes has been put on rhizobia inoculation. Although the role of phosphorus fertilizers in nodulation, nitrogen fixation and growth of soybean has been reported information is scanty regarding the role of phosphate-solubilizing bacteria in phosphorus bioavailability, growth promotion and also their interaction

with N_2-fixing bacteria. The isolation of native root endogenous PSB has not been reported in Kenya and their influence on growth promotion of common bean have not been documented. The present study was therefore designed to (1): isolate and identify PSB from common bean root nodules and (2): evaluate the effect of co-inoculation of *Bacillus sp* and *Rhizobium sp* for improving growth and nodulation of common beans in a low P soil.

4.3 Materials and methods

4.3.1 Isolation and identification of PSB

The experiment was conducted in pots in the greenhouse using soils from different locations of Nakuru County to trap bacteria from the soil using common bean (Lyamungu-85 variety) as a trap crop. Five soils (from Rongai, Bahati, Ngata, Egerton and Lare regions in Nakuru County) of medium fertility were used. Soils were obtained from fields in which common bean had recently been cultivated. Top soil was sampled from different points in the farm and mixed to obtain a composite sample. The soils were air dried and sieved through 2 mm sieve. Two kilograms of each soil were weighed into white, perforated plastic pots (volume = 2.5 L), and placed on plastic plates. New common bean variety Lyamungu-85 was used as the trap crop. The pot trials were laid out following a completely randomized design with 3 replicates **(**5 soils x 3 replicates = 15 pots).

4.3.2 Seed sterilization, planting and thinning

Seeds were surface-sterilized by soaking in 3.5% NaOCl solution for 5 minutes, then thoroughly washed with sterile, distilled water. Three pre-selected healthy seeds of uniform size were then planted per pot, and thinned to one plant per pot of comparable height and vigor between 1 – 2 weeks after planting. Plants were watered daily using distilled water, and twice daily (when necessary) during later growth stages to avoid water stress to maintain the soil at field capacity.

4.2.3 Basal nutrient addition

Macronutrients P, K, Mg, Ca, and S, and micronutrients Mn, Zn, Cu, B, Mo, and Co were added at optimal rates (750 mg K, 270 mg Ca, 165 mgMg, 60 mg S, 36 mg Mn, 1.5 mg Zn, 0.6 mg Cu, 0.9 mg B, 0.15 mg Mo and 0.15 mg Co pot^{-1}) to allow conditions for the rhizobia to demonstrate their maximal capacity to enhance N nutrition. The standard nutrient solution was

prepared as described by Broughton & Dillworth (1971) and 10ml added at planting and 10 ml three weeks after planting in each pot.

4.3.4 Harvesting

Crops were grown under greenhouse conditions until flowering stage of growth (7-8 weeks). At harvest, shoots were cut using a clean, sharp knife at 1 cm above the soil surface. Thereafter, the pots were emptied on a 2 mm sieve and soil was washed away gently to isolate the roots. Nodulation and fresh total root weight were assessed. Nodule samples were taken for laboratory analysis.

4.3.5 Laboratory isolation of nodule endophytes strains

The nodule endophyte strains were isolated from crushed nodules stored at –20° C in glycerol by streaking onto yeast mannitol agar (YMA) plates (Vincent, 1970). Purity of colonies was checked for by repeated streaking on YMA plates and by microscopic examination of living cells. Microscopic observations were performed to investigate some characteristics of the isolates such as shape and gram reaction. Catalase test was also carried out where bacterial cultures (24 h) were used. Single bacterial colony was placed on glass slide and a drop of 30% hydrogen peroxide (H_2O_2) was added. Appearance of gas bubbles indicated the presence of catalase enzymes in the bacteria.

4.3.6 DNA extraction

DNA was extracted from seven isolates that showed positive results for the PSB as described by Wilson (1987). Liquid culture from the isolation step (1.2 ml) was centrifuged for 5 min at 13000 rpm at room temperature. The supernatant was poured out and the pellet suspended by adding 500 µl of TE 1 X. The suspension was then centrifuged for 5 min at 13000 rpm at room temperature and the supernatant poured out. The pellet was re-suspended in 540 µl of TE 5 X and incubated for 15 min at 70^{o}C. Two microliters of proteinase K, 30 µl of 10% SDS (w/v) were added and incubated for 15 min at 70^{o}C. Six hundred microliters of phenol: chloroform:isoamylalcohol 25:24:1 (v/v/v) was added and centrifuged at 13000 rpm for 5 min at room temperature. The supernatant was transferred to a clean tube and extracted with an equal volume of choloroform: isoamylalcohol 24:1 (v/v) to remove residual phenol. It was centrifuge for 5 min at 13000 rpm at room temperature, and the supernatant transferred into another clean Eppendorf tube and the volume noted. The DNA was precipitated by the addition of 100 µl of ice-cold isopropanol and incubated overnight at -20^{o}C. The precipitated DNA was centrifuged

for 5 min at 13000 rpm at 4^{o}C, the supernatant poured out the pellet cleaned with 500 µl of 70% Ethanol. It was then centrifuge for 10 min at 13000 rpm at 4^{o}C and the supernatant poured out. The pellet was air dried and then dissolved in 50 µl double distilled water and stored at -20^{o}C for further analysis (COMPRO-II, 2014).

4.3.7 Molecular characterization of strains

The 16S-23S rDNA intergenic region was amplified through PCR. Primers used for amplification were 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'TACGGCTACCTTGTTACGACTT 3') (Lane 1991). The PCR mix for one sample included: PCR master 12.5 µl, Forward primer 27F 1 µl, Reverse primer 1 µl, sterile distilled water 7.5 µl and 3 µl DNA template. Amplification was performed in a Bio-Rad PCR system thermal cycler adjusted to the following program: initial denaturation for 5 min at 94 $^{\circ}$C, 35 cycles of denaturation for 30 sec at 94 $^{\circ}$C, annealing for 30 sec at 58 $^{\circ}$C, extension for 30 sec at 72 $^{\circ}$C and final extension for 7 min at 72 $^{\circ}$C. The PCR products were visualized by electrophoresis of 3 µl of the amplified DNA on 1% (w/v) horizontal agarose gel (SIGMA®) in TBE buffer (1.1 w/v Tris-HCL; 0.1% w/v Na_2EDTA $2H_2O$; 0.55% w/v Boric acid), pre-stained with 3.5 µl of ethidium bromide. The gel was photographed under UV illumination with Gel Doc (BIO-RAD) Software (USA). Products with a single band were selected as suitable for purification.

4.3.8 Purification of PCR product and sequencing

GeneJET PCR Purification Kit was used to purify the DNA. This kit includes binding buffer, washing buffer and elution buffer. PCR product was mixed well with binding buffer in a ratio of 1:1, centrifuged for 30 sec at 13000rpm and flow through was discarded. Then, 700 µl of washing buffer were added each to the mixture and centrifuged for 30 sec at 13000 rpm and flow-through was discarded. Then the PCR product was centrifuged for 30 sec at 13000 rpm to get rid of all the ethanol. Finally, 50 µl of elution buffer was added, centrifuged for 1min at 13000rpm and purified PCR products were collected. The purified PCR product was submitted for sequencing.

4.4 Co-inoculation effect of the PSB with rhizobia strains on growth of common bean

4.4.1 Plant material and pot volume

The test crop used was common beans; AFR 708 (AFR 708) and GLP 2 (GLP 2) varieties. AFR 708 is a newly released common bean variety by Egerton University with special

attributes of being high yielding, and pest and disease resistant (Njoka *et al*., 2009). GLP 2 is a high yielding variety suitable for medium altitudes (KALRO, 2008). Soils from Chuka were collected from the 0-20 cm top layer, air-dried, sieved to pass 2 mm and thoroughly homogenized. Pot volume was 5.3 L (15.0 cm inner diameter and 30 cm length), and contained 4 kg of soil from Chuka. PVC tubes were closed at the bottom using a nylon mesh, and placed on plastic plates.

4.4.2 Bacterial strains and treatment structure

The *Paenibacillus polymyxa* and *Bacilllus megaterium* used were isolated from Nakuru area, while the rhizobial strains IITA-PAU987 and IITA-PAU983 are from work done on indigenous rhizobia from Ethiopia. The reference strain CIAT 899 (*Rhizobium tropici*) was obtained from the University of Nairobi soil microbiology laboratory. The experiment was laid out in a completely randomized design (CRD) with three replicates in the greenhouse and the pots were rotated regularly on the benches to reduce the effect of sunlight intensity at the different times of the day. A detailed treatment structure is represented in Table 4.1. The soil characteristics are similar with the one used in Chapter 3 for the Nitisol (Table 3.4).

Table 4.1: Treatment structure for testing PGPR

Factor	**number**	**Description**
Variety	2	AFR 708, GLP 2
Soil type	1	Nitisol
Inoculation	11	IITA-PAU983, IITA-PAU987, CIAT 899, *P. polymyxa* + IITA-PAU983, *B. megaterium* +IITA-PAU983, *P. polymyxa* + IITA-PAU987, *B. megaterium* + IITA-PAU987, *P. polymyxa* + CIAT 899, *B. megaterium* + CIAT 899, positive control (no inoculation + N-fertilizer), Negative control (no inoculation, no N-fertilizer)
Reps	3	
No. of pots		2×1×11×3=**66**

4.4.3 Nutrient addition

Nutrient rates were based on common bean plant requirements. Macronutrients P, K, Mg, Ca, and S, and micronutrients Mn, Zn, Cu, B, Mo, and Co was added at optimal rates (750 mg K, 270 mg Ca, 165 mg Mg, 60 mg S, 36 mg Mn, 1.5 mg Zn, 0.6 mg Cu, 0.9 mg B, 0.15 mg Mo and 0.15 mg Co pot^{-1}) as a basal application . For N-control, 100 kg N $ha^{-1}$was applied as 384 kg ha^{-1} Calcium Ammonium Nitrate (CAN = 0.64 g pot^{-1}). A quarter (25%) of N (0.16 g) was applied at planting and the remaining 75% (0.48 g) after three weeks (Woomer *et al*., 2011). Stock solutions were prepared separately and mixed at the required application rate when required for fertilization following procedure described by Broughton and Dillworth (1971) and 10ml of the nutrient was added at planting and 10 ml three weeks after planting.

4.4.4 Inoculum preparation

For the inoculation study rhizobia strains were grown in yeast mannitol broth (YMB), in flasks and shaken at 28^{o}C at 200 rpm in a rotary shaker for 5-7 days until (turbid) when logarithmic phase is attained (approximately 1 x 10^{9}cells ml^{-1}) (Woomer *et al*., 2011). Bacillus and Paenibacillus strains were cultured in nutrient broth medium in 250 ml flasks and shaken at 200 rpm at 37˚C for 24 h. The 5 day old culture of Rhizobia strains and 24 h old cultures of the other strains were used as inoculants (Atieno *et al*., 2012).

4.4.5 Planting, thinning and water addition

Seeds were surface-sterilized by soaking in 3.5% NaOCl solution for 5 minutes and then thoroughly washed with distilled water. Two healthy seeds of uniform size were then planted per pot, and thinned to one plant per pot of comparable height and vigour at 7 days after planting. The pots were watered regularly to maintain the soil at field capacity.

4.4.6 Inoculation

One ml of log phase bacterial culture was inoculated in the treatment pot seven days after planting. For the co-inoculation treatments, a cocktail consisting of the two strains was prepared in the ratio of 1:1 and 1 ml of the mixture inoculated to the crop seven days after planting (Wasike *et al.*, 2009).

4.5 Data collection

The data collected included nodulation and shoot and root dry weights of the crops. At mid podding after planting, plants were carefully uprooted from the pots and placed on sieves to

avoid loss of nodules during cleaning. The soil was then gently washed off the roots under a stream of running tap water. Roots were inspected for nodulation using a 0 to 5 scoring system (Howeison & Dilworth, 2016) where 0 = no nodules, 1 = 1 to 5 nodules (rare), 2 = 6 to 10 nodules (few), 3 = 10 to 20 nodules (moderate), 4 = 20 to 50 nodules (abundant) and 5 = >50 nodules (extra abundant). The nodules were then carefully removed from the roots, counted and weighed. The above ground biomass and root dry weight was taken after drying to a constant weight at 65 ºC.

4.6 Data analyses

Data were collected in replicates of three and analysed using SAS Statistical Package Version 9.3. To determine the effects due to inoculation, analysis of variance at 95% confidence limit was done and means were separated using Duncan Multiple Range Test (DMRT) at $\alpha = 0.05$. Orthogonal contrast was also done to test for significance between the single and co-inoculation and between inoculation and the controls.

Statistical model

$\mathbf{Y_{ijk} = \mu + \alpha_i + \beta_j + R_k + \alpha\beta_{ij} + \Sigma_{ijk}}$

$\mathbf{Y_{ijkl}}$ = Observation

$\mathbf{\mu}$= overall mean

$\mathbf{\alpha_i}$= effect due to the i^{th} variety

$\mathbf{\beta_j}$ = Effect due to the j^{th} treatment

$\mathbf{R^k}$ = effect due to the k^{th} replication

$\mathbf{\alpha\beta_{ij}}$ = effect due to the i^{th} variety in the j^{th} treatment

$\mathbf{\Sigma_{ijkl}}$ = Random experimental error

4.7 Results

4.7.1 Morphological and biochemical characteristics of the isolated bacterial strains

Out of the 19 morph types obtained, seven of the colonies showed positive results for gram staining and catalase test. The isolates were found to be first growers (1-2 days). All the isolates produced round shaped and raised colonies having smooth shiny surface with smooth margin. All the isolates were rod shaped (Bacilli) and Gram positive in reaction (Table 4.2).

Table 4.2: Morphological and biochemical properties of the bacteria isolates

S.No	Isolate	Colony shape	Colour	Elevation	Size	catalase	Cell Shape	Gram stain
1	HK1	Circular	White	Raised	Small	+	Rod	+
2	HK2	Circular	Creamy white	Raised	Medium	+	Rod	+
3	HK3	Circular	White	Raised	Medium	+	Rod	+
4	HK4	Circular	White	Raised	Small	+	Rod	+
5	HK5	Circular	Creamy white	Raised	Small	+	Rod	+
6	HK6	Circular	White	Raised	Small	+	Rod	+
7	HK7	Circular	White	Raised	Medium	+	Rod	+

4.7.2 Amplification of 16S rRNA by PCR

PCR amplification of 16S rRNA gene yielded DNA fragments of single bands for each isolates (Figure 4.1).

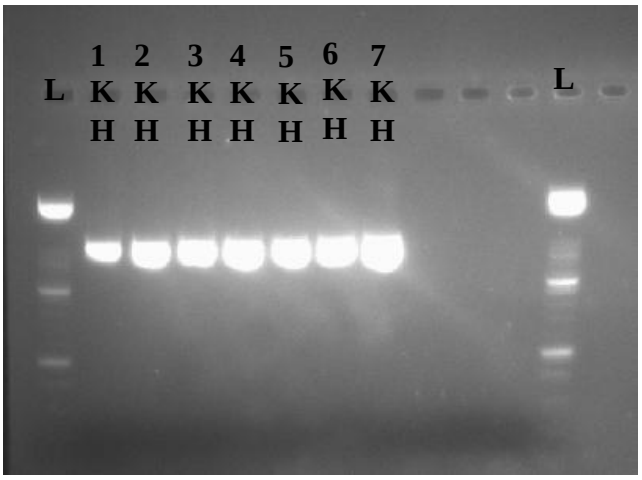

Figure 4.1: PCR product from DNA samples submitted to the SegoliP unit of BeCA-ILRI Hub for sequencing. L- ladder (1000bp)

4.7.3 Partial Sequencing of 16S rRNA Gene and Sequence Analysis

Seven bacterial isolates were successfully isolated from the nodules of common bean. They were characterized by 16S rDNA partial gene sequencing. There was no dominant species within 7 isolates of *Bacillus* sp. based on bioinformatics analysis using BLASTN program.

Maximum identities for each isolate were 98-99% with E-value 0. The distributions were genetically diverse on several species of *Bacillus* sp., such as *B. megaterium*, *B. subtilis*, *B. aryabhattai* and *P. polymyxa* (Table 4.3).

Table 4.3: Molecular identification of common bean nodule endophytes by 16S rDNA sequencing

Sequence ID	Query length	Blast-related sequence	Accession	E- value	Identity
HK1	1447	*Paenibacillus polymyxa*	NC_014483.1	0.0	98%
HK 2	1492	*Bacillus megaterium strain*	KF658192.1	0.0	98%
HK3	1457	*Bacillus aryabhattai strain*	KJ009477.1	0.0	98%
HK4	1400	*Bacillus megaterium*	JF496300.1	0.0	99%
HK5	1437	*Bacillus subtilis*	KJ496376.1	0.0	99%
HK6	1441	*Bacillus megaterium*	KC441754.1	0.0	99%
HK7	1427	*Bacillus sp*	AB508884.1	0.0	98%

4.7.4 Effect of the treatments on the common bean growth parameters

The nodule numbers, nodule weight, and shoot and root dry weight were significantly affected by inoculation. However, there was no significant variety effect on all the parameters tested. There was no significant variety*treatment interactions (Appendix III).

4.7.5 Effect of rhizobia-PSB co-inoculation on the nodulation of common bean

Inoculation of common beans with rhizobial strains significantly increased the number of nodules of common bean over the control (Figure 4.2 and Table 4.4). Co-inoculation of the rhizobia strains with the phosphate solubilizing bacteria (PSB) furthur enhanced the nodulation compared to single rhizobial inoculation except for IITA-PAU987 and CIAT 899. Application of mineral nitrogen recorded the least number of nodules (Figure 4.2 and Table 4.4).

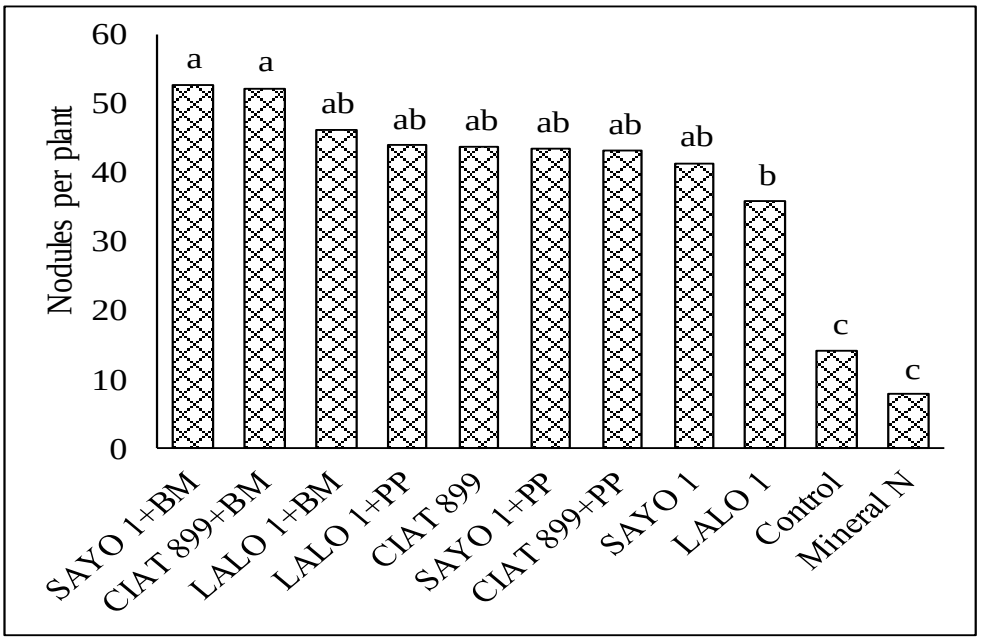

Figure 4.2: Effect of co-inoculation on the number of nodules of common bean. PP- *Paenibacillus polymyxa*, BB- *Bacillus megaterium.* Means followed by the same letter are not significantly different at $\alpha = 0.05$

Table 4.4: Effect of rhizobia-PSB co-inoculation on the nodulation of common beans

Treatment	Nodule number	Score	Nodulation
IITA-PAU987 + *B. megaterium*	53	5	Extra abundant
CIAT 899 + *B. megaterium*	52	5	Extra abundant
IITA-PAU983 + *B. megaterium*	46	4	Abundant
IITA-PAU983 + *P. polymyxa*	44	4	Abundant
CIAT 899	44	4	Abundant
SAYO + *P. polymyxa*	43	4	Abundant
CIAT 899 + *P. polymyxa*	43	4	Abundant
IITA-PAU987	41	4	Abundant
IITA-PAU983	36	4	Abundant
Control	14	3	Moderate
Mineral N	8	2	Few

2 = 6 to 10 nodules, 3 = 10 to 20 nodules, 4 = 20 to 50 nodules and 5 = >50 nodules (extra abundant) - (Howeison & Dilworth, 2016)

4.7.6 Effect of rhizobia-PSB co-inoculation on the nodule weight, shoot and root dry weight of common bean

Common bean showed varied responses to co-inoculation of rhizobia strains with the PSB on the nodule weight as compared to single rhizobia inoculation. Co-inoculation of IITA-PAU987 + *B. megaterium* recorded the highest nodule weight (405.2 mg) compared to IITA-PAU987 alone (324.8 mg), followed by CIAT 899 + *B. megaterium* (401.2 mg) compared to CIAT 899 alone (337.2 mg). However, CIAT 899 alone recorded a higher nodule weight as compared to CIAT 899 + *P. polymyxa* (Table 4.5). Inoculation with rhizobia in combination with the PSB recorded significantly higher nodule fresh weight compared to the mineral fertilizer application and control. Rhizobia inoculation resulted in a higher nodule fresh weight compared to the control and the mineral nitrogen application (Table 4.5).

Co-inoculation of rhizobia strains and PSB had varied effect on shoot dry weight compared to single rhizobia inoculation and was dependent on the specific rhizobium-PSB combination. For instance CIAT 899 + *B. megaterium* recorded a significantly a higher shoot dry weight percentage over the control (124.7 %) compared to CIAT 899 alone (81 %). However, there was no significant difference between CIAT 899 + P. polymyxa (84.5 %) and CIAT 899 alone (81 %) increase over the control. Similarly, combination of IITA-PAU987 and *B. megaterium* led to significantly higher shoot dry weight over the control compared to IITA-PAU987 alone (112.6 5% and 65.2% respectively) but no significant difference was observed when co-inoculated with *P. polymyxa*(Table 4.5). However IITA-PAU983 in combination with *P. polymyxa* led to significantly higher shoot dry weight (7.1 g) compared to IITA-PAU983 alone (5.1 g) and IITA-PAU983 + *B. megaterium* (5.7 g) (Table 4.5). There was no significant difference among the three rhizobia strains under study on the shoot dry weight of the common bean. However, the three rhizobia strains led to a significant increase in shoot dry weight over the negative control (65.2 % and 59.1 % increases). Mineral nitrogen application recorded the highest shoot dry weight while the control recorded the least (Table 4.5).

In terms of root dry weight, co-inoculation of rhizobia strains with the PSB did not significantly increase the root dry weight compared to single rhizobial inoculation. Apart from IITA-PAU983 and IITA-PAU987 + *P. polymyxa*, all the other inoculation treatments performed at par with the mineral nitrogen application (Table 4.5). Co-inoculation of the rhizobia strains and PSB

significantly increased the root dry weight compared to the control apart from CIAT 899 + *P. polymyxa* and IITA-PAU987 + *P. polymyxa* (Table 4.5).

4.7.7 Orthogonal contrast of the factors affecting common bean growth parameters

The results showed a significant contrast between inoculation versus mineral N application, inoculation versus control and control versus the others in terms of the nodule number and nodule fresh weight (Table 4.6). However there was no significant difference between the single and dual inoculation. In terms of shoot dry weight and root dry weight of common beans, all the other contrasts were significant except for the inoculation versus mineral N application. In soybean all the contrasts were significant except for the root dry weight in the single versus dual inoculation and inoculation versus mineral N application (Table 4.6).

Table 4.5: Effect of co-inoculation on the nodule fresh weight, shoot and root dry weight of common bean

Treatment	NFW (mg)	SDW (g)	% Increase over control	RDW (g)	% Increase over control
CIAT 899	337.2ab	5.8cde	81.0	0.9ab	115.7
CIAT 899 + *B. megaterium*	401.2a	7.2b	124.7	1.0ab	127.3
CIAT 899 + *P. polymyxa*	331.3ab	5.8cde	84.5	0.8abc	97.1
IITA-PAU987	324.8ab	5.3e	65.2	0.8abc	70.2
IITA-PAU987 + *B. megaterium*	405.2a	6.8bcd	112.6	1.0ab	123.5
IITA-PAU987 + *P. polymyxa*	340.0ab	5.9bcde	85.5	0.9ab	108.3
IITA-PAU983	268.6b	5.1e	59.8	0.7bc	43.8
IITA-PAU983 + *B. megaterium*	354.8ab	5.7de	80.0	0.9ab	97.1
IITA-PAU983 + *P. polymyxa*	336.7ab	7.2bc	77.1	0.9ab	120.1
Control	137.3c	3.2f	0.0	0.5c	0.0
Mineral N	47.2c	9.3a	188.6	1.2a	168.3

Means followed by the same letter within a column are not significantly different at $\alpha = 0.05$. NFW- nodule fresh weight, SDW- Shoot dry weight, RDW- root dry weight

Table 4.6: Mean square table of orthogonal contrast of the treatments on the growth parameters of common bean

Contrast	NN	NFW	SDW	RDW
Single vs Dual	126.8^{ns}	24390.1^{ns}	110.5^{***}	1.7^{***}
Inoculation vs +N	6183.5^{***}	341562.7^{***}	5.0^{ns}	0.01^{ns}
Inoculation vs control	11646.0^{***}	652998.7^{***}	0.3^{*}	0.1^{*}
Control vs others	1561.7^{***}	53798.1^{***}	75.6^{***}	1.5^{***}

NN- number of nodules, NFW- Nodule fresh weight, SDW- shoot dry weight, RDW- Root dry weight, +N- mineral nitrogen treatment. *- significant at 0.05 level, ***- significant at 0.001 level, ns- not significant

4.8 Discussion

4.8.1 Diversity of nodule endophytic phosphate solubilizing bacteria

Endophytic non rhizobial root endophytes were isolated from root nodules of common bean. An increasing number of α, β and γ*_Proteobacteria* have been isolated from root nodules of a wide range of legumes and are reported as nodule associated bacteria or nodule endophytes (Zakhia *et al.*, 2006; Kan *et al.*, 2007). Such nodule associated bacteria may be endophytic or free living rhizobacteria and may establish neutral or beneficial interactions with plants (Pandya *et al*., 2015; Li *et al*., 2008; Muresu *et al*., 2008 and Stajkovic, 2009). Results from this study are in line with the studies by Rajendran *et al*. (2008) who reported the presence of high proportion of gram positive endophytes within the root nodules of pigeon pea.

Root nodules also accommodate various non-nodulating bacteria having definite influence on the survival, nodulation and grain yield of crop and their densities are reported to be very high (Mishra *et al*. 2009; Tariq *et al*., 2014). Hung *et al.* (2007) reported that the isolation of *Paenibacillus polymyxa* HKA-15, a Gram-positive bacterium from root nodules of soybean. A total of 75 endophytic bacteria roots and nodules of field pea (Narula *et al*., 2013) and 88 from roots and nodules of chickpea showed that 50% in roots and 93.4% in nodules were Gram positive. Diep *et al*. (2016) also reported the presence of endophytic bacteria in soybean root nodules.

4.8.2 Effect of co-inoculation of rhizobia and PSB on nodulation, shoot and root dry weight of common bean

In this study, inoculations either singly or combined resulted in increased growth of common bean. All plant factors measured were positively affected by inoculation with rhizobial strains IITA-PAU983, IITA-PAU987 and CIAT 899. Rhizobial strains were able to increase nodule number and weight, shoot dry weight, and root dry weight over uninoculated control plants. Gicharu *et al*. (2013) also reported a significant increase in nodulation when three climbing bean cultivars were inoculated with CIAT 899 rhizobia strains compared to the control treatments. Similar results were reported by Majid *et al*. (2009) and Salih *et al*. (2015) on inoculation of legumes with appropriate rhizobia strains. On-field inoculation of *P. vulgaris* with strains 8a3 and 4H41 showed that nodule number and shoot dry weight of common bean were increased significantly compared to the control plants (Trabelsi *et al*., 2011). Mehrpouyan (2011) also reported significant increase in nodule number and dry weight in common bean cultivars when inoculated with *Rhizobium leguminosarum* strain Rb117. Javaid & Mahmood (2010) reported that inoculation of soybean with specific Bradyrhizobium strains improved the plant dry matter, nitrogen concentration, nitrogen accumulation, and grain yield.

Co-inoculation, frequently, increase growth and yield, compared to single inoculation, provided the plants with more balanced nutrition, and improved absorption of nitrogen, phosphorus, and mineral nutrients (Araujo *et al*., 2009). In this study, co-inoculation of *Rhizobium* and *B. megaterium and P. polymyxa* enhanced nodulation, shoot and root dry weight as compared to the control. This may be due to direct and indirect enhancement of plant growth by a variety of mechanisms such as production of growth promoting substance and solubilization of minerals such as P (Verma *et al*., 2012). Phosphorous availability increases the number and size of nodules, the amount of nitrogen assimilated per unit weight of nodules, the percent and total amount of nitrogen in the harvested portion of the host legume and improving the density of Rhizobia bacteria in the soil surrounding the root (Bashir *et al*., 2011). Phosphorous deficiency has been shown to affect symbiosis by decreasing the supply of photosynthates to the nodule, which reduces the rate of bacterial growth and the total population of legume-nodulating microorganisms (Moreira *et al*., 2010). The positive effect of combined inoculation of some endophytic bacteria with *Rhizobium* spp. can be attributed to an early nodulation, an increase in the number of nodules, or a general improvement in root development (Sa *et al.*, 2012)

The results from this study showed increased number of nodules and nodule weight due to co-inoculation with *P. polymyxa* and *B. megaterium* in compared to the inoculation with *Rhizobium* alone in both common bean varieties. Similarly, Stajkovic *et al*. (2011) reported that co-inoculation of bean with *Bacillus* strains SNji and Bx was found to positively influence nodule number (106.67 and 76.67 nodule number plant^{-1}, respectively) compared to inoculation with *Rhizobium* alone (54 nodule number plant^{-1}). Enhancement in nodule number, nodular mass due to combined inoculation might be the expansion in root length and mass, thus more number of active sites for nodulation by the rhizobial strains.

In this study, co-inoculation of common bean with *Rhizobium* and the PSB, significantly increased shoot and root dry weights of plants in respect to *Rhizobium* inoculation alone and the uninoculated control showing the potential of these strains to improve dry matter accumulation over *Rhizobium* alone. Similarly, Figueiredo *et al*. (2008) recorded a higher shoot and root dry weight when CIAT 899 rhizobia strains were coinoculated with *Paenibacillus polymyxa* strain DSM 36 than single inoculation with CIAT 899 strain in common bean. Additionally, Elkoca *et al*. (2012) reported an increased shoot dry weight as a result of co-inoculation of common bean with *Bacillus megaterium* (M-3) strain and *Rhizobium* strain.

In studies with other legumes, synergism between Bacillus and Bradyrhizobium in the rhizosphere has been shown to increase nodulation and plant biomass (Medeot *et al*., 2010; Tsigie *et al*., 2012). In soybean, pot experiments were conducted by Fatima *et al*. (2006) to evaluate the effects of *Rhizobium leguminosarum* strain alone and in combination with PSB (*Bacillus* and *Pseudomonas*). The plants grown with combination showed increased root and shoot weight suggesting a promising way for enhancing the growth of legume crops. The results from field and greenhouse chickpea experiments by Verma *et al*. (2012) showed significant results with the combination of *Mesorhizobium* sp. BHURC02 with *B. megaterium*, to be superior over uninoculated control.

4.9 Conclusion

The results from this experiment showed that rhizobia inoculation and co-inoculation with PSB enhances nodulation of both common bean over the control treatment (185.7% and 235.7% respectively). It also showed that co-inoculation of the rhizobial strains with the tested phosphate solubilizing bacteria further enhances the growth of common bean in a phosphorous

deficient soil. Therefore *P.polymyxa* and *B. megaterium* strains can be used together with the tested indigenous rhizobia strains and *Bradyrhizobia japonicum* to improve growth of common beans and soybean respectively in soils that are low in phosphorous. *B. megaterium and P. polymyxa* strains can be used in further investigations as potential agents of new biofertilizer for improved common bean and soybean production. Also further studies will be needed to validate promoting effects of inoculation under field conditions.

References

Araújo, F., Sérgio, A., and Figueiredo, M. V. B. (2009). Role of plant growth-promoting bacteria in sustainable agriculture chapter 10 In: *Sustainable Agriculture: Technology, Planning* Editor: Salazar, A. and Rios, I.

Atieno, M., Hermann, L., Okalebo, R. & Lesueur, D. (2012). Efficiency of different formulations of *Bradyrhizobium japonicum* and effect of co-inoculation of *Bacillus subtilis* with two different strains of *Bradyrhizobium japonicum. World Journal of Microbiology and Biotechnology* 28:2541–2550.

Bai, Y., D'Aoust, F., Smith, D. L. & Driscoll, B. T. (2002). Isolation of plant growth promoting *Bacillus* strains from soybean root nodules. *Canadian Journal of Microbiology* 48: 230 238.

Bai-YuMing, Zhou-Xiao, M. & Smith, D. L. (2003). Enhanced soybean plant growth resulting from coinoculation of Bacillus strains with *Bradyrhizobium japonicum*. *Crop Science* 43:1774–1781.

Bashir, K., Ali, S. & Umair, A. (2011). Effect of different phosphorus levels on xylem sap components and their correlation with growth variables of mash bean. *Sarhad Journal of Agriculture* 27(4): 1-6.

Broughton, W. J. & Dilworth, M. J. (1971). Control of leghaemoglobin synthesis in snake beans. *Journal of Biochemistry* 125:1075-1080

COMPRO-II (2014). DNA extraction from liquid bacterial cultures. Retrieved from http//:www.compro2.org/compro2-publications/Sops/category/8-molecular-biology.html (Accessed on 5th February, 2015).

Camacho, M., Santamaria, C., Temprano, F., Rodriguez-Navarro, D. N. & Daza, A. (2001). Co-inoculation with Bacillus sp. CECT 450 improves nodulation in *Phaseolus vulgaris* L. *Canadian Journal of Microbiology* 47:1058–1062.

Collavino, M. M., Sansberro, P. A., Mroginski, L. A. & Aguilar, O. M. (2010). Comparison of *in vitro* solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. *Biology and Fertility of Soils* 46 (7): 727-738.

Diep, C.N., My, N. T. X & Nhu, V. T. P. (2016). Isolation and characterization of endophytic bacteria in soybean root nodules. *World Journal of Pharmacy and Pharmaceutical Sciences* 5(6): 222-241

Elkoca, E., Turan, M. & Donmez, M. F. (2012). Effects of single, dual and triple inoculations with Bacillus subtilis, *Bacillus megaterium* and *Rhizobium leguminosarum* BV. Phaseoli on nodulation, nutrient uptake, yield and yield parameters of common bean (*Phaseolus vulgaris* L. CV. 'ELKOCA-05'). *Journal of Plant Nutrition* 33(14): 2104-2119.

EI-Yazeid, A. A. & H. E. Abou-Aly. (2011). Enhancing growth, productivity and quality of tomato plants using phosphate solubilizing microorganisms. *Australian Journal of Basic and Applied Sciences* 7:371-379.

Fatima, Z., Muhammad, Z., & Fayyaz, C. (2006). Effects of Rhizobium Strains and Phosphorus on Growth of Soy Bean (*Glycin Max*) and Survival of Rhizobium and P Solubilizing Bacteria. *Pakistan Journal of Botany 38* (2): 459-464.

Figueiredo, M. V. B., Martinez, C. R., Burity, H. A. & Chanway, C. P. (2008). Plant growth-promoting rhizobacteria for improving nodulation and nitrogen fixation in the common bean (*Phaseolus vulgaris* L.). *World Journal of Microbiology and Biotechnology* 24:1187–1193.

Gicharu, G. K., Gitonga, N. M., Boga, H., Cheruiyot R. C. & Maingi, J. M. (2013). Effect of inoculating selected climbing bean cultivars with different rhizobia strains on nitrogen fixation.*International Journal of Microbiology Research* 1(2): 25-31.

Guiñazu, L. B., Andres, J. A., DelPapa, M. F., Pistorio, M. & Rosas, S. B. (2010). Response of alfalfa (*Medicago sativa* L.) to single and mixed inoculation with phosphate-solubilising bacteria and *Sinorhizobium meliloti*. *Biology and Fertility of Soils* 46:185–190.

Goldstein, A. H. (2007). Future trends in research on microbial phosphate solubilization: one hundred years of insolubility. In: Velazquez E, Rodriguez-Barrueco, C. (eds). First International Meeting on Microbial phosphate solubilization. Springer, Dordrecht, pp 91–96.

Howieson, J. G. and Dilworth, M. J. (2016). *Working with Rhizobia*. ACIAR publishing, Canberra, ACT.

Hung, P.Q., Kumar, S.M., Govindsamy, V. & Annapurna, A. (2007). Isolation and characterization of endophytic bacteria from wild and cultivated soybean varieties. *Biology and Fertility of Soils* 44: 155-162.

Javaid, A. & Mahmood, N. (2010). Growth, nodulation, and yield response of soybean to biofertilizers and organic manures. *Plant Journal of Botany* 42 (2): 863-871.

Jorquera, M. A., Hernández, M. T., Rengel, Z., Marschner, P. & Mora, M. L. (2008). Isolation of culturable phosphobacteria with both phytate mineralization and phosphate-solubilization activity from the rhizosphere of plants grown in a volcanic soil. *Biology and Fertility of Soils* 44:1025–1034.

Kan, F. L., Chen, Z. Y., Wang, E. T., Tian, C. F., Sui, X. H., & Chen, W. X. (2007). Characterization of symbiotic and endophytic bacteria isolated from root nodules of herbaceous legumes grown in Qinghai_Tibet plateau and in other zones of China. *Archives of Microbiology* 18: 103–115.

Khan, M. S., Zaidi, A. & Wani, P. A. (2006). Role of phosphate solubilizing microorganisms in sustainable agriculture-A review. *Agronomy for Sustainable Development* 26: 1-15.

Lane, D. J. (1991). *16S/23S rRNA sequencing*. *In Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.

Li, J. H., Wang, E. T., Chen, W. F. & Chen, W. X. (2008). Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. *Soil Biology and Biochemistry* 40: 238-246.

Majid, M. T., Kaleem, M., Khaliq, A. & Kazmi, M. H. (2009). Effect of Rhizobium inoculation and NP fertilization on growth, yield and nodulation of soybean (Glycine max). *African Journal of Biotechnology* 8 (22): 6191-6200.

Medeot, D. B., Paulucci, N. S., Albornoz, A. I., Fumero, M. V., Bueno, M. A., Garcia, M. B., Woelke, M. R., Okon, Y. & Dardanelli, M. S. (2010). Plant growth promoting rhizobacteria improving the legume-rhizobia symbiosis. 2010. In: Khan MS, Zaidi A, Musarrat J (eds) *Microbes for legume improvement*. Springer-Verlag/Wien, Berlin, pp 473–494.

Mehrpouyan, M. (2011). Nitrogen Fixation Efficiency in Native Strains Compared with Non-Native Strains of Rhizobia *Leguminosarum. 2011 International Conference on Environment Science and Engineering IPCBEE. IACSIT Press, Singapore, Vol.8* pp *216-219.*

Mishra, P.K., Mishra, S., Selvakumar, G., Bishr, J.K., Kundu, S. & Gupta, H.S. (2009). Co-inoculation of *Bacillus thuringeinsis*-KR1 with *Rhizobium leguminosarum* enhances plant growth and nodulation of pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.) *World Journal of Microbiology and Biotechnology* 25: 753-761.

Moreira, F. M. S., Carvalho, T. S. & Siqueira, J. O. (2010). Effect of fertilizers, lime, and inoculation with rhizobia and mycorrhizal fungi on the growth of four leguminous tree species in a low-fertility soil. *Biology and Fertility of Soils* 46: 771-779.

Muresu, R., Polone, E., Sulas, L., Baldan, B., Tondello, A., Delogu, G., Cappuccinelli, P., Alberghini, S., Benhizia, Y., Benhizia, H., Benguedouar, A., Mori, B., Calamassi, R., Dazzo, F. B. & Squartini, A. (2008). Coexistence of predominantly nonculturable rhizobia with diverse, endophytic bacterial taxa within nodules of wild legumes. *FEMS Microbiology Ecology* 63: 383-400.

Narula, S., Anand, R.C., and Dudeja, S.S. (2013). Beneficial traits of endophytic bacteria from field pea nodules and plant growth promotion of field pea. *Journal of Food Legume* 26(4): 73-79.

Njoka E. M**.,** Kimurto P. K**.,** Towett, B. K. Ogumu, M. O., Ngolo, A., Macharia, J. K. & Gatongi, I**.** (2009). Development and Release of 3 bean varieties *(AFR 708, Lyamungu-85 and Ciankui*) for Commercial Production in high ad medium altitudes of Kenya. *KEPHIS Reports, 2009 and Kenya Gazette Vol 9 (3), 2009.*

Olivera M, Tejera N, Iribarne C, Ocana A, LIuch C (2004). Growth, nitrogen fixation and ammonium assimilation in common bean (*Phaseolus vulgaris*): effect of phosphorus. *Physiologia Plantarum* 121: 498-505.

Pandya, M., Rajput, M. & Rajkumar, S. (2015). Exploring Plant Growth Promoting Potential of Non Rhizobial Root Nodules Endophytes of *Vigna radiate. Microbiology* 84(1): 110-119.

Rajendran, G., Sing, F., Desai, A. J. & Archana, G. (2008). Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* sp. *Bioresource technology* 99: 4544-4550.

Remans, R., Croonenborghs, A., Gutierrez, R. S., Michiels J. & Vanderleyden, J. (2007). Effects of plant growth-promoting rhizobacteria on nodulation of *Phaseolus vulgaris* L. are dependent on plant P nutrition. *European Journal of Plant Pathology* 119: 341-351.

Sa, A. L. B., Dias, A. C. F., Teixeiria, M. A. & Vieira, R. F. (2012). *Contribution of N_2 fixation for the world agriculture*. Chapter 17 in Bacteria in Agrobiology: Plant Probiotics. Springer-Verlag. Berlin Heidelberg, 2012 pp 33-42

Salih S. H, Hamd, S.A.M., & Dagash, Y. M. I. (2015). The Effects of Rhizobium, Mycorrhizal Inoculations and Diammonium Phosphate (DAP) on Nodulation, Growth, and Yield of Soybean. *Universal Journal of Agricultural Research* 3(1): 11-14.

Shiri-Janagard, M., Raei, Y., Gasemi-Golezani, G. & Aliasgarzad, N. (2012). Influence of *Bradyrhizobium japonicum* and phosphate solubilizing bacteria on soybean yield at different levels of nitrogen and phosphorus. *International journal of Agronomy and Plant Production* 3(11), 544-549.

Silva, V. N; Silva, L.E.S.F., Martinez, C.R., Seldin, L., Burity, H. A., Figueiredo, M. V. B. (2007). Effect of *Paenibacillus* strains on specific nodulation in *Bradyrhizobium*-bacillus symbiosis. *Acta Scientific and Agronomy*, 29: 331-338.

Stajkovic, O. (2009). Isolation and characterization of endophytic non-rhizobial bacteria from root nodules of alfalfa (*Medicago sativa* L.). *Botany Serbia* 33: 107-114.

Stajkovic, O., Delic, D., Josic, D., Kuzmanovic, D., Rasulic, N., Vukcevic, K.J. (2011). Improvement of common bean growth by co-inoculation with *Rhizobium* and plant growth-promoting bacteria. *Romanian Biotechnological Letters* 16(1): 5919-5926.

Tariq, M., Hameed, S., Yasmeen, T. & Ali, A. (2012). Non-rhizobial bacteria for improved nodulation and grain yield of mung bean [*Vigna radiata* (L.) Wilczek]. *African Journal of Biotechnology* 11(84): 15012-15019.

Tariq, M., Hameed, S., Yasmeen, T; Zahid, M; and Zafar, M. (2014) Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (Pisum sativum L.). *World Journal of Microbiology and Biotechnology* 30: 719-725.

Trabelsi, D., Mengoni, A., Ammar, H. B & Mhamdi, R. (2011). Effect of on field inoculation of *Phaseolus vulgaris* with rhizobia on soil bacterial communities. *FEMS Microbiology and Ecology 77*: 211–222.

Tsigie, A., Tilak, K. V. B. R. & Anil, K. S. (2012) Field response of legumes to inoculation with plant growth-promoting rhizobacteria. *Biology and Fertility Soil*s 47:971–974.

Veena, K. & Poonam, S. (2011) Potential for enhancing lentil (*Lens culinaris*) productivity by co-inoculation with PSB, plant growth-promoting rhizobacteria and Rhizobium. *The Indian Journal of Agricultural Sciences* 81: 932–934.

Verma, J.P., Yadav, J. & Tiwari, K. N. (2012). Enhancement of Nodulation and Yield of Chickpea by Co-inoculation of Indigenous *Mesorhizobium spp*. and Plant Growth–Promoting Rhizobacteria in Eastern Uttar Pradesh. *Communications in Soil Science and Plant Analysis* 43(3): 605-621.

Vincent, J. M. (1970). *A manual for the practical study of root-nodule bacteria*. IBP Handbook Number 15, Blackwell, Oxford.

Walpola, B. C & Yoon, M. (2013). Phosphate solubilizing bacteria: Assessment of their effect on growth promotion and phosphorous uptake of mung bean (*Vigna radiata* [L.] R. Wilczek)**.** *Chilean Journal of Agricultural Research* 73(3): 275-281.

Wasike, V., Vanlauwe, B., Mungai, N., Wachira, F., Mumera, L., Sanginga, N., Herrmann, L., Mburu, H, Malala, P., Wasilwa, L. & Lesueur, D. (2009). The Competitiveness of Indigenous *Bradyrhizobium Spp* and *Bradyrhizobium Elkanii* Strains on Promiscuous and Specific Soybean Varieties in Kenyan Soils. *Plant and soil* 322:25-35.

Woomer, P.L., Karanja, N., Kisamuli, S.M., Murwira, M. & Bala, A. (2011). A revised manual for rhizobium methods and standard protocols available retrieved from: www.N2Africa.org (Accessed on 18th April, 2015).

Zakhia, F., Jeder, H., Willems, A., Gillis, M., Dreyfus, B. & De Lajudie, P. (2006). Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for *nifH*_lik gene within the genera *Micro bacterium* and *Starkeya. Microbology and Ecology* 51: 375–393.

CHAPTER FIVE: GENERAL CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSIONS

1. The inoculant tested had variable quality with Biofix (common bean) containing more than one microorganism.
2. The inoculant showed positive results in nodulation and plant biomass relative to control. However this was dependent on soil type and legume variety.
3. Common bean nodules contained endopytic bacteria other than rhizobia in the nodules showing a degree of coexistence of rhizobia and other beneficial bacteria which could be involved in promoting plant growth
4. Rhizobia strains and reference strain (CIAT 899) strain enhanced the growth of common bean relative to uninoculated control. Co-inoculation of the rhizobia strains with *Paenibacillus polymyxa* and *Bacillus megaterium* further enhanced the growth of common bean compared to single inoculation with rhizobia strains. However, this was dependent on the specific Rhizobia-PSB combination.

5.2 RECOMMENDATIONS

1. There is need for the manufacturing companies to give full details of their product on the labels including the minimum number of viable microorganisms and the different types of microorganism present in their products
2. Recommendation for common bean and soybean inoculation should be variety and soil type specific. For example, farmers in Chuka (Nitisol) can plant the new Lyamungu-85 bean variety and inoculate with Biofix to replace GLP 2 variety.
3. There is need for further research to identify indigenous rhizobia strains from the study area that can elicit more nodulation and promote better plant growth than the commercial available inoculants.

6.0 APPENDICES

Appendix I: Mean square for the factors affecting common bean growth parameters

		ANOVA Mean square values					
Source of variation	df	NN	NFW	SDW	RDW	N	BNF
Soil	1	4.4^{***}	11.2^{***}	347.2^{***}	207.6^{***}	249.1^{ns}	174.5^{ns}
Variety	2	0.3^{ns}	2.5^{**}	5.9^{ns}	3.7^{ns}	10.5^{ns}	10.5^{ns}
Inoculation	2	8.8^{***}	18.4^{***}	274.0^{***}	23.2^{***}	1568.0^{***}	1568.0^{***}
Soil*variety	2	0.3^{*}	2.1^{ns}	22.9^{*}	0.7^{ns}	72.8^{ns}	72.8^{ns}
Soil*inoculation	2	0.5^{***}	2.7^{*}	55.5^{***}	5.7^{ns}	31.9^{ns}	31.9^{ns}
Variety*treatment	4	0.1^{*}	2.4^{*}	2.3^{ns}	1.1^{ns}	54.8^{ns}	54.8^{ns}
Soil*variety*inoculation	4	0.5^{**}	2.4^{*}	3.9^{ns}	4.6^{ns}	41.4^{ns}	41.4^{ns}
C.V (%)		36.4	82.4	23.0	32.8	24	39.3
Mean		67	1.0	10.9	4.1	37.1	23.5

NN- number of nodules, NFW- Nodule fresh weight, SDW- shoot dry weight, RDW- Root dry weight.

*- significant at 0.05 level, **- significant at 0.01 level, ***- significant at 0.001 level, ns- not significant

Appendix II: Mean square table for the factors affecting soybean growth parameters

		ANOVA Mean square values					
Source of variation	df	NN	NFW	SDW	RDW	N	BNF
Soil	1	18.8^{ns}	42781.0^{*}	230.6^{***}	66.8^{***}	144.5^{ns}	144.5^{ns}
Variety	1	363.0^{***}	28372.7^{ns}	120.9^{***}	19.9^{***}	24.5^{ns}	24.5^{ns}
inoculation	1	1845.4^{***}	71078.7^{***}	115.8^{***}	4.6^{*}	710.9^{**}	710.9^{**}
Soil*variety	3	1.3^{ns}	540.0^{ns}	33.6^{**}	19.1^{***}	680.0^{ns}	680.0^{ns}
Soil*inoculation	3	181.8^{ns}	6619.5^{ns}	3.1^{ns}	0.5^{ns}	370.6^{ns}	370.6^{ns}
Variety*inoculation	3	83.4^{*}	4708.5^{ns}	7.4^{ns}	3.4^{ns}	38.7^{ns}	38.7^{ns}
Soil*variety*inoculation	3	22.4^{ns}	2097.1^{ns}	1.0^{ns}	2.7^{ns}	668.3^{ns}	668.3^{ns}
C.V (%)		28.0	91.6	27.1	52.2	26.5	36.9
Mean		17.0	92.2	5.9	5.9	47.7	32.4

NN- number of nodules, NFW- Nodule fresh weight, SDW- shoot dry weight, RDW- Root dry weight, *- significant at 0.05 level, **- significant at 0.01 level, ***- significant at 0.001 level, ns- not significant.

Appendix III: Mean square table of the factors affecting common bean growth

	ANOVA Mean Square values				
Source of variation	df	NN	NFW	SDW	RDW
Variety	1	45.8^{ns}	2400.0^{ns}	0.02^{ns}	0^{ns}
Inoculation	10	1263.2^{***}	72942.2^{***}	14.0^{***}	0.3^{*}
Variety*inoculation	10	89.2^{ns}	5622.0^{ns}	2.0^{ns}	0.1^{ns}
C.V (%)		27.9	27.8	17.1	37
Mean		38.5	298.6	6.1	0.9

NN- number of nodules, NFW- Nodule fresh weight, SDW- shoot dry weight, RDW- Root dry weight.

*- significant at 0.05 level, ***- significant at 0.001 level, ns- not significant

Appendix IV: THESIS OUTPUT

1. Korir, H., Mungai, N. W., Thuita, M., Hamba, Y. and Masso, C. (2016). Co-inoculation of rhizobia and phosphate solubilizing bacteria effects on common bean production in a low phosphorus soil. *Paper presented at the 10th International Conference and Agriculture Summit*. 30th March-1st April, 2016. Egerton University.

2. Korir, H., Mungai, N. W., Thuita, M., Hamba, Y. and Masso, C. Co-Inoculation effect of Rhizobia and Phosphate Solubilizing Bacteria on Common Bean Growth in a Low Phosphorus Soil. *Frontiers in Plant Science.* **Under review**.

3. Korir, H., Mungai, N. W., Thuita, M. and Masso, C. Efficacy Assessment of Commercial Rhizobia Inoculants on Common Bean and Soybean Growth in Two Soil Types- **Manuscript**