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Isolatation, authentication and evaluation of symbiotic effectiveness of elite indigenous rhizobia nodulating phaseolus vulgaris l. in Hai district, Northern Tanzania

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ISOLATATION, AUTHENTICATION AND EVALUATION OF SYMBIOTIC EFFECTIVENESS OF ELITE INDIGENOUS RHIZOBIA NODULATING *PHASEOLUS VULGARIS* L. IN HAI DISTRICT, NORTHERN TANZANIA

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A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master's in Life Sciences and Engineering of the Nelson Mandela African Institution of Science and Technology

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GENERAL ABSTRACT

Phaseolus vulgaris L. production in Sub Saharan Africa is affected by nitrogen deficiency in the soils. Rhizobium inoculants are considered as the best solution to curb the problem of soil nitrogen deficiency. However, accessibility of rhizobia inoculants in most African countries is a challenge, hence leads to most inoculants being imported from abroad. The estimation of the number of indigenous rhizobia nodulating *P.vulgaris* in the soils of Hai District, northern Tanzania as well as isolation, authentication and evaluation of the symbiotic effectiveness of those indigenous rhizobia strains was conducted. The most probable number infection method was used to estimate the population of indigenous rhizobia in the soils. Yeast Extract Manitol Agar containing congo red was used to grow the isolated rhizobia strains. Koch's postulates were employed in authentication, while the number of nodules per plant, chlorophyll content and other plant growth parameters was used to test effectiveness of the isolated strains. The Shapiro Wilk's W test was used for testing normality of the data, one way ANOVA and Kruskal-Wallis Ranksum test was used for analysis of the data which are normally distributed and those which are not normally distributed respectively while Fisher's Least Significant Difference were used as a post hoc significance test. The 61% of the soil in the study area had low rhizobia populations (<100 cells g^{-1} of soil). Moreover, 18 bacterial strains were isolated and proved to be rhizobia. Two isolated strains (NR12 and NR13) showed higher effectiveness in nitrogen fixation than the commercial strain CIAT 899.

Key words: Common bean, indigenous rhizobia, Nitrogen fixation, MPN, symbiotic effectiveness**.**

DECLARATION

I, **Yusuph Namkeleja** do hereby declare to the Senate of The Nelson Mandela African Institution of Science and Technology that, this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution**.**

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Signature and name of candidate Date

The above declaration is confirmed

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DR. KELVIN MTEI  _____ 24th April, 2017

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DR. FREDERICK P. BAIJUKYA  _____ 24th April, 2017

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CERTIFICATION

The undersigned certify that he has read and hereby recommend for acceptance by The Nelson Mandela African Institution of Science and Technology a dissertation entitled "**Isolation, authentication and evaluation of symbiotic effectiveness of elite indigenous rhizobia nodulating *Phaseolus vulgaris* L. in Hai District, Northern Tanzania**"

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DEDICATION

I dedicate this work to my mother Ms. Zuhura Nguhwe and my father Mr. Salum Namkeleja. Their efforts and prayers in up bringing me in a lovely and peaceful manner up to this moment are what define me and this work.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA-Analysis of Variance

BNF-Biological Nitrogen Fixation

BTB-Bromothyl Blue

Chl-Chlorophyll

CR-Congo red

DMSO- Dimethyl Sulphoxide

MLSA-Multi Locus Sequence Analysis

MPN-Most Probable Number

MPNES-Most Probable Number Enumeration System

NM-AIST- Nelson Mandela African Institution of Science and Technology

SSA- Sub Saharan Africa

TN- Total Nitrogen

YEMA-Yeast Extract Manitol Agar

YEMB-Yeast Extract Manitol Broth

URT- United Republic of Tanzania

CHAPTER ONE

1.0. GENERAL INTRODUCTION AND BACKGROUND

1.1. Background of the study

Poor soil fertility is the major challenge that deters crop production in the tropics and sub-tropics (Giller *et al.*, 1998; Mwandemele and Nchimbi, 1990). Legumes including *Phaseolus vulgaris* are among the crops whose production is lowered by the soil nutrient deficiencies. In Tanzania soil fertility loss and increased demand for food production because of high population pressure has led to increased use of inorganic fertilizers in most cropping systems. But excessive use of inorganic fertilizers has an adverse impact on the environment. Suprapta (2012) reported that, excessive use of inorganic fertilizers has undesirable effects on agriculture, food, biodiversity and health status of communities. Use of organic resources such as animal manure and compost is a traditional practice of farmers in Northern Tanzania to restore soil fertility, but its continued use is limited by the availability of these resources. Incorporation of grain legumes, including *P. vulgaris* in combinations with inorganic fertilizers is a practical way to achieve sustainable food production, taking into consideration of annual population increase rate of 2.7% (National Bureau of Statistics, 2012).

Phaseolus vulgaris is the important sources of dietary protein, food for human, fodder for livestock and source of soil nitrogen (Nyasasi and Kisetu, 2014; Ribeiro *et al.*, 2013). *Phaseolus vulgaris* and other grain obtain nitrogen through a process known as biological nitrogen fixation (BNF), which is done by the soil bacteria found in their root nodules known as rhizobia (Giller, 2001). Although *P. vulgaris* yield in Tanzania is below its production potential (< 3 t ha^{-1}), the crop is widely grown in most regions of the country (Hillocks *et al.*, 2006; Kisetu *et al.*, 2013; Ndakidemi *et al.*, 2006; Ndakidemi and Semoka, 2006). Of recent, there is renewed interest in the use of BNF technology in agricultural systems mainly to overcome problems associated with depletion of soil nitrogen and as an alternative to excessive use of inorganic nitrogen fertilizers (Loganathan *et al.*, 2014a). Apart from the soil fertility aspect, BNF helps to improve food safety and enhances conservation of biodiversity since it poses no adverse impact to the environment (Ghimire, 2002; Haru and Ethiopia, 2012; Jonah *et al.*, 2012; Mfilinge *et al.*, 2015).

Inoculation of legumes with rhizobia is the oldest and mostly used BNF technology in agriculture (Bull *et al.*, 2002; Chisholm *et al.*, 2006; Deshwal and Chaubey, 2014; Lindström and Mousavi, 2010; Lindström *et al.*, 2010). *Rhizobium* is a genus of gram negative bacteria that lives symbiotically in root nodules of legumes which converts atmospheric nitrogen to ammonia and provide organic nitrogenous compounds to the plants (Giller, 2001; Pawar *et al.*, 2014). This study was conducted to estimate the population of indigenous rhizobia nodulating *P. vulgaris* as well as to isolate, authenticate and to evaluate the symbiotic effectiveness of the indigenous rhizobia strains nodulating *P. vulgaris* as a means to advance the BNF technology, which is useful to enhance availability of N in the soil.

1.2. Statement of the Problem and Justification

Smallholder *P. vulgaris* production in SSA is threatened by poor soil fertility exacerbated by the depletion of essential nutrients including nitrogen and phosphorous, thereby jeopardizing the efforts to sustain food security (Bationo *et al.*, 1998; Ndakidemi *et al.*, 2006; Okalebo *et al.*, 2007; Pereira *et al.*, 2006). Feasible and adaptable options to address the problem of depleted soil nitrogen include, among others, the use of BNF technology (Bull *et al.*, 2002; Chisholm *et al.*, 2006). BNF through rhizobia inoculants is one of the best alternatives to increase N in the system because apart from increasing crop yield it also enhances environmental conservation (Bloem *et al.*, 2009). Unfortunately, in most African countries, awareness and the accessibility of the inoculants is not well established. In many African countries, most of rhizobial inoculants are imported from abroad. Moreover, where produced (e.g. in Kenya and Zimbabwe), inoculants are manufactured using imported commercials which are necessarily not as effective as some of the land race strains (Bala *et al.*, 2011). This study isolated, authenticated and evaluated symbiotic effectiveness of indigenous rhizobia strains as a means towards making the inoculants available and accessible to smallholder farmers. This was done by taking into consideration that, there is still a high host diversity of rhizobia in tropical and subtropical soils.

1.3. Objectives of the Study

1.3.1. Overall objective

The overall objective of this study was to isolate, authenticate, and evaluate symbiotic effectiveness of elite rhizobia strains for *P. vulgaris* production in different agro-ecological zones of Hai District, Northern Tanzania.

1.3.2. Specific objectives

The specific objectives were;

(i) To determine the population size of indigenous rhizobia for *P. vulgaris* in relation to altitude in Hai District.

(ii) To isolate, authenticate and evaluate symbiotic effectiveness of elite rhizobia strains for *P. vulgaris* isolated in selected soils of Hai District.

1.4. Research questions

(i) What is the abundance of indigenous rhizobia capable of nodulating *P. vulgaris* in selected soils of Hai District and how do the populations vary with altitude?

(ii) Do local rhizobia strains have the same N-fixation potential when compared with the commercial strains?

1.5. Significance of the Study

This study will help to unravel the bottleneck of knowledge on improving legume production by isolating elite rhizobia strains that will be used in manufacturing the rhizobia inoculants to enhance BNF of *P. vulgaris* in Tanzania.

CHAPTER TWO

2.0. ISOLATION AND MOLECULAR CHARACTERIZATION OF ELITE INDIGENOUS RHIZOBIA NODULATING PHASEOLUS BEAN (*PHASEOLUS VULGARIS* L.)

Abstract

The application of biological nitrogen fixation (BNF) through rhizobia inoculums is promoted as a solution to solve the problem of poor soil fertility in areas where legumes are cultivated. This is due to the fact that, rhizobia enhance nitrogen fixation, induce disease resistance, reduce levels of heavy metals in the soil, facilitate bioavailability of iron in soil and is environmental friendly. To get rhizobia strains which are suitable for inoculants production, isolation and molecular characterization of elite rhizobia is needed. Molecular characterization act as a spark plug for discovery of many microbes including Rhizobia. Multi Locus Sequence Analysis (MLSA), 16S rRNA gene sequence analysis, DNA-DNA hybridization and SDS-PAGE analysis of the whole-cell proteins are the molecular techniques mostly used in characterizing rhizobia. But before deciding to use or not to use rhizobia inoculants in certain areas, knowing the population size of indigenous rhizobia found in that area is very important, because this is a major factor which determine inoculums responses as well gives clues on which areas need or do not need inoculation. The most probable number (MPN) method is mostly used in enumerating rhizobia population of the soil. Given that, most of the developing countries, including Tanzania, Biological nitrogen fixation (BNF) technology is not fully flourished; more efforts in isolation, molecular characterization of elite rhizobia and estimation of indigenous rhizobia population in various areas are required.

Key words: Biological Nitrogen fixation, Inoculums, Rhizobium-legume symbiosis, *Phaseoulus* bean

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[1] Namkeleja *et al* (2016). *American Journal of Plant sciences*, 7(14), 10.4236/ajps.2016.714175

2.1. Introduction

Common bean *(Phaseolus vulgaris* L.) is the third most important legume crop grown worldwide superseded by soy bean and peanuts (Van Berkum *et al.*, 1996; Aserse *et al.*, 2012; Faghire *et al.*, 2012; Cao *et al.*, 2014). It is the major and the most important staple food legumes in Eastern, Central and Southern Africa (Amijee and Giller, 1998; Ndakidemi *et al.*, 2006; Aserse *et al.*, 2012; Ribeiro *et al.*, 2013). It is among the most important sources of dietary protein in human (Ribeiro *et al.*, 2013).

Bean yield in Tanzania including Hai District is below its production potential (< 3 t ha^{-1}), but the crop is still widely grown in most regions of the country (Hillocks *et al.*, 2006; Ndakidemi *et al.*, 2006; Kisetu *et al.*, 2013). Productions of *Phaseolus* bean in the tropics and sub-tropics regions are deterred by poor soil fertility (Mwandemele and Nchimbi, 1990; Giller *et al.*, 1998; Ndakidemi *et al.*, 2006; Pereira *et al.*, 2006; Okalebo *et al.*, 2007). This is due to the fact that this crop is mostly grown by the resource-poor small scale farmers (Graham, 1981; Ndakidemi *et al.*, 2006) who are not able to purchase expensive inorganic fertilizers.

Currently, more emphasizes are on the use of BNF technology in agricultural systems in order to overcome the problems associated with depletion of soil fertility as well as reducing excessive use of inorganic N fertilizers (Loganathan *et al.*, 2010; Tairo and Ndakidemi, 2013a; Nyoki and Ndakidemi, 2014). BNF by rhizobia is considered as an inexpensive and environmentally friendly alternative to improve crop yield in comparison to its counterpart chemical nitrogen fertilizers (Meade *et al.*, 1985; Ghimire, 2002; Gaurav *et al.*, 2009; Haru and Ethiopia, 2012; Jonah *et al.*, 2012; Nyoki and Patrick, 2013; Mfilinge *et al.*, 2015). Inoculation of legumes with rhizobia inoculants is the most used BNF technology in agriculture as evidenced in previous studies (Bull *et al.*, 2002; Chisholm *et al.*, 2006; Lindström *et al.*, 2010; Deshwal and Chaubey, 2014) but in most African countries including Tanzania, this technology is not well established (Bala *et al.*, 2011). BNF technology may be successful if isolation and characterization of indigenous rhizobia found in the soil are done properly as may foster the discovery of stronger strains for inoculants production (Lindström *et al.*, 2010). Also, it has been emphasized that prior to the application of *Rhizobium* inoculants in the field, it is necessary to determine population size of indigenous rhizobia found in that soil as indigenous rhizobia population influence

inoculums responses (Thies *et al.*, 1991a). The lower the population size of the indigenous rhizobia, the higher the responses of inoculums if other factors remain constant (Singleton and Tavares, 1986; Thies *et al.*, 1991a, b). Singleton and Tavares (1986) have reported that, it is not possible to enhance nitrogen fixation when indigenous soil rhizobia populations are above the threshold number of 10^2 cells of bacteria per gram of soil. Therefore, there is a need of doing isolation, molecular characterization of elite rhizobia strains and determining population of indigenous rhizobia nodulating *Phaseolus* beans in various places. This will help to identify the suitable rhizobia strains for inoculants production aiming in enhancing crop yield and identifying right places where inoculation is needed.

2.2. Rhizobia legume symbiosis

Rhizobia-legume symbiosis is the most studied plant-bacteria mutualism (Subramanian *et al.*, 2009). Rhizobia are soil bacteria that are well known for their symbiotic relationship with legumes even though they are also found in soil devoid of legumes (West *et al.*, 2002; Denison and Kiers, 2004a, b; Lindström and Mousavi, 2010). Rhizobia are grouped into two major groups which are fast grower and slow growers (Somasegaran and Hoben, 2012). Fast grower takes 3-5 days to grow on the media and when grown in media containing bromothymol blue (BTB) indicator, they undergo alkaline reaction. While slow growing rhizobia takes about 7 to 10 days to grow on the media and show acidic reaction on BTB (Bala *et al.*, 2010). Most of rhizobia which nodulate *Phaseoul*s beans are from genus *Rhizobium*, and species belong to genus *Rhizobium* are fast growers (Somasegaran and Hoben, 2012). Rhizobia range from symbiotic which nodulate legumes, to non-symbiotic which are unable to nodulate legumes at all. Symbiotic rhizobia are divided into two groups which are mutualists and parasites. Mutualist rhizobia supply their hosts with nitrogen at a reasonable carbon cost while parasite rhizobia infect legume plants but then fix little or no nitrogen inside their nodules (Denison and Kiers, 2004a). This review is based on Mutualist rhizobia.

Mutualist rhizobia convert atmospheric nitrogen to ammonia and provide organic nitrogenous compounds to the plants (Giller, 2001; Pawar *et al.*, 2014). Through the symbiotic relationship between rhizobia and legume, rhizobia provide combined form of nitrogen to plant by converting free atmospheric nitrogen into ammonia a form of Nitrogen which can be directly utilized by the

plants while the plant provide shelter and energy to rhizobia (West *et al.*, 2002; Denison and Kiers, 2004a, b; Lindström and Mousavi, 2010). Some findings explained that, the drivers of rhizobia-legume interaction are dryness and infertility of the soils (Schrire *et al.*, 2005). That means the rhizobia nodulate legume in order to get shelter from hostile environments and legume accepts rhizobia in order to get access to combined nitrogen through a process of nitrogen fixation by rhizobia hence be able to survive and colonize the area which are dry with low nitrogen (infertile areas). But the real origin of rhizobia legume symbiosis is not known up to moment (Lindström and Mousavi, 2010). What is known is that, symbiosis between rhizobia and legumes is genetically controlled. Rhizobia have a nodulation gene (*nodABC*) which encode enzyme responsible for core structure of the signal molecule (Nod factor, NF) needed to induce nodule formation in host plant (Lindström and Mousavi, 2010). Initial interaction consists of stimulation of biochemical activity in the rhizobial strains by flavonoid and isoflavonoid molecules in the plant root exudates (Ndakidemi and Dakora, 2003). These compounds stimulate the activity of *nod* (nodulation) genes, the gene whose products are required to enable nodulation of the cognate legume host (Giller, 2001).

2.3. Significance of Legume-Rhizobia symbiosis

Rhizobia are found to have many benefits in agriculture industry. Some of the documented benefits include; promotion of plant growth through nitrogen fixation, solubilization of insoluble phosphate, controlling crop diseases, chelation of iron and bioremediation of heavy metals (Zaidi *et al.*, 2009; Loganathan *et al.*, 2010; Kala *et al.*, 2011; Bhattacharyya and Jha, 2012; Loganathan *et al.*, 2014a; Pawar *et al.*, 2014;).

2.3.1. Nitrogen fixation

All living organisms need nitrogen for their survival (Giller, 2001). It is the nutrient most required for plant growth and is a key for good yield of agriculturally important crops (Dall'Agnol *et al.*, 2014). It is a primary nutrient for plant growth and survival due to the fact that nitrogen is a source of cell proteins, enzymes and chlorophyll (Matiru and Dakora, 2004). Nitrogen is efficiently utilized by many organisms (all eukaryotes) when it is in the form of ammonium NH_4 or nitrate NO_3 (Giller, 2001; Loganathan *et al.*, 2014a). But high amount of

nitrogen that is found in the earth is atmospheric dinitrogen gas (N_2) which cannot be directly used by the plants. Some Bacteria (including rhizobia) and *Archaea* are the only organisms that can reduce atmospheric nitrogen to ammonia through a process known as biological nitrogen fixation (Giller, 2001; Lindström *et al.*, 2010).

Legumes including *Phaseolus* bean acquire nitrogen by living symbiotically with rhizobia which are capable of fixing atmospheric nitrogen (N_2) in root nodules and supplying it to the plant (Dall'Agnol *et al.*, 2014). Genera *Rhizobium, Mesorhizobium, Allorhizobium, Sinorhizobium, Arzorhizobium and Bradyrhizobium* which all together form rhizobia, play a vital role in converting free nitrogen in the soil into ammonia (Loganathan *et al.*, 2014a). Also, some species of bacteria that belongs to genus *Bacillus* like *B.cereus, B.fusiformis, B.marisflavi and B.alkalidiazotrophicus* are involved in nitrogen fixation (Sorokin *et al.*, 2008) but rhizobia remains as the most effective nitrogen fixing bacteria (Doignon-Bourcier *et al.*, 2000; Botha *et al.*, 2004; Lindström and Mousavi, 2010; Lupwayi *et al.*, 2011). It was reported that, in the tropics and subtropics, legume-rhizobia symbiosis is a key player in the nitrogen cycle and is a major contributor of nitrogen to terrestrial biosphere (Lindström *et al.*, 2010). Additionally, Lindström and Mousavi (2010) reported that BNF by rhizobia is the most efficient system with a mean annual fixation rate of 55140 kg N per hectare, compared with 0.330 kg N per hectare for other biological systems. It has been estimated that symbiotic interaction between microbes and legumes worldwide reduce about 100 million metric tons of atmospheric nitrogen per year hence saving about US$ 8 billion per year in fertilizer (Hassen *et al.*, 2014).

Moreover, BNF is considered as relatively inexpensive in comparison to its counterpart chemical nitrogen fertilizers as Jonah *et al.* (2012) expounded that, the cost of inoculants required for one hectare is about US$ 4 while the cost of chemical nitrogen fertilizers required for the same area (one hectare) is about US$45. Therefore, there is a need of providing knowledge on the significance of BNF and enhancing availability and use of inoculants through isolation and characterization of rhizobia in various legumes. If BNF is efficiently utilized, it will enhance legume production as well as reducing cost of production.

2.3.2. Disease control

Apart from nitrogen fixation, rhizobia are able to induce plant resistance against disease (Van Loon, 2007; Zaidi *et al.*, 2009). They are reported to have the ability of controlling crop diseases through elimination of the plant's enemies, including microbial pathogens, insects and weeds (Lugtenberg and Kamilova, 2009; Pawar *et al.*, 2014). They induce defensive enzymes and chemicals that suppress pathogens and enabling a process called Induced Systematic Resistance (ISR) to plants (Ramamoorthy *et al.*, 2001). Findings from Ehteshamul-Haque and Ghaffar (1993) proved that *Rhizobium meliloti* can inhibit growth of pathogens like *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium solani* while *Bradyrhizobium japonicum* can inhibit pathogens like *M. phaseolina* and *R. solani* in legumes (soybean, mung bean) and non-leguminous (sunflower and okra) plants. Pawar *et al.* (2014), reported that *Rhizobium* secrete antifungal compounds which selectively inhibit the growth of pathogenic fungi only and not rhizospheric bacteria. Due to these findings, there is a need of continued searching for rhizobia strain for *Phaseolus* bean that will be efficient in nitrogen fixation as well as in providing resistance to diseases. This will be possible through isolation and characterizations of indigenous rhizobia with dual purpose characteristics. By doing so, it will improve production of both legumes and non-legumes crops because rhizobia will reduce pathogen infections to all crops.

2.3.3. Solubilization of insoluble phosphate

Phosphorus (P) is one of the most essential macronutrients for the growth and development of plants (Rodríguez and Fraga, 1999; Chen *et al.*, 2006; Son *et al.*, 2006). The natural source of phosphorous (P) in the soil is organic and mineral phosphates. But both of them, organic and mineral phosphates in large quantity are found in the form of insoluble phosphate, which cannot be utilized by the plant, a situation, making soluble phosphate (a form that can be utilized by the plants) to be always scarce in the soil (Goldstein, 1994; Son *et al.*, 2006; Richardson *et al.*, 2009). Due to the scarcity nature and importance of soluble phosphate for plant growth and crop production, this leads to the excess use of chemical P fertilizers which have economic and environmental burdens (Son *et al.*, 2006). To reduce excessive use of industrial chemical P fertilizers, rhizobia-legume symbiosis is considered as among the most powerful solution. Rhizobium-legume symbiosis enhances utilization of naturally available insoluble phosphate to

make phosphorous available for plants. Research evidence has established that *Rhizobium* is among the most powerful phosphate solubilizer (Rodríguez and Fraga, 1999; Son *et al.*, 2006). *Rhizobium leguminosarum* biovar *viceae* and *Rhizobium meliloti* are the few examples of *Rhizobium* species which are capable of solubilizing insoluble phosphate (Halder and Chakrabartty, 1993). In that context determining and isolation of rhizobia species which are suitable for inoculants production are activities of special vitality for enhancing crop production and environmental protection.

2.3.4. Chelation of iron and Bioremediation of heavy metals

Iron is the essential element for plant growth (Compant *et al.*, 2005) as it is responsible for the formation of chlorophyll. Most iron in the soil is in the form of ferric ion (F^{3+}) in which plant can't utilize it, hence leads to scarcity of bioavailable iron in soil and plant surface (Compant *et al.*, 2005; Loganathan *et al.*, 2014b). Under the iron-limiting conditions, rhizobia tend to produce low-molecular-weight compounds called siderophores to acquire enough ferric ions (Berraho *et al.*, 1997; Compant *et al.*, 2005). Siderophore (iron bearer) have the ability to convert ferric iron F^{3+} into soluble F^{3+} and they transfer soluble F^{3+} and delivering it to the plant roots surfaces where becomes reduced to Fe^{2+} and absorbed by plants (Nakamura *et al.*, 1997; von Wirén *et al.*, 2000). Apart from iron, Rhizobacteria also have ability to chelate other heavy metals such as cadmium, lead, nickel, arsenic, aluminium, magnesium zinc, copper, cobalt, and controlling its mobility and availability to the growing plant through the release of chelating agents, acidification, phosphate solubilization and redox changes (Sayyed *et al.*, 2013). For instance study done by Saleh and Saleh (2006) indicated that dual inoculation with Arbuscular mycorrhiza (AM) fungus and *Rhizobium* on the host plant cowpea (*Vigna sinensis*) lead to increased tolerance against high concentration of Zinc and Cadmium. Therefore rhizobia–legume symbiosis is important in reducing heavy metals in the soil and it has been proposed as a proper tool for bioremediation of heavy metals in the soil.

2.4. Indigenous rhizobia population

Inoculation of legumes with introduced rhizobia strain is a common agricultural practice intended to promote nitrogen fixation and increasing crop yield (Thies *et al.*, 1991a). But for

Rhizobium inoculants to be efficient in fixing nitrogen as well as increasing crop yield, population size of indigenous rhizobia strains play an important role (Meade *et al.*, 1985). The size of the indigenous rhizobia population is the most powerful environmental factors that determine the competitive success of inoculated rhizobia versus indigenous rhizobia strain found in the area (Thies *et al.*, 1992). It has been found that the likelihood of a response to inoculation with *Rhizobium* strains decreased as the numbers of indigenous rhizobia increased (Thies *et al.*, 1991a, b). Singleton and Tavares (1986) found the same inverse correlation between rhizobia inoculants with native rhizobia population. According to Singleton and Tavares (1986), introduced rhizobia strains (inoculants) are always outcompeted with the native rhizobia strains, thus, it is not possible to enhance N_2-fixation when indigenous soil rhizobia populations were above threshold number (10^2 rhizobia cells per gram of soil) and had some effective strains.Amijee and Giller (1998) reported that, th response of *Phaseolus vulgaris* to *Rhizobium* inoculants is not common in areas with a large number of indigenous rhizobia. Furthermore, Meade *et al.* (1985) explained that the number of indigenous rhizobia present in the soil before inoculation affect the concentration of inoculants required in that area. A Study done by Weaver and Frederick (1974) indicated that for rhizobia inoculums to be able to form 50% or more of the nodules must be applied in a rate of 1,000 times higher than the size of the indigenous population in the soil.

Also increase in economic yield due to inoculation is a function of indigenous rhizobia present in the soil (Thies *et al.*, 1991b). That means, economic yield due to inoculums application increase as a number of indigenous rhizobia per gram of soil decrease and vice versa (figure 1). It is important to note that the indigenous rhizobia population is not the only factor that determines inoculums responses as there are other factors such as soil N availability, physiochemical constraints (like soil pH and salinity) and climatic conditions such as temperature, moisture content and oxygen stresses (Singleton and Tavares, 1986; Thies *et al.*, 1991a; Giller *et al.*, 1998; Hungria and Vargas, 2000). However, the population of indigenous rhizobia is among of the most important factor. For that reason, many findings recommended that for successful use of rhizobia inoculants, there should be prior knowledge of the size of the indigenous rhizobia population (Meade *et al.*, 1985; Peterson and Loynachan, 1981; Singleton and Tavares, 1986). Therefore, in order to know which areas need inoculation and at which quantity, knowing the population of indigenous rhizobia found in that area is of importance.

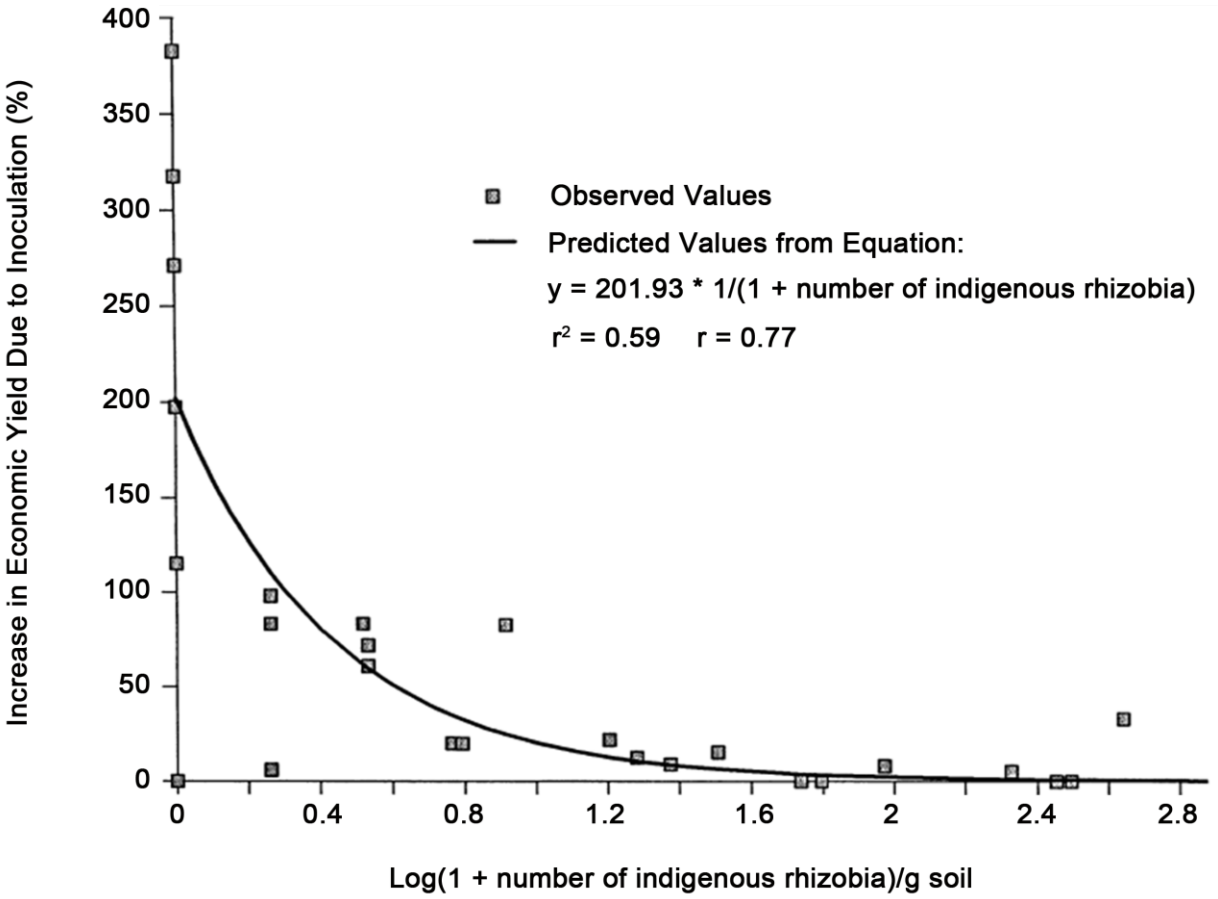

Figure 1: Comparison of the fit of the observed to estimated inoculation responses by use of a hyperbolic equation to describe the relationship between numbers of indigenous rhizobia and legume inoculation responses. Only 26 of the 29 total species-site combination is presented.

Source: Thies *et al.* (1991b).

2.4.1. Method for determining the population of indigenous rhizobia in the soil

Most probable number (MPN) method is usually used to determine the indigenous rhizobia population found in the soil. (Woomer *et al.*, 1990; Olsen *et al.*, 1996; Ndakidemi *et al.*, 2014). The MPN method of population estimate is based on the equation:

$$\text{Population Estimate} = \frac{1}{\text{Inoculant volume}} \times \text{Tabular estimate}$$

Additionally, computer software called Most Probable Number Enumeration System (MPNES) is employed to perform the same task (Woomer *et al.*, 1990).

2.5. Isolation of elite rhizobia strains

Rhizobia strain isolation is done by taking nodules from the host legume, sterilizing them by using ethanol and sodium hypochlorite, followed by crushing it in a sterile Petri dish by using blunt tipped sterilized forceps and lastly streaking drop of the nodule suspension on the media. Yeast-Mannitol agar (YMA) and peptone glucose agar are used as growth media while bromothymol blue (BTB) or Congo Red (CR) is used as indicators (Bala *et al.*, 2010; Somasegaran and Hoben, 2012). In most cases, YMA containing CR or BTB indicators are used as evidenced in many studies (Zahran *et al.*, 2013; Deshwal and Chaubey, 2014; Hassen *et al.*, 2014). The use of indicator media reported to camouflage real morphologies and distort the growth rate of the rhizobia (Bala *et al.*, 2010). Because of this drawback, some isolation is done on YMA plates without indicator media. Rhizobia isolation which does not involve the use of indicator media, aims at investigating uniformity of colonies growth across the plates, uniformity indicate a pure culture while non-uniformity indicate contamination.

Isolation of rhizobia is a valuable process to maximize agricultural production (Berrada *et al.*, 2012; Berrada and Fikri-Benbrahim, 2014; Simon *et al.*, 2014). It helps to get strong strain for nitrogen fixation, because effectiveness in nitrogen fixation by soil rhizobia population does vary widely between species (Singleton and Tavares, 1986) and the number of rhizobia that are not yet known is big and exceed the known one (Giller, 2001; Wolde-meskel *et al.*, 2005). Therefore, isolation of indigenous rhizobia is a stepping stone towards discovering effective strain that will be more efficient in fixing nitrogen for various legumes. Specifically isolation of rhizobia strain from nonspecific (promiscuous) legumes gives a wider chance of identifying new effective strains for such legumes. Common bean (*Phaseolus vulgaris* L.) is amongst the promiscuous legume hosts (Valverde *et al.*, 2006; Aserse *et al.*, 2012) and several rhizobia species have been reported to nodulate this legume, although not always effective in terms of fixing N_2 (Dall'Agnol *et al.*, 2014). Since *Phaseolus* bean is a most important legume crop in most African countries as earlier mentioned, there is a need of isolating elite rhizobia strains nodulating it in areas where its production is practiced but yield potential has never been realized.

2.6. Molecular characterization of Rhizobia

The use of molecular techniques in the characterization of microorganisms steered discovery of several new bacterial phylogenetics that were previously unknown (Whitman *et al.*, 1998; Macrae, 2000; Lindström *et al.*, 2010). Molecular characterization came into practice because classification based on morphological and physiological characters did not reflect true evolutionary relationship (Giller, 2001). Among the molecular techniques used in the characterization of the bacteria includes; Multi Locus Sequence Analysis (MLSA), Sequence analysis of 16S rDNA, 16S rRNA gene sequence analysis, DNA-DNA hybridization and SDS-PAGE analysis of the whole-cell proteins (Yao *et al.*, 2002; Hassen *et al.*, 2014).

The sequence analysis of 16S rRNA and DNA-DNA hybridization has been used frequently in bacteria taxonomy (Aserse *et al.*, 2012). 16S rRNA gene sequencing enables classification to genus level, while DNA-DNA hybridization helps the classification to species level (Peter *et al.*, 1996). The 16S rRNA gene is mostly used in characterization and classification of bacteria because it contains conserved small sub-unit which does not undergo mutation easily with time (Peter *et al.*, 1996; Giller, 2001). The 16S rRNA gene is found in almost all bacteria, and is large (1500bp) enough for informatics purposes (Patel, 2001; Schröder, 2014). But it has been confirmed that, the use of 16S rRNA gene sequencing alone as a phylogenetic marker for characterising bacteria is difficult and may give wrong results (Aserse *et al.*, 2012). This is due to the reason that 16S rRNA gene has low divergence between closely related species (Vinuesa *et al.*, 2005; Germano *et al.*, 2006) hence cannot distinguish between recently distinguished species (Reller *et al.*, 2007). Also, it is susceptible to genetic recombination and horizontal gene transfer and it is found in multiple copies in the genome of some bacteria (Haukka *et al.*, 1996; Van Berkum *et al.*, 1996). To solve this problem, the DNA-DNA hybridization came as a solution to the weaknesses of 16S rRNA gene sequence analysis.

DNA-DNA hybridization is able to differentiate between closely related species (Stackebrandt and Goebel, 1994; Yao *et al.*, 2002), but it has been reported that, its results tend to vary between laboratories where DNA-DNA hybridization was done, a situation which may lead to conflicting result for the same sets of strains (Rosselló-Mora, 2006; Aserse *et al.*, 2012). Nowadays, the multilocus sequence analysis (MLSA) is considered as the best in species identification and delineation than 16S rRNA gene sequence analysis and DNA–DNA hybridization (Martens *et*

al., 2008). Despite the drawbacks they have, 16S rRNA gene sequence analysis and DNA-DNA hybridization are still the most frequently used molecular techniques in taxonomy of bacteria as evidenced in many studies. The use of such molecular techniques to characterize elite rhizobia strains will help to identify exactly what species of rhizobia are effective for nodulating and fixing nitrogen and performing other functions in *Phaseolus* bean, thus making easy in inoculants production. Unfortunately, in Tanzania there are very few studies concerning molecular characterization of rhizobia isolated from the natural environments, hence more researches in this area are needed.

2.7. Conclusion

Isolation, molecular characterization of elite rhizobia strains nodulating *Phaseolus* bean and enumeration of indigenous rhizobia population in soil where beans are cultivated or expected to be cultivated may help to broaden knowledge on rhizobiology as well as to prosper the discovery of unknown effective and efficient strain of rhizobia and ultimately enhancing inoculums production. More efforts and interest must be directed in this area so as to increase the productivity of legumes considering that legumes are the major source of dietary protein and carbohydrates for human being.

CHAPTER THREE

3.0. POPULATION SIZE OF INDIGENOUS RHIZOBIA NODULATING *PHASEOLUS VULGARIS* L. IN SELECTED SOILS OF HAI DISTRICT, NORTHERN TANZANIA

Abstract

The estimation of the population size of indigenous rhizobia nodulating *Phaseolus vulgaris* and soil analysis was conducted in 18 soils samples collected across three altitudes (Higher, middle and lower) of Hai district-Tanzania, so as to assess the need of inoculation to improve bran yields. The randomized block design was used to sample soils from Hai District across three different altitudes. *P. vulgaris* "Masai red variety" with trade name 'USHINDI' was used as host crop. The bean was planted in sterile growth pouches containing N-free plant nutrient solution and then infected with soils from the study site. The most probable number (MPN) method was used to estimate the population size of indigenous rhizobia. Analysis of Variance (ANOVA) was used in analysis of the number of indigenous across three altitude of the study area while linear regression analysis was used for analysis of the influence of the soil components and structure on population size of indigenous rhizobia. The rhizobia population nodulating *P.vulgaris* in Hai District is between $2.3x10^0$ and$1.0632x10^4$ rhizobia cellsg^{-1} of soil. Out of all soil samples, 61% had a low rhizobia population (<100 cells g^{-1} of soil). This suggests that the application of *Rhizobium* inoculants is proper for *P.vulgaris* production in Hai District. Also, the study revealed that the indigenous rhizobia population does not differ significantly across the three altitudes of the Hai District, $F(2, 15) = 0.123$, $p=0.885$) but slightly differ with amount of soil Nitrogen, soil pH, available P, Ca and K.

***Key words*:** Population, Indigenous rhizobia, Most Probable Number (MPN), Nitrogen fixation, Nodule.

3.1. Introduction

Loss of soil fertility is a major factor hindering legume production (Ndakidemi *et al.*, 2006; Pereira *et al.*, 2006; Okalebo *et al.*, 2007). Biological Nitrogen Fixation (BNF) technology is considered as the best alternative to solve this problem (Loganathan *et al.*, 2014a), especially under smallholder conditions. However, for BNF to be efficient in fixing nitrogen as well as increasing crop yield, population size of indigenous rhizobia play an important role (Vlassak *et al.*, 1997; Lindström *et al.*, 2010). The size of the indigenous rhizobia population is the most powerful environmental factors that determine the competitive success of inoculated rhizobia (Thies *et al.*, 1991a). The higher the population sizes of the indigenous rhizobia, the lower the performance on inoculated rhizobia and vice versa (Singleton and Tavares, 1986; Thies *et al.*, 1991a, b). Indigenous Rhizobia are those which are naturally found in the soil. They fix atmospheric nitrogen to ammonia hence makes N readily available for plants (Giller, 2001; Pawar *et al.*, 2014). Due to increased loss of nutrients in most soils, indigenous rhizobia become insufficient to fix enough amount of nitrogen required by the plants, as it was reported that indigenous rhizobia are more competitive, but less efficient in nitrogen fixation (Vlassak *et al.*, 1997; Michiels *et al.*, 1998; Hungria *et al.*, 2000). This gives a loophole for *Rhizobium* inoculant to be used in areas where nitrogen is found to be scarce. Rhizobium inoculants are used as the best alternative to solve the problem of soil nitrogen deficiency and overtaking chemical nitrogen fertilizers. Biofertilizers are considered as the best solution to the soil nitrogen loss because it is cost efficient, climate change resilience, it boosts water availability and does not have collateral destruction of the indigenous flora and fauna found in the area where it is applied (Zhuang *et al.*, 2007; Khanal, 2009; Sharma *et al.*, 2013; Muthini *et al.*, 2014).

Despite the effectiveness in fixing nitrogen, rhizobia inoculants are always outcompeted with indigenous rhizobia. It has been reported that for rhizobia inoculants to work better, the number of indigenous rhizobia found in that soil should be lower at $<10^2$ rhizobia cells g^{-1} of soil (Singleton and Tavares, 1986). In this study, population size of indigenous rhizobia across three different altitudes was estimated by using the MPN method. Evaluation of the soil nutrients such as total N, available P, Mg, Ca, Na, K, and soil organic matter content was conducted to see how these components influence population size of indigenous rhizobia.

3.2. Materials and Methods

3.2.1. Study area description

This study was conducted at Hai District, Northern Tanzania. Hai is one of the districts in Kilimanjaro region in northern Tanzania, which lies between latitudes 2° 25' and 4° 15' and between Longitudes 36° 25' 30" and 38° 10' 45". The area experiences bimodal rainy seasons and the average rainfall varies from 500mm per year in low altitude to 2000 mm per year in high altitude. The average temperature ranges from 15^{o} C to 30^{o} C in high altitude while in low altitude temperatures can be as high as 40° C. The District is divided into four agro-ecological zones based on altitude such as a Mount Kilimanjaro peak zone, which lies from 1800m and above, upper zone from 1351m–1800m, middle zone from 900m–1350m and the lower zone which is below 900m above the sea level. But this study was done in only three zones such as upper, middle and lower zone.

3.2.2. Selection of sample village

The sample villages were randomly selected from three agro-ecological zones (upper, middle and lower zone). In each zone, six villages were randomly selected by using tossing method in which the names of all villages in the respective zone were written on small pieces of paper, then folded and mixed up well, then six pieces were randomly picked and name found in picked pieces were taken as sample villages for that zone.

3.2.3. Soil sample collection

Soil samples were collected from three agro-ecological zones of Hai District, which are lower, middle and upper agro-ecological zone. A total of 18 soil samples (six samples per zone) of 2kg each were collected from study site at a depth of 0-20cm. In every Agro ecological zone, six villages were randomly selected for sample collection in which one composite soil sample was collected from each selected village. Prior to soil sampling, the area where samples were to be taken was cleared and all debris was removed. To avoid contamination, all soil sample collection tools were sterilized by spraying with 3% Sodium hypochlorite at every point prior to soil sample collection at that point. The coordinates of each area where samples are collected were recorded by using GPS in WGS 84 systems. From each site, soils were collected at different

three points using sterilized spade in sterilized polyethylene bags and then mixed up well as one composite sample. Then 2 kg of each soil were taken from this composite sample. Then soil samples were taken to the NM-AIST laboratory divided into two portions, one dried under the sun and sieved to pass a 2 mm sieve ready for physical chemical characterisation. The other portion was stored at a room temperature of 10^{o} C for MPN counts.

3.2.4. Soil analysis

Portions of collected soil samples (about 250 g) was dried under the sun, grinded to pass a 2 mm sieve and analyzed for texture, pH, total nitrogen (TN), available phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg) and organic matter (OM) at the Mlingano Agricultural Research Institute Tanga according to methods described in Okalebo *et al.* (1993) and Gathua *et al.* (2002). These soil components are among of the factors determining the number of indigenous rhizobia in the soil (Thies *et al.*, 1991a). Soil organic matter was obtained by multiplying organic carbon (OC) by a factor of 1.72 as described by Walkey and Black (1934) cited by Ndakidemi and Semoka (2006). The soil textural classes were obtained from percentage of clay, silt and sand by using soil texture triangle as described by Shirazi and Boersma (1984) as well as by using the soil texture calculator developed by the United State Department of Agriculture in Natural Resource Conservation Services (USDA-NRCS, 2009).

The soil texture triangle is a diagram that is used to visualize and understand the meaning of soil texture names and it is used to get soil texture classes based on the percent of sand, silt, and clay (Shirazi and Boersma, 1984; Bonan, 2002). The use of soil texture triangle enable to classify soil into twelve (12) textural classes which are sand, clay, silt, loam, sandy clay, silty clay, sandy clay loam, clay loam, silty clay loam, loamy sand, sandy loam, and silt loam as shown in Figure 2 bellow. On the left of the triangle there are numbers corresponding to the percentage of clay, and on the right, there are numbers corresponding to the percentage of silt and at the bottom of the triangle chart there are numbers correspond to percentages of sand. The percentage of clay is ready by following grid lines in horizontal direction from left to right, the percentage of silt is ready by following grid lines from top right to bottom left and the percentage of sand is ready

by following grid lines from bottom right to top left. The intersection point where three lines meet is what determines the soil textural class in the soil texture triangle.

In addition to that the U.S department of Agriculture-Natural Resource Conservation Services (USDA-NRCS) have developed a soil texture calculator which is an excel sheet with blanks required to be filled with percentage of clay, sand and silt, then after being filled with those percentage value it automatically gives the name of the textural class of the soil sample (USDA-NRCS, 2009). In this study both, soil texture triangle and soil texture calculator were used.

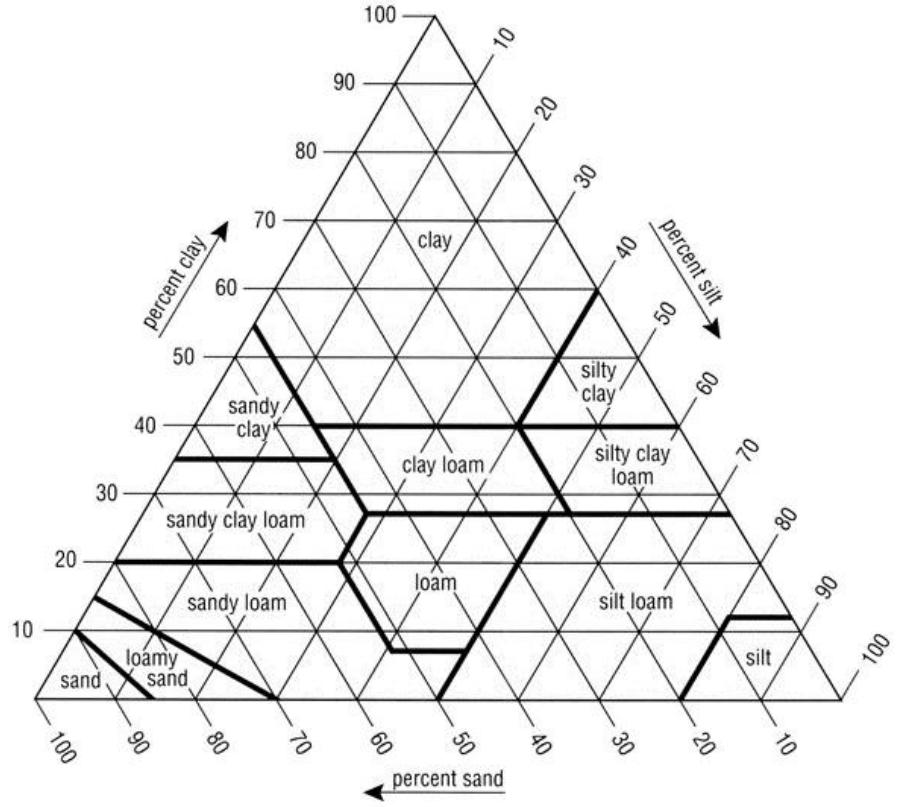

Figure 2: Soil Texture Triangle. Source: USDA-NRCS, (2009)

3.2.5. Estimation of indigenous rhizobia population by MPN method

Population size of indigenous rhizobia was done by using the Most Probable Number (MPN) method which involves preparation of N-free plant nutrient solution, seed surface sterilization and pre-germination, preparation of racks for placing growth pouches and preparation of soil dilutes samples.

a. N-Free plant nutrient solution preparation

The nutrient solution was prepared by mixing various nutrient elements which are in stock solutions as directed by Olsen *et al.* (1996) in Table 1 below.

Table 1. List of Nutrient elements used to prepare N-free plant nutrient solution

Stock solution	Chemical	Quantity g per litre	Quantity of stock per litre
1	K_2SO_4	93	3 mL
2	$MgSO_4.7H_2O$	493	1 mL
3	KH_2PO_4	23	1 mL
4	$CaCl_2$	56	1 mL
5	$CaSO_4$		1 g
6	$FeCl_3$	6.5	1 mL
7	H_3BO_3	0.23	1 mL
8	$MnSO_4.H_2O$	0.16	1 mL
9	$ZnSO_4.7H_2O$	0.22	1 mL
10	$CuSO_4.5H_2O$	0.08	1 mL
11	$Na_2MoO_4.2H_2O$		1mL

Source: Olsen *et al.* (1996)

b. Seed surface sterilization and pre-germination

P.vulgaris seeds (Masai red variety) with the trade name USHINDI collected from the Seliani Research Centre were used. The seeds were washed with distilled water then placed in a sterilized one litre conical flask up to one fourth of the flask. Then the seeds were rinsed with 95% alcohol for 10 seconds, followed by 3% Sodium hypochlorite for 4 minutes. After that, seeds were rinsed with eight (8) changes of sterile water. After the eighth rinse, sufficient amount of water was poured to submerge the seeds and left in the refrigerator at 4°C for 4 hours in order to allow seeds to imbibe. After 4 hours the seeds were rinsed with three (3) changes of water and the seeds were placed in 0.75% (w/v) agar water in Petri dishes. Then the Petri dishes were incubated at 28°C for 48 hours to allow germination.

c. Setting growth pouches in its racks

Locally made racks were used to hold the growth pouches as shown in Plate 1. Each rack had 28 slots that hold 2 growth pouches. For each soil sample 1 rack with 50 growth pouches were used.

Plate 1: *Phaseolus vulgaris* grown in the growth pouches arranged in locally made racks

d. Putting the nutrient solution into growth pouches

Growth pouches arranged in racks, then were filled up with 30 ml of N-free plant nutrient solution by using sterile syringe. The N-free plant nutrient solution was added in growth pouches any time when wetness starts to disappear in the growth pouches.

e. Placing the seeds on growth pouches

The sterilized seeds of common beans were placed in growth pouches containing N-free plant nutrient solution by using sterile forceps. Each seed was planted in separate growth pouch (one seed per growth pouch).

f. Wetting the soil sample

Portions of soil samples meant for MPN, which were collected from the respective sites in Hai district were wetted at 30% w/v in which 70 mg of soil was mixed up with 30 ml of distilled water, and then incubated for 7 days to facilitate the soil to acquire the field condition.

g. Preparation of soil dilutes sample

Prior to conducting MPN count, dirt material from the all 18 soil samples were removed, then clean soil samples were wetted at 30% and incubated at 28^{o}C for seven days. Soil sample was diluted at a tenfold dilution with four replicates. Dilution was done by adding 1 grams of soil in 9 ml of distilled water sequentially, then 1ml of diluents added in 9 ml of sterile water to get the second diluents and the same procedure followed up to tenth dilution step. By using pipette 1 ml of diluents from each dilution level (from 10^{-1} to 10^{-10}) was inoculated in the growth pouches containing 30 ml of nitrogen free solution in four replicates. The aliquots were taken from the highest dilution and low with the same pipette. The pipette tip was changed for every soil sample to avoid contamination

3.2.6. Statistical Analysis

Most Probable Number, Enumeration System (MPNES) and STATISTICA software were used in the analysis. Analysis of Variance (ANOVA) was used to see if there are significant differences in the number of indigenous rhizobia, between upper, middle and lower zone of the study area, while linear regression analysis was used to determine if soil components and structure have an influence on population size of indigenous rhizobia.

3.3. Results

MPN results showed that, both highest and lowest population sizes of indigenous rhizobia were observed in the upper zone**.** The highest rhizobia population was 1.0632×10^{4} cells g^{-1} of soil, while the lowest was 0 cells g^{-1} of soil observed at Ng'uni village and Wari village respectively. Also, there were no significant differences in population size of indigenous rhizobia between all three altitudes of the study area F (2, 15) = 0.123, P = 0. 885). In addition, the soil analysis showed that, the study area has high deficiency in nitrogen and phosphorous

3.3.1. Soil Analysis results

The soil analysis results show that, pH values were above the critical level in 94% of the all sampled soils. Organic matter was below the critical level by 78 %, while only 22% of the soil has organic matter above the critical level, so there is a low level of organic matter in the study

area. Concerning total nitrogen 50% of the soils had nitrogen below the critical level, 5.56% within the critical level and 44.44% are above the critical level. The results of the analyzed soil components are presented in Table 2.

3.3.2. Population size of indigenous rhizobia

The population size of indigenous rhizobia was estimated by using the Most Probable Number Method and the results were presented in Table 3. In upper zone the lowest population of indigenous rhizobia was observed in Wari village where by population was below detection levels while high population was 1.06 x 10^4 cells g^{-1} of soil observed in Ng'uni Village. In this agro-ecological zone, about 67% had populations less than 10^2 rhizobia cells g^{-1} of soil, 17% had a population size between 10^2 to 10^3 cells g^{-1}, and 16% have populations greater than 10^3 cells g^{-1} of soil (Table 3).

In middle agro-ecological zone, 66% of all soil samples had a population of indigenous rhizobia which is below 10^2 cells g^{-1}, while 34% had population ranged 10^2 to 10^3 cells $g^{-1}$of soil. The lowest population in this zone observed in the Roo village with 2 rhizobia cells g^{-1} of soil, while the highest population within the zone observed in a Mamba village with 3.23x10^2 rhizobia cells g^{-1} of soil (Table 3).

In the lower agro ecological zone, out of all soil samples, 50% had a population which is below 10^2 rhizobia cells g^{-1} of soil, 33.3% had a population ranged 10^2 rhizobia cells g^{-1} of soil to 10^3 rhizobia cells g^{-1} of soil, and 16.7% had population above 10^3 cells g^{-1}. Highest population was 1.14x10^3rhizobia cell g^{-1} of soil observed in the Kikavu chini village while the lowest was observed in the Longoi Village with 5 rhizobia cells g^{-1} of soil as displayed in Table 3.

Table 2. Some chemical and physical properties of soils collected from Hai District of the Kilimanjaro Region, Tanzania compared with critical values of Tanzanian soils.

Village name	pH	OM (g/kg)	N (%)	Av. P (mg/kg)	K(meq/100g)	Ca (meq/100g)	Na (meq/100g)	Mg (meq/100g)	Texture
Kawaya	7.9	1.01	0.13	1.56	2.37	16.3	2.04	6.03	CL
Kikavu chini	7.2	1.35	0.15	3.87	2.18	17.1	0.52	5.8	SiL
Longoi	6.5	0.74	0.09	3.92	3.31	10.71	0.21	4.08	Clay
Mbatakero	6.4	1.02	0.13	3.01	2.27	9.52	0.16	3.39	SCL
Ngosero	6.3	0.82	0.08	4.45	3.31	9.12	0.26	3.92	Clay
Rundugai	8.4	2.17	0.17	0.29	12.31	37.46	7.48	13.29	L
Mamba	6.2	2.71	0.32	3.42	1.89	14.81	0.19	3.77	L
Mudio	6.5	4.04	0.27	4.74	2.46	22.19	0.31	6.06	CL
Nshara	6.8	1.82	0.22	6.71	1.89	16.4	0.26	3.34	Clay
Roo	4.8	3.56	0.35	0.41	1.61	5.2	0.21	1.29	Clay
Sonu	6.3	2.53	0.27	4.11	0.53	13.51	0.26	4.76	Clay
Uduru	6.4	1.95	0.23	6.82	1.42	10.31	0.17	3.28	CL
Foo	5.8	4.91	0.34	1.78	1.04	8.42	0.22	2.75	L
Kyeeri	5.9	7.18	0.29	1.13	0.74	6.42	0.12	2.19	SL
Mboreni	6	1.76	0.12	2.76	0.24	2.73	0.14	3.36	Clay
Ng'uni	6	2.42	0.27	1.29	0.81	11.21	0.17	3.82	L
Shari Uraa	5.8	1.99	0.25	0.78	0.62	7.92	0.17	2.75	CL
Wari	6.2	2.57	0.33	3.95	0.32	4.53	0.14	0.96	L
Critical level	**5.5**	**3**	**0.25**	**15**	**0.22**	**4.0**	**<1**	**2**	

OM=Organic matter, N=Nitrogen, P=Available phosphorous, K=potassium, Ca=calcium, Na=sodium, Mg=magnesium, EC=Electric Conductivity, L=loam, S=Sandy, SiL=Silt Loam, SCL=sandy clay loam, CL=clay loam, SL=sandy loam

Table 3. Population of Indigenous rhizobia estimated by the MPN method in Hai District

Village name	Zone	Total nodulating units in 4 replicates of each dilution step										MPN (cells g^{-1})	Confidence Factor	Confidence Interval P(0.95)
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10			
Foo	Upper zone (1351-1800m)	1	1	0	0	0	0	0	0	0	0	**$4.61x10^{0}$**	3.445636	$1.34x10^{0}$-$1.59x10^{1}$
Kyeeri		4	4	2	0	0	0	0	0	0	0	**$4.49x10^{2}$**	3.445636	$1.30x10^{2}$-$1.55x10^{3}$
Mboreni		4	1	0	0	0	0	0	0	0	0	**$3.03x10^{1}$**	3.445636	$8.78x10^{0}$-$1.04x10^{2}$
Ng'uni		4	4	4	3	1	0	0	0	0	0	**$1.06x10^{4}$**	3.445636	$3.09x10^{2}$-$3.66x10^{4}$
Shari Uraa		4	0	0	0	0	0	0	0	0	0	**$1.98x10^{1}$**	3.445636	$5.74x10^{0}$-$6.84x10^{1}$
Wari		0	0	0	0	0	0	0	0	0	0	-	-	-
Mamba	Middle zone (900-1350m)	4	3	3	2	0	0	0	0	0	0	**$3.23x10^{2}$**	3.445636	$9.39x10^{1}$-$1.11x10^{3}$
Mudio		4	4	1	0	0	0	0	0	0	0	**$4.42x10^{1}$**	3.445636	$1.28x10^{1}$-$1.52x10^{2}$
Nshara		3	3	3	3	0	0	0	0	0	0	**$6.29x10^{1}$**	3.445636	$1.83x10^{1}$-$2.17x10^{2}$
Roo		1	0	0	0	0	0	0	0	0	0	**$2.26x10^{0}$**	3.445636	6.61^{-1}-$7.79x10^{0}$
Sonu		3	2	0	0	0	0	0	0	0	0	**$1.88x10^{1}$**	3.445636	$5.45x10^{0}$-$6.47x10^{1}$
Uduru		4	4	1	0	0	0	0	0	0	0	**$2.72x10^{2}$**	3.445636	$7.89x10^{1}$-$9.38x10^{2}$
Kawaya	Lower Zone (>900M)	4	2	3	1	0	0	0	0	0	0	**$1.62x10^{2}$**	3.445636	$4.69x10^{1}$-$5.57x10^{2}$
Kikavu Chini		4	4	3	1	0	0	0	0	0	0	**$1.15x10^{3}$**	3.445636	$3.31x10^{2}$-$3.93x10^{3}$
Longoi		2	0	0	0	0	0	0	0	0	0	**$5.29x10^{0}$**	3.445636	$1.54x10^{0}$-$1.82x10^{1}$
Mbatakeo		4	2	1	0	0	0	0	0	0	0	**$7.40x10^{1}$**	3.445636	$2.15x10^{1}$-$2.55x10^{2}$
Ngosero		3	2	2	0	0	0	0	0	0	0	**$2.89x10^{1}$**	3.445636	$8.39x10^{0}$-$9.97x10^{1}$
Rundugai		4	3	1	0	0	0	0	0	0	0	**$1.26x10^{2}$**	3.445636	$3.66x10^{1}$-$4.35x10^{2}$

D1=dilution 1, D2=dilution 2, D3=dilution 3, D4=dilution 4, D5=dilution 5, D6=dilution 6, D7=dilution 7, D8=dilution 8, D9=dilution 9, D10=dilution 10

The population size of the indigenous rhizobia in the whole study area ranged from 0 rhizobia cell g^{-1} of soil to 1.0632 x 10^4 rhizobia cells g^{-1} of soil (Table 3). The lowest population was observed in the Wari village (upper zone) in which indigenous rhizobia was below detection level and the highest population was observed in the Ng'uni village (Upper zone). The 61% of the whole study area had a low rhizobia population size (<10^2 rhizobia cells g^{-1} of soil), 28% had moderate population (10^2 to10^3 rhizobia cells g^{-1} of soil) and 11% had higher population (>10^3cells g^{-1} of soil).

The study also revealed that the indigenous rhizobia population was not statistically different across all agro-ecological zones (F (2, 15) = 0.123, P=0. 885) as reflected in figure 3.

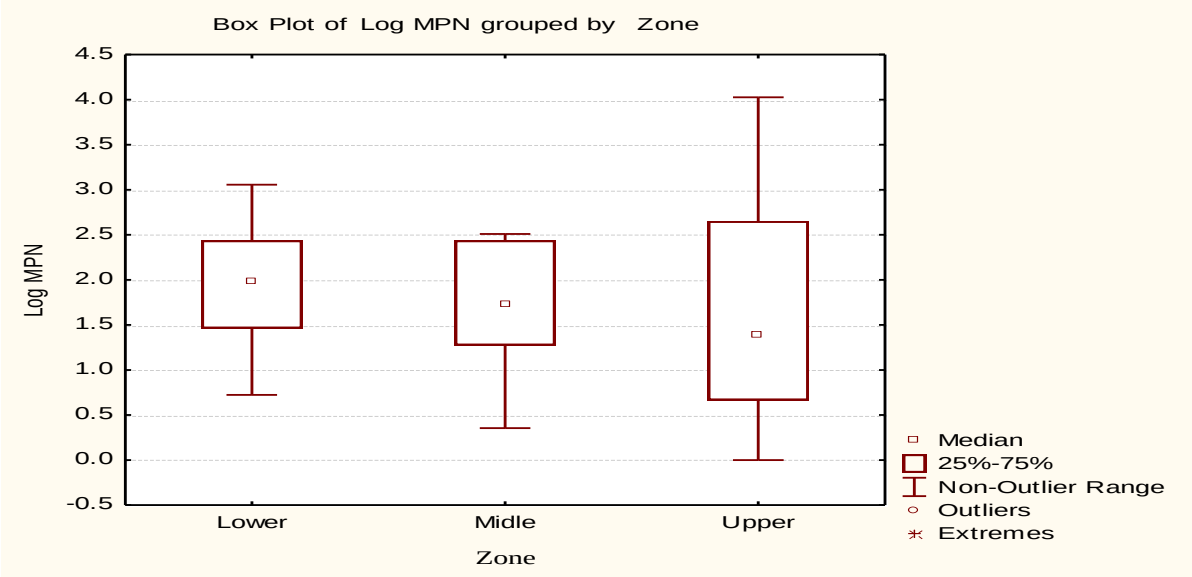

Figure 3: The box plot for distribution of indigenous rhizobia in three agro-ecological zones

3.3.3. Association of indigenous rhizobia with soil components

The association between number of indigenous rhizobia and soil components was determined by using regression analysis. The influence of soil components to the number of indigenous rhizobia not statistically different as the coefficient of determination (R^2) showed below (Table 4).

Table 4. The influence of Soil components on the number of indigenous rhizobia

Soil components	Coefficient of Determination(R^2) of soil components to the Number of indigenous rhizobia	P-value
Total nitrogen (TN)	0.0307	0.486
Available phosphorous (P)	0.0008	0.907
pH	0.1246	0.150
Organic Matter (OM)	0.0063	0.920
Calcium (Ca)	0.1015	0.197
Magnesium (Mg)	0.1023	0.195
Sodium (Na)	0.1757	0.600
Potassium (K)	0.0087	0.713

3.4. Discussion

3.4.1. Population Trend across the altitude

The study indicated that, the number of indigenous rhizobia in Hai District does not differ significantly between altitudes. This is against the view that, the number of rhizobia is favoured by the soil moisture content (Thies *et al.*, 1991a; Thies *et al.*, 1992; Giller *et al.*, 1998;) and at high altitude (upper zone) have higher moisture content than the lower zone (Zephania *et al.*, 2015). Therefore, under normal circumstances upper zone was expected to have a large number of indigenous rhizobia in comparison to lower zone. Additionally, this study found that in general the soils of Hai District has low number of indigenous rhizobia (less than 10^2 rhizobia cells g^{-1} of soil), indicating that the area may give positive responses to the rhizobia inoculants if the population size of indigenous rhizobia available in the soil is the only factor affecting inoculants responses. This finding corresponds with other results (Zephania *et al.*, 2015) which found that Northern Tanzania have low numbers of native rhizobia. But the small population observed in this study may be associated with season factor since the soil sample was collected during the dry season and there was no beans in the farms where soil samples were collected but have been harvested in three to six months back, except in Mudio village where the soil was collected in a field where there were beans.

3.4.2. Influence of soil nutrients in population size of the indigenous rhizobia

This study found that the soils of Hai District have nitrogen deficiency, similar to the findings of the previous studies in other parts of Tanzania (Mwandemele and Nchimbi, 1990; Ndakidemi

and Semoka, 2006). In all soil samples available phosphorous (P) found to be below the critical level, this concurs with the reported data by Ndakidemi and Semoka (2006) which indicated that the area of northern Tanzania including Hai District in Kilimanjaro region have low phosphorous level. Potassium (K) was above the critical level in all sampled soils. 94.4% of the sampled soils have Ca above the critical level and only 5.6% are below the critical level. Sodium was above the critical level by 89%, while 11% were below the critical level. The probable reasons for K, Ca, and Na to be high in the area may be due to excessive use of industrial chemical fertilizers. About Soil Texture; 33.3% of the sites were clay, 27.7% loam, 22.2% clay loam, 5.6% Silt loam, 5.6% silt clay loam and 5.6% sandy loam.

Total Nitrogen (TN)

Total nitrogen showed slight influence on the population of indigenous rhizobia where R^2= 0.0307 which means the indigenous rhizobia population size was negatively influenced by the amount of total N in the soil for 3.07%. But this influence was not statically significant at α=0.05, p= 0.486626 (Table 4). Negative association between total N and indigenous rhizobia were also reported by Thies *et al.* (1991a) who found that higher concentration of total N in the soil tend to inhibit nodulation and ultimately a lower number of indigenous rhizobia hence decrease amount of plant N derived from Nitrogen fixation. In this study the influence of total N was not significant due to the fact that there is no large variation of total N level between all sampled soils.

Available Phosphorous (P)

Available P in all soil samples ranged between 0.29 to 6.82 mg kg^{-1}, which is below the critical level 15 mg kg^{-1} (Ndakidemi and Semoka, 2006; Okalebo *et al.*, 2007; Muthini *et al.*, 2014; Zephania *et al.*, 2015). Due to P deficiency, there was a very minor negative relationship between P and the number of indigenous rhizobia at R^2= 0.000868 (Table 4). This is contrary to the report by Giller *et al.* (1998) which showed a significant positive relationship between available P and the number of indigenous rhizobia. The probable reason of having minor or no significant relation between available P and the number of indigenous rhizobia is the extremely low level of P which doesn't exceed even half of the critical level in all sampled soils of the study site (Table 2).

Soil pH

Soil pH is known as the major determinant factor for population size of the indigenous rhizobia in the soil (Ndakidemi *et al.*, 2014). Rhizobia population tends to be low in soil with low pH (Shahzad *et al.*, 2012) and be high in moderate pH value ranging from 6.0 up to 6.8 (Bordeleau and Prévost, 1994). But in some literature, it was expressed that rhizobia can survive better in pH 6 up to 8 (Zahran *et al.*, 2012). In this study, pH ranged from 4.8 to 8.4 and higher population was observed at pH value 6.0 and there are positive relationship between pH and indigenous rhizobia population at R^2=0.1246 (Table 4), but this relationship was not statistically significant at *P*=0.15

Soil Texture

In case of soil texture in areas with loam soil showed the higher rhizobia population, compared with other textural classes (Table 2 and 3). In Loam soil, rhizobia population ranged from 0 to 1.063 x 10^4 cells/g of soil with an average population of 2.217 x 10^3 cells/g of soil. In clay soil, rhizobia population ranged from 2.26x10^0 to 6.287x10^1 cells g^{-1} of soil with an average population of 2.473 x 10^1 cells g^{-1} of soil. In clay loam soil, rhizobia population ranged from 1.978x10^1 to 2.722 x 10^2 cells g^{-1} of soil with an average population of 1.244 x 10^2 cells g^{-1} of soil. Silt loam, sand clay loam and sandy loam occurred only in one sample village with rhizobia population of 1.139 x 10^3 cells g^{-1} of soil, 7.4 x 10^1cells g^{-1} of soil, 4.48 x 10^2 cells g^{-1} of soil respectively. There was no statistical difference in size of the indigenous rhizobia population amongst soil texture classes at F (5, 12) = 0.90038, p = 0.51161.

Available K, Ca and Mg

Potassium (K) and Calcium (Ca) were reported as among of the limiting factors for bean production in East Africa (Ndakidemi and Semoka, 2006). In the study area K was above the critical level in all soil samples, while Ca was above the critical level in seventeen soil samples (94%), therefore there are no deficient in neither K nor Ca, this is contrary to the results of Ndakidemi and Semoka, (2006) who found that 53% of the area have the deficient in K while 50% have deficiencies in Ca. Furthermore available K, Ca and Mg have showed a positive relation with the number of indigenous rhizobia at R^2 of 0.0087 at p=0.7127, 0.1015 at p=0.1974,

and 0.1023 at p= 0.195 respectively even though their relationships are not statistically significant (Table 4).

3.5. Conclusion

This study found that the large area of Hai District in Kilimanjaro Region-northern Tanzania have generally low population of indigenous rhizobia, low total N level and extreme deficiency of the available Phosphorous (P). In that perspective, use of rhizobia inoculants and P fertilizes may be the proper means to recover soil fertility in Hai District and other areas which have soil properties resembling these soils. Also, this study found that there are no statistical significant differences in a number of indigenous rhizobia in different altitude. Additionally, this study found that soil properties have a slight influence on the population size of indigenous rhizobia; this may be due to the reasons that, there is no big difference in soil properties across all areas where soil samples was collected. This study does recommend that intensive studies across all seasons are required so as to establish a benchmark of the size of indigenous rhizobia population in the soil, due to the fact that rhizobia do vary unevenly, two soils within few centimeters may have very huge difference in numbers of rhizobia in it.

CHAPTER FOUR

4.0. ISOLATATION, AUTHENTICATION AND EVALUATION OF SYMBIOTIC EFFECTIVENESS OF ELITE RHIZOBIA STRAIN NODULATING *PHASEOLUS VULGARIS* L. BEAN IN HAI DISTRICT, NORTHERN TANZANIA

Abstract

Isolation of rhizobia strain from nonspecific (promiscuous) legumes gives a wider chance of identifying new effective strains for such legumes. *Phaseolus vulgaris* is amongst the promiscuous legume hosts and several rhizobia species have been reported to nodulate this legume. This study isolated indigenous rhizobia strains from root nodule of *P.vulgaris* planted in the soil of Hai District in Kilimanjaro region, northern Tanzania and its effectiveness in nitrogen fixation was tested. Yeast Extract Mannitol Agar containing Congo red was used as the growth media. Koch's postulates were used to authenticate isolated strains while plant growth parameters were used to measure symbiotic effectiveness. Shapiro Wilk's W test was used to test normality of the data, one way ANOVA for analysis of the symbiotic effectiveness data which seemed normally distributed. Kruskal- Wallis Ranksum test was used for analysis of the symbiotic effectiveness data which are not normally distributed and Fisher LSD was used as a post hoc significance test. Eighteen (18) isolates were proved to be rhizobia through authentication experiment. Isolated Strain RN12 and RN13 showed higher symbiotic effectiveness than the commercial strain CIAT 899 a situation which gives possibilities of getting native elite strains which can be used in manufacturing of inoculants for beans.

Key words: Isolation, Authentication, symbiotic effectiveness, rhizobia strains.

4.1. Introduction

Populations of Rhizobium contain a number of species and within a species, strains exhibit a range of symbiotic effectiveness with a legume (Singleton and Tavares, 1986). Isolation of indigenous rhizobia prospers the discovery of a new rhizobia strain which is more effective than the available commercial strains (Giller, 2001). Rhizobium is a group of soil bacteria which are capable of fixing atmospheric nitrogen through a process called Biological Nitrogen Fixation (Giller, 2001; Pawar *et al.*, 2014). Biological Nitrogen Fixation (BNF) is the process in which atmospheric dinitrogen gas (N_2) is converted to ammonia (a form which can be utilized by the plant) and is considered as the main route in which combined nitrogen enter into the soil (Lindström and Mousavi, 2010; Lindström *et al.*, 2010). Due to the advancement of biotechnology nowadays, these bacteria called rhizobia are used to produce what is called inoculants as biofertilizers. Rhizobium inoculants are considered the best among the preferred solution to combat a problem of N deficiency as it was reported that, nitrogen is the most limiting nutrient in most small holder farmers (Chemining'wa *et al.*, 2007; Howieson and Committee, 2007; Jonah *et al.*, 2012; Zahran *et al.*, 2013).

In most African countries including Tanzania, most of the inoculants are imported from the developed countries (Bala *et al.*, 2011). The searching for strong rhizobia strain native to African soil, which letter on may be used to produce inoculants are very crucial as reported inoculants made from native strains to that soil are more effective in improving crop yield (Giller *et al.*, 1994). This study isolated elite indigenous rhizobia strains nodulating *P.vulgaris*, authenticated the isolated strains and then tested its symbiotic effectiveness.

4.2. Material and Methods

4.2.1. Soil sample collection

Soil samples were collected from three agro-ecological zones of Hai District. A total of 18 soil samples of 2 kg each was collected from study site at a depth of 0-20 cm. Prior to the soil sample collection, the history of the farm was taken and the sample was collected from the farms which had beans or beans were harvested in three to six months back and had no history of applying *Rhizobium* inoculants.

The area where sample soils were taken was cleared and all debris was removed. In each zone (lower, middle and upper) six soil samples in of 2 kg each, were collected. In each agro-ecological zone, six (6) villages were randomly selected for sample collection and one (1) soil sample was collected from each selected village. To avoid contamination, the soil samples were taken using sterilized spade and stored in sterilized polyethylene bags (Ribeiro *et al.*, 2013). The coordinates of each sampling point were recorded by using GPS in WGS 84 systems. At each site soil were collected from three points then mixed to make a one composite sample of about 2 kg. The soil samples were taken to the greenhouse at Nelson Mandela African Institution of Science and Technology and poured in 2 kg plastic pots. 5-6 seeds of *P.vulgaris* were planted in those pots containing the soil sampled from the field. Growing bean plants were periodically watered with distilled water until they reached 50% flowering stage.

4.2.2. Collection of nodules

Nodule collection was done as described by Bala *et al.*, (2010) whereby at half flowering period healthy, dark, pinkish red and medium sized nodules were directly collected from *P.vulgaris* grown in different soils of Hai District in the green house. Soil was gently and carefully removed from the root material by hands. In case of heavy soil, root clump was carefully immersed in a bucket of water for easy removal of the soil. Then nodules were observed at the lateral and main roots and careful taking the sample by cutting the root about 0.5 cm on each side of the nodules (Somasegaran and Hoben, 2012). Only effective nodules were taken as a sample, and nodule effectiveness was determined by looking on colour and size. Dark or pinkish red and medium sized nodules were taken as a sample as these are easily sterilized during isolations than the bigger nodules and with green or white colour.

4.2.3. Isolation of rhizobia strains from nodules

Isolation was done following procedures described by Bala *et al.*, (2010) and Somasegaran and Hoben, (2012). A total of 90 healthy root nodules from 18 soil samples (5nodules per sample) were thoroughly washed with normal water five times to remove all soils and other particles. Then nodules were immersed in 95% ethanol for 10 seconds to break surface tension and removing air bubbles in the nodules.

The nodules were surface sterilized by using 3% sodium hypochlorite by immersing in it for 5 minutes, followed by dipping in sterile water in eight changes. To avoid contamination, sterile forceps were used for transfer of nodules, and between transfers, the forceps were sterilized by dipping in alcohol and flaming. Each surface sterilized nodule was crushed in large drop of sterile water in the Petri dishes. One loopful drop which is about 0.1 ml of the nodule suspension was streaked on a yeast extract mannitol agar (YEMA) plate containing Congo-red (CR). The plates were incubated at 28^{o}C for 2-7 days and observation for appearance of typical colonies of rhizobia was done on a daily bases. Slow growing colonies appeared in seven (7) to ten (10) days while fast-growing rhizobia were seen two (2) to five (5) days. Contamination was detected if CR were absorbed, because typical rhizobia colon does not absorb CR. Well isolated typical single colonies were re-streaked on fresh YMA containing BTB and YMA containing CR in order to obtain pure cultures.

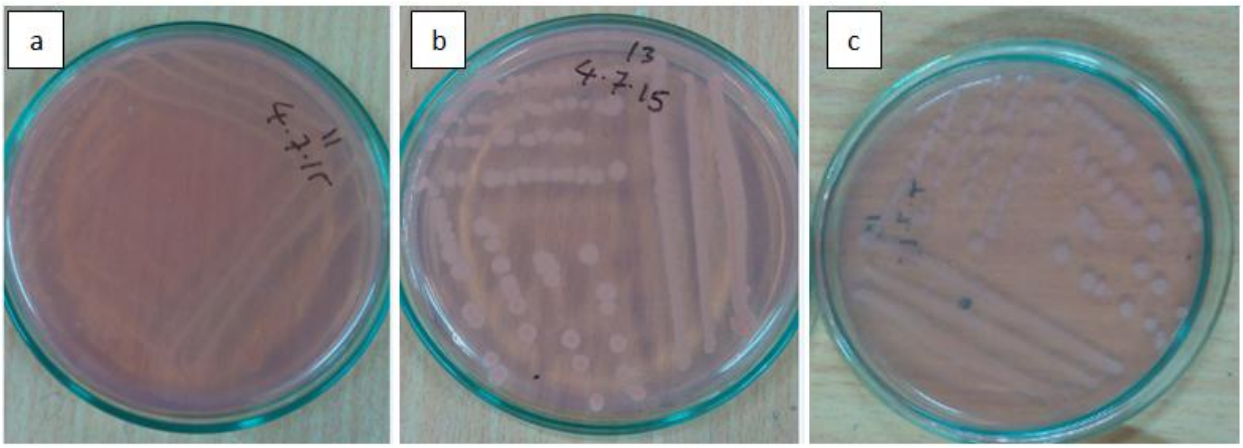

Plate 2: Pure colonies of the indigenous rhizobia nodulating *P.vulgaris*;

(a) Indigeneous rhizobia from Mboreni village (NR11), (b) Indigeneous rhizobia from Rundugai village (NR13), (c) Indigeneous rhizobia from Ng'uni village (NR12)

4.2.4. Authentication of isolated *rhizobium* strains

Sterile Growth pouches were used to test the ability of the isolated rhizobia strains to form nodules on the legume species from which it was originally obtained as stipulated by Somasegaran and Hoben (2012).

The authentication was done by following Koch's postulates where by *P.vulgaris* grown in rhizobia free media were inoculated with pure rhizobia strains (rhizobia strains isolated from *P.vulgaris* grown in sampled soils) under sterile conditions (Bala *et al.*, 2010; Hassen *et al.*, 2014) while other *P.vulgaris* was grown in rhizobia free media and remain non-inoculated as a control. Strains which managed to nodulate *P.vulgaris* grown in sterile media were proved to be rhizobia and those failed to nodulate considered as non-rhizobia for *P.vulgaris*.

4.2.5. Storage of authenticated rhizobia strains

Authenticated rhizobia strains were kept in 2 mls Eppendorf tube containing Yeast Mannitol Broth (YMB) and stored in -80°C deep freezer at NM-AIST laboratory.

4.2.6. Evaluation of symbiotic effectiveness of new rhizobia strains

Symbiotic effectiveness of the new rhizobia strains was evaluated through greenhouse experiments conducted at the Nelson Mandela African Institution of Science and Technology, where by sterile seed of *P.vulgaris* with the same size were grown in rhizobia free media and inoculated with isolated rhizobia strains. The autoclaved river sand was used as sterile media. Chlorophyll content, number of nodules per plant, and other plant growth parameters were used for testing the effectiveness of the isolated strain in nitrogen fixation. The symbiotic effectiveness testing involves preparation of sterile media, preparation of isolated strains, seed sterilization, planting, and taking measurements of various growth parameters.

a. Preparation of sterile media

The river sand was collected and all debris in it was removed, washed, dried and autoclaved at 121°C for 30 minutes, so as to kill all soil rhizobia and pathogens. Then autoclaved soil was left for 24 hours so that to allow it to cool. After cooling, the sterile soil was put in 2 kg plastic pots ready for growing the *P.vulgaris*.

b. Preparation of isolated strains for inoculation

The authenticated strains of indigenous rhizobia and standard rhizobia CIAT 899 were re-grown on plates containing Yeast Extract Mannitol Agar (YEMA) with Congo red (CR) and incubated at 28°C. After three days, by using an inoculation loop, rhizobia strains in the same amount for each strain, were taken from the plates and inoculated in 100 ml sterile beakers containing 60 ml

of Yeast Extract Mannitol Broth (YEMB). The inoculated beakers of YEMB were covered with autoclaved foil paper and kept in a shaker incubator for 72 hours at 28°C and 120 rpm (rotation per minute) until broth colour changed from colourless to the milky indicating presence of rhizobia strains. Then 10 mls of YEMB containing rhizobia were applied in 2 kg plastic pots containing sterile sand and germinating seeds of *P.vulgaris*.

c. Seed sterilization and planting

Seed of *P.vulgaris* with the same size were sterilized by rinsing with 95% alcohol for 10 seconds, followed by 3% Sodium hypochlorite for 4 minutes, then sterilant drained off. After that seeds were rinsed with eight changes of sterile water. After that seed were submerged in sterile water left in the refrigerator at 4°C for 4 hours in order to allow seeds to imbibe. After 4 hours, the seeds were rinsed with three changes of sterile water and left at room temperature for 24hours, then sown in pots containing sterilized sand. Then some pots were inoculated with the isolated *rhizobium* strains, while some was inoculated with standard rhizobia inoculants "CIAT 899" as positive control and other were left non- inoculated as negative control (Plate 3a). After 5 weeks, growth parameters such as number of nodules, stem girth, number of leaves, number of branches, shoot length, biomass, plant vigour and chlorophyll content were measured and compared as described by Bala *et al.* (2010) and Mhango *et al.* (2013).

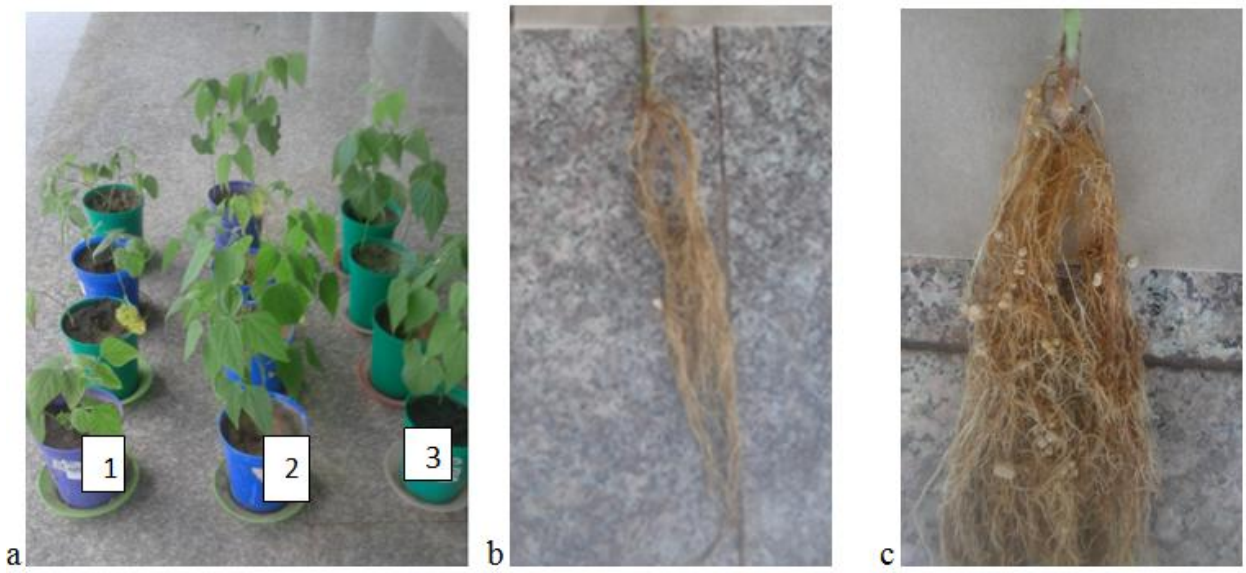

Plate 3: Various stage in testing the symbiotic effectiveness of the isolated rhizobia strains

(a) 1- *P.vulgaris non inoculated* 2- *P.vulgaris inoculated with one of the isolates of indigenous rhizobia strain* 3- *P.vulgaris inoculated with commercial inoculant CIAT 899*, (b) *Root of non-inoculated P.vulgaris* (c) *Roots of P.vulgaris inoculated with the indigenous rhizobia strain.*

d. Chlorophyll content determination

Chlorophyll content was determined by following procedures described by Hiscox and Israelstam (1979) and referring previous studies done by Richardson *et al.* (2002) and Elisante *et al.* (2013). A one hundred milligram (100 mg) of green leaf slices of *P.vulgaris* from each treatment and control was dissolved in 7 mls of Dimethyl Sulphoxide (DMSO) placed in 15 mls vials and then incubating at 4°C for 72 hours. Then the extract was diluted by adding 3 mls of DMSO hence making it 10 mls. Then 2ml of the leaf extract were transferred to disposable polystyrene cuvettes ready for absorbance determination in spectrophotometer. The spectrophotometer was calibrated to zero absorbance by using pure DMSO. The absorbance of sample and blank (pure DMSO) was measured at 645nm and 663nm wavelength. Then the total chlorophyll content was calculated by using equation suggested by Arnon (1949), $\mathbf{Chlt\ (gl^{-1}) = 0.0202D_{645} + 0.00802D_{663}}$ where **D** is the density value measured at the respective wavelength as measured in UV spectrophotometer.

e. Nodule count and measuring stem girth, shoot length and shoot dry mass

Nodule count, measuring of stem girth, shoot length and dry mass were conducted in the fifth week after planting. Stem girth was measured by using digital calliper in millimetre (mm); shoot length was measured by using a tape measure in centimetre (cm). Dry biomass of the shoot were measured in milligram (mg) by using weighing balance after drying those shoots in microwave at 60°C for 48 hours. Nodules were directly counted after removing soils in the roots.

f. Colour ranking of plant leaves

Plant colour ranking was done by looking colour of the *P.vulgaris* leaves and ranking them in a scale of 1 to 5 whereby 1 = severely chlorotic leaves, 2 = pale green, 3 = green, 4 = light green and 5 = deep green healthy leaves as done by Simon *et al.* (2014).

4.2.7. Statistical analysis

The data for symbiotic effectiveness were first subjected to Shapiro Wilk's W test for normality. One way analysis of variance (ANOVA) was used for analysis of the data which seemed normally distributed, while Kruskal-Walls-Ranksum Tests were used in analysis of the data which were not normally distributed at a significance level of $p = 0.05$. Treatment means of the growth parameters such as shoot length, stem girth, number of leaves per plant, number of nodules per plant, leaf chlorophyll contents, number of branches per plant and shoot dry biomass were compared by using Fisher LSD as the post hoc significance test. All above analysis was done by using STATISTICA software.

4.3. Results

4.3.1. Isolated rhizobia strain

A total of 18 bacterial strains were isolated from the nodules of *P.vulgaris* grown in soil collected from Hai District in Kilimanjaro Region Tanzania. All isolated strains were fast grower having taken 2 – 5 days to grow in Yeast Extract Mannitol Agar (YEMA) after inoculation. All strains were authenticated and have been proved to be rhizobia by inducing nodulation after inoculating in *P.vulgaris* grown in rhizobia free media. The characteristics of the isolated strains were presented in the Table 5 and as seen in plate 2.

Table 5. Characteristics of the Isolated Indigenous Rhizobia Strains

Isolates	**Characteristics**							
	Colony shape	Colony colour	Colony transparency	Colony appearance	Colony texture	Congo red absorption	Colony elevation	Colony margin
NR1	Circular	Cream yellow	Opaque	Dull	Firm dry	Not absorbing	Convex	Entire
NR2	Oval	Cream yellow	Opaque	Dull	Smooth Viscous			
NR3	Circular	Milky white	Opaque	Shiny	Smooth Viscous			
NR4	Oval	Cream yellow	Translucent	Shiny	Smooth Viscous			
NR5	Oval	Milky while	Opaque	Shiny	Smooth Viscous			
NR6	Oval	Milky while	Opaque	Shiny	Smooth Viscous			
NR7	Oval	Cream white	Translucent	Shiny	Smooth Viscous			
NR8	Oval	Milky while	Opaque	Shiny	Smooth Viscous			
NR9	Oval	Milky white	Opaque	Shiny	Smooth Viscous			
NR10	Oval	Cream white	Translucent	Shiny	Smooth Viscous			
NR11	Circular	Cream yellow	Opaque	Dull	Firm dry			
NR12	Circular	Cream yellow	Opaque	Shiny	Firm dry			
NR13	Oval	Milky white	Opaque	Shiny	Smooth Viscous			
NR14	Oval	Milky white	Opaque	Shiny	Smooth Viscous			
NR15	Circular	Cream yellow	Translucent	Shiny	Smooth viscous			
NR16	Oval	Milky white	Opaque	Shiny	Smooth viscous			
NR17	Circular	Cream yellow	Opaque	Shiny	Smooth viscous			
NR18	Circular	Cream yellow	Opaque	Shiny	Firm dry			

NR=Native Rhizobium, Number after NR represent village in which soil was collected, 1=Nshara, 2=Uduru, 3=Wari, 4=Foo, 5=Mamba, 6=Kiyeri, 7=Shari Uraa, 8=Sonu, 9=Roo, 10=Mudio, 11=Mboreni, 12=Ng'uni, 13=Rundugai, 14=Mbatakero, 15=Ngosero, 16=Longoi, 17=Kikavu chini, 18=Kawaya

4.3.2. Symbiotic effectiveness of the isolated indigenous rhizobia strains

Isolated rhizobia strains showed significant influence on various growth parameters. They have shown a significant influence on the number of nodules per plant and shoot length at $p < 0.001$, shoot dry mass, plant vigour and stem girth is significant at $p < 0.01$, leaf chlorophyll content and number of branches/plant at $p < 0.05$. Isolates didn't show statistical differences ($p \leq 0.05$) in the number of leaves/plant (Tables 6 and 7). Amongst the plant growth parameters which showed highly significant differences due to inoculation of the isolated strains in *P.vulgaris* are the number of nodules and shoot length. There is a high significance difference ($p < 0.001$) in chlorophyll content and shoot dry mass between inoculated and non-inoculated *P.vulgaris*. The mean number of root nodules per plant ranged from 0 nodules plant^{-1} observed in non inoculated pots control to 90 nodules plant^{-1} in pots inoculated with NR13 as shown in Table 7. The lowest mean shoot length was 25cm and observed in non-inoculated pots while the highest was 42cm observed at CIAT899. The indigenous rhizobia strains which performed better similar to or more than the CIAT899 in shoot length was, RN13, RN12, RN4, and RN17 (Table 6)

Table 6. Influence of isolated indigenous rhizobia on shoot length, stem girth, dry biomass and chlorophyll content

Treatments	Parameters Measured			
	Shoot length (cm)	**Stem girth (mm)**	**Shoot Dry biomass (g)**	**Chlorophyll content ($\mu gChlg^{-1}$)**
-ve control	25.25 ± 3.19^{a}	2.64 ± 0.18^{a}	0.573 ± 0.045^{a}	0.0124 ± 0.0005^{a}
+N	33.38 ± 3.13^{bcde}	3.57 ± 0.21^{bcd}	1.068 ± 0.343^{bcdef}	0.0715 ± 0.0080^{hi}
CIAT 899	41.93 ± 1.60^{g}	3.69 ± 0.21^{bcde}	1.471 ± 0.349^{fg}	0.0611 ± 0.0024^{h}
NR1	38.3 ± 3.19^{cdefg}	3.89 ± 0.31^{cde}	1.327 ± 0.206^{defg}	0.0257 ± 0.0019^{abcdef}
NR2	31.75 ± 1.95^{abc}	3.40 ± 0.22^{bcd}	0.793 ± 0.112^{ab}	0.0332 ± 0.0111^{cdefg}
NR3	38.37 ± 1.63^{cdefg}	3.82 ± 0.08^{bcde}	0.875 ± 0.171^{abcd}	0.0307 ± 0.0049^{bcdefg}
NR4	40.77 ± 4.19^{efg}	3.20 ± 0.09^{ab}	1.231 ± 0.176^{bcdefg}	0.0248 ± 0.0026^{abcde}
NR5	38.38 ± 3.54^{cdefg}	3.84 ± 0.20^{bcde}	1.323 ± 0.183^{cdefg}	0.0251 ± 0.0014^{abcde}
NR6	39.58 ± 1.24^{defg}	3.91 ± 0.29^{de}	0.847 ± 0.291^{abc}	0.0417 ± 0.0062^{fg}
NR7	36.53 ± 1.21^{bcdefg}	3.63 ± 0.22^{bcde}	1.139 ± 0.162^{bcdef}	0.0447 ± 0.0107^{g}
NR8	34.50 ± 3.66^{bcdef}	3.46 ± 0.19^{bcd}	0.923 ± 0.051^{abcde}	0.0248 ± 0.0051^{abcde}
NR9	36.00 ± 4.76^{bcdefg}	3.57 ± 0.21^{bcd}	0.798 ± 0.065^{ab}	0.0242 ± 0.0045^{abcde}
NR10	29.85 ± 0.83^{ab}	3.23 ± 0.05^{abc}	0.997 ± 0.131^{abcdef}	0.0164 ± 0.0007^{ab}
NR11	38.88 ± 2.01^{cdefg}	4.30 ± 0.46^{e}	1.132 ± 0.091^{bcdef}	0.0209 ± 0.0010^{abcd}
NR12	41.28 ± 1.94^{fg}	3.94 ± 0.17^{de}	1.679 ± 0.104^{g}	0.0780 ± 0.0018^{i}
NR13	41.45 ± 3.20^{fg}	3.94 ± 0.29^{de}	1.179 ± 0.046^{bcdef}	0.0370 ± 0.0068^{defg}
NR14	35.73 ± 1.91^{bcdefg}	3.57 ± 0.14^{bcd}	1.048 ± 0.027^{abcdef}	0.0261 ± 0.0060^{abcdef}
NR15	32.40 ± 3.39^{abcd}	4.07 ± 0.31^{de}	0.797 ± 0.012^{ab}	0.0194 ± 0.0016^{abc}
NR16	29.88 ± 0.77^{ab}	4.27 ± 0.29^{e}	1.372 ± 0.131^{efg}	0.0367 ± 0.0062^{defg}
NR17	$4\ 0.58\pm1.33^{efg}$	3.84 ± 0.31^{bcde}	1.284 ± 0.125^{cdefg}	0.0315 ± 0.0056^{bcdefg}
NR18	33.48 ± 2.23^{bcde}	3.68 ± 0.17^{bcde}	1.389 ± 0.143^{efg}	0.0372 ± 0.0077^{ef}
	P=0.00048	**P=0.002648**	**P=0.0018**	**P=0.0249**

*Values represent Mean±SE, values with dissimilar letter(s) in a column are significant by Fisher LSD at P=0.05,

NR = Native Rhizobium, Number after NR represent village in which soil was collected, 1 = Nshara, 2 = Uduru, 3 = Wari, 4 = Foo, 5 = Mamba, 6 = Kiyeri, 7 = Shari Uraa, 8 = Sonu, 9 = Roo, 10 = Mudio, 11 = Mboreni, 12 = Ng'uni, 13 = Rundugai, 14 = Mbatakero, 15 = Ngosero, 16 = Longoi, 17 = Kikavu chini, 18 = Kawaya

Table 7. Influence of isolated indigenous rhizobia on Number of nodules, Branches, leaves and plant leaves colour

Treatments	Parameters measured			
	Nodules plant^{-1}	**Branches plant^{-1}**	**Leaves plant^{-1}**	**Plant leaves colour**
-Ve control	0.00±0.00^{a}	1.25±0.25^{abc}	13.25±1.03^{a}	2.25±0.25^{a}
+N	0.00±0.00^{a}	1.25±0.63^{abc}	12.50±1.04^{a}	4.75±0.25^{e}
CIAT 899	32.50±11.48^{abcde}	2.75±0.25^{d}	15.00±1.29^{a}	4.25±0.25^{cde}
NR1	50.75 ±9.94^{cdefg}	1.50±0.29^{bc}	14.25±1.76^{a}	3.75±0.25^{bcd}
NR2	26.75±8.89^{abcd}	0.75±0.25^{ab}	12.25±0.85^{a}	3.00±0.00^{ab}
NR3	8.50±2.11^{abc}	2.00±0.41^{cd}	12.00±0.41^{a}	3.00±0.41^{ab}
NR4	14.50±4.01^{abc}	1.50±0.29^{bc}	12.50±0.65^{a}	4.00±0.41^{cde}
NR5	61.25±17.27^{defg}	1.00±0.41^{abc}	11.00±0.91^{a}	3.75±0.48^{bcd}
NR6	40.75±6.14^{abcdef}	1.75±0.75^{bcd}	12.75±1.65^{a}	3.75±0.25^{bcd}
NR7	22.75±7.74^{abcd}	1.50±0.29^{bc}	13.50±0.65^{a}	3.75±0.25^{bcd}
NR8	37.75±1.89^{abcde}	1.25±0.25^{abc}	12.75±0.75^{a}	3.50±0.29^{bc}
NR9	15.50±5.61^{abc}	0.75±0.48^{ab}	12.75±0.75^{a}	4.25±0.25^{cde}
NR10	3.75±1.93^{ab}	1.00±0.41^{abc}	12.00±0.41^{a}	4.50±0.29^{de}
NR11	4.75 ±1.49^{ab}	1.75±0.48^{bcd}	14.00±1.41^{a}	4.50±0.50^{de}
NR12	84.00±5.96^{fg}	1.50±0.29^{bc}	16.00±0.91^{a}	4.75±0.25^{e}
NR13	90.25±6.49^{g}	0.75±0.48^{ab}	12.00±1.22^{a}	4.25±0.25^{cde}
NR14	34.50±6.36^{abcde}	0.75±0.48^{ab}	11.00±0.71^{a}	4.25±0.48^{cde}
NR15	13.50±5.69^{abc}	1.00±0.41^{abc}	11.25±0.75^{a}	4.25±0.25^{cde}
NR16	26.00±8.03^{abcd}	1.50±0.29^{bc}	12.50±0.65^{a}	4.50±0.29^{de}
NR17	71.75±4.27^{efg}	1.25±0.25^{abc}	13.00±1.35^{a}	4.75±0.25^{e}
NR18	47.00±22.73^{bcdefg}	0.25±0.25^{a}	13.50±1.55^{a}	4.25±0.48^{cde}
	P=0.0001	**P=0.0372**	**P=0.315**	**P=0.0026**

*Values represent Mean ± Standard error (SE). Values with dissimilar letter(s) in a column are significant by Fisher LSD at $p = 0.05$.

NR=Native Rhizobium, Number after NR represent village in which soil was collected, 1=Nshara, 2=Uduru, 3=Wari, 4=Foo, 5=Mamba, 6=Kiyeri, 7=Shari Uraa, 8=Sonu, 9=Roo, 10=Mudio, 11=Mboreni, 12=Ng'uni, 13=Rundugai, 14=Mbatakero, 15=Ngosero, 16=Longoi, 17=Kikavu chini, 18=Kawaya

4.4. Discussion

4.4.1. Isolated strain

The characteristics of the isolated rhizobia strains presented in table 5 above resemble to the standard characteristics of rhizobia, and hence proved that the isolated strains were rhizobia. These findings are in line with the study done by Muthini *et al.* (2014) and Simon *et al.* (2014) who isolated the indigenous rhizobia from nodules of *Phaseoulus* bean and the colony characteristics was similar with those found in this study. These findings gave hopeful indicator towards production of inoculants for *Phaseoulus* bean native to Tanzania since most of the isolated strains showed ability to induce nodulation on the roots of *P.vulgaris*.

4.4.2. Influence of isolated rhizobia strains on nodule number and growth parameters of *P.vulgaris* as a measure of symbiotic effectiveness

The results revealed that, to a large extent isolated rhizobia strains have a positive influence on nodule number per plant, shoot length (cm), shoot dry mass (mg), colour rank of the plant leaves, stem girth (mm) and chlorophyll content per gram of leaf ($\mu gChlg^{-1}$), but don't have significant influence on the number of leaves per plant. Some of the isolated rhizobia strain such as NR12 and NR13 showed outstanding performance in symbiotic effectiveness exceeding the standard strain CIAT 899 in various growth parameters.

Number of nodules and shoot length

Basing on the mean number of nodules, NR13 and NR12 strains are more effective in nitrogen fixation than the standard strain CIAT 899 (Table 7). These findings agree with Rodriguez-Navarro *et al.* (2000), Muthini *et al.* (2014) and Simon *et al.* (2014) whereby both of them found that, some of the indigenous isolates had higher ability of forming effective nodules in the roots of *P.vulgaris* than the commercial/standard strains. Also, this study demonstrated that nodule can be the best measure of the symbiotic effectiveness of rhizobia since all highly nodulated plants in this study showed higher shoot length, shoot dry mass, plant leaves colour and higher chlorophyll contents as opposed to Sharma and Kumawat (2011) who demonstrated that nodule number is not an appropriate measure of effectiveness in rhizobia–legume symbiosis. The shoot was longer in the inoculated beans compared to shoot of non-inoculated beans.

Shoot dry mass and chlorophyll content

Shoot dry mass is considered as the best measure of the symbiotic effectiveness between rhizobia and legumes while the chlorophyll is considered as the indirect measure of nutrient status due to the fact that nitrogen is incorporated in chlorophyll (Filella *et al.*, 1995; Moran *et al.*, 2000; Sharma and Kumawat, 2011). Therefore, the shoot dry mass and chlorophyll content is the main parameters which determine the effectiveness of the inoculants. In this study, isolated strains showed significance efficiency in fixing nitrogen by enhancing shoot dry mass and chlorophyll content at a significance level of $p < 0.01$ and $p < 0.05$ respectively. In both shoot dry mass and chlorophyll content the highest value was obtained in plants inoculated with indigenous strain RN12 and the lowest was obtained in non-inoculated plants (negative control). This corresponds with previous studies (Muthini *et al.*, 2014; Simon *et al.*, 2014). This implies that there is high possibility of having indigenous rhizobia strain which is more effective in fixing nitrogen than the current standard strain CIAT 899.

Colour of plant leaves

The plant colour ranking was done by looking at the greenish colour of the leaves. By taking into consideration that greenish colour of plants is from the chlorophyll pigment, looking at plant colour is one of the indirect way of measuring chlorophyll and nitrogen status of the plant. The ability of the isolated rhizobia strains to fix nitrogen was shown by significant differences in the colour of inoculated plant and non-inoculated plants at $P < 0.01$. The plant inoculated with isolated strain RN12, RN17, RN10, RN11, RN16, CIAT899 and those inoculated with nitrogen only (+N) had a deep green colour, while non-inoculated plant had a pale green colour. These results suggest that, there is a great hope of having stronger native strains that will be used as inoculants since most of the isolated strains have the same or more ability of fixing nitrogen as the standard strain CIAT899.

Stem girth, number of branches and number of leaves

The isolated strain shows significant influence ($p \leq 0.05$) on the colour ranking stem girth and the (Table 6 and 7). Inoculation with the rhizobia did not give any significant ($p \leq 0.05$) differences in the number of leaves per plant $p = 0.315$. This gives a clue that the number of leaves is not a good measure of the symbiotic effectiveness. This was against the findings by

Simon *et al.* (2014) who reported significant result in the number of leaves due to application of rhizobia strains.

4.5. Conclusion

The study confirmed that all isolated strains were rhizobia due to their ability of inducing nodules in the *Phaseoulus* bean after re-inoculation in authentication experiment. Symbiotic effectiveness tests found that, the isolated RN12 showed extraordinary efficiency in fixing nitrogen in *P.vulgaris* than standard/commercial rhizobia strain CIAT8999 which was used as a positive control. Plants inoculated with RN12 shows higher nodulation, highest shoot dry mass, deep green, healthy leaves and highest chlorophyll content while those inoculated with CIAT 899 showed the highest value in only one growth parameter which is the shoot length. Those strains which showed higher symbiotic effectiveness than the commercial strain (CIAT 899) enhances the hope of having inoculants derived from *Rhizobium* which are native to Tanzanian soil. However, the field trial to test symbiotic effectiveness and molecular characterization of the best isolated strain is of importance for the confirmation of its effectiveness and for proper genotypes records of those strains.

CHAPTER FIVE

5.0. GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1. General Discussion

This study aimed at isolating, authenticating and evaluating symbiotic effectiveness of indigenous rhizobia as well as estimating population size of those indigenous rhizobia. This was done purposely as an effort towards tackling the problem of nitrogen deficiency in the soils of smallholder farmers in Africa. Additionally, analysis of soil components such as N, P, K, Na, Ca, Mg, organic matter, and pH was done.

The results showed that, the soils in Hai district had a deficit in soil N and P while exchangeable cations (Ca, Mg and Na) are in normal level. The population size of indigenous rhizobia in the study was generally low (less than 100 cells g^{-1} of soil) and the total of 18 strains of indigenous rhizobia strain was isolated and proved to be rhizobia in an authentication experiment. Out of the 18 isolated strains two strains NR12 and NR13 outperformed the standard strain CIAT 899 in symbiotic effectiveness test.

The observed lower levels of N and P in soils of Hai district implies that, in order to improve crop production in this area effort should be directed at finding the means of improving nitrogen and phosphorous in these soils. The use of rhizobia inoculants may partly solve this problem as it has been reported that *Rhizobium* are able to solubilize the insoluble phosphate hence releasing available phosphorus (Halder *et al.*, 1990; Halder and Chakrabartty, 1993; Rodriguez-Navarro *et al.*, 2000; Son *et al.*, 2006). Also *Rhizobium* have the ability to convert atmospheric dinitrogen gas (N_2) into ammonia (NH_3), a form which plant can be utilized by the plants, hence solving a problem of nitrogen deficiency (Giller, 2001; Lindström and Mousavi, 2010; Lindström *et al.*, 2010; Dall'Agnol *et al.*, 2014; Loganathan *et al.*, 2014a).

The MPN results showed that the number of indigenous rhizobia population at 61% of the study area was below 10^2cells g^{-1} of soil, which means the area have generally low indigenous rhizobia, so application of rhizobia inoculants in this area is of importance. Also, there were no significant differences in the number of the indigenous rhizobia between lower, middle and upper agro ecological zone. This differs with Simon *et al.* (2014) who found significant differences in the number of indigenous rhizobia between lower and upper agro-ecological zone,

with upper zone having higher number of indigenous rhizobia than the lower. The large number of rhizobia population in the upper zone soils was due to high amount of rainfall in the upper zone compared with lower zone, hence upper zone have an environment that support rhizobia survival. The MPN result of this study provides an indication that, a number of indigenous is not the limiting factor for inoculants response in the study area just as reported by Singleton and Tavares (1986) that for the rhizobia inoculants to increase crop yield, size of indigenous rhizobia should be lower than 100cells g^{-1}soil.

In isolation, 18 indigenous rhizobia strains were authenticated and found to be rhizobia through their ability to form nodules on the roots of *P.vulgaris*. Strain NR12 from Ng'uni Village in the upper zone and RN13 from Rundugai village in lower zone showed an outstanding performance in symbiotic effectiveness than the standard/commercial strain CIAT 899. RN12 came up as the best strain by influencing nodulation in the roots of the *P.vulgaris* than CIAT 899 which was used as control. RN12 is the strain which is considered as the best in this study since it have shown higher effectiveness in fixing nitrogen by enhancing shoot dry mass, leaf chlorophyll content and plant vigour in higher efficiency than the standard strain CIAT 899. This sent a message that if deliberate efforts are vested in promoting BNF technology through rhizobia inoculants there is higher possibility of having best inoculants made from rhizobia strains indigenous to Tanzanian soil.

5.2. General Conclusion

Rhizobia are very crucial for enhancing nitrogen availability in the soil. This study found that, the area of Hai District has a low population size of indigenous rhizobia (<100cells g^{-1} of soil) as well high deficiency of soil nitrogen and phosphorous. Therefore the use of *rhizobium* inoculants in this area is highly recommended to rescue the situation of low nutrient in the soil of this area. Also in isolation and evaluation of symbiotic effectiveness a total of 18 indigenous rhizobia strains were isolated and two strains NR12 and NR 13 demonstrated higher effectiveness in nitrogen fixation in the greenhouse experiment than the tested standard strain CIAT 899. This gives reflection that in Tanzanian soil there is a vast range of rhizobia species which may be used to produce local inoculants for *P.vulgaris* and other legumes.

5.3. General Recommendations

This study recommends further study, especially to involve the field trial to test symbiotic effectiveness and molecular characterization of the strains which showed outstanding performance in the greenhouse trial. This is to prove the symbiotic effectiveness of the indigenous rhizobia strains which performed better in greenhouse experiment as well as getting their phylogenetic records for life time references. Also there is a need to conduct bigger studies on indigenous rhizobia populations as well as isolation studies across different agro-ecological zones of Tanzania

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