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Characterization and effectiveness of indigenous rhizobia nodulating common bean (phaseolus vulgaris l.) in Hai district, Northern Tanzania

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CHARACTERIZATION AND EFFECTIVENESS OF INDIGENOUS RHIZOBIA NODULATING COMMON BEAN (*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 of the Nelson Mandela African Institution of Science and Technology

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ABSTRACT

A study to characterise indigenous rhizobia nodulating bean (*Phaseolus vulgaris* L) was conducted from April, 2018 to November, 2018 in Hai District. Thirty rhizobia organisms were isolated from bean root nodules and grown on Yeast Extract Mannitol Agar containing Congo Red (YEMA w/Congo Red). Morphological identification and molecular characterization based on 16srRNA and *gyrase* subunit B (*gyrB*) genes and evaluation of the rhizobia isolates for their symbiotic effectiveness on bean was conducted. The results showed that all rhizobia isolates were fast growing (2 – 5 days) on YEMA w/congo red, failed to absorb Congo Red, and displayed whitish or creamy colour on Yeast Manitol Broth (YMB) indicating that they were rhizobia. Results also showed that DNAs from 18 out of 30 rhizobia isolates were amplified by 16S rRNA primers, and only 9 out of 30 rhizobia isolates were amplified by *gyrase* subunit B primers. DNAs from eight of the isolates were neither amplified by 16S rRNA nor *gyrB* primers indicating they were different from those that the primers could amplify. Nevertheless, in testing the effectiveness, all 30 isolates were able to induce nodules on host plant and most of them had relative effectiveness index (RI) which was as high as that of the commercial CIAT 899 strain. Isolate IR1, IR3, IR10 and IR12 had RI above 100%, higher than CIAT 899. Isolate IR20 had the lowest HI (78%). Such results implied that indigenous rhizobia identified can be used for bean inoculation instead CIAT 899 in Tanzania.

DECLARATION

I, Grace Lucian Kilambo 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**.**

Grace Lucian Kilambo _____ _____

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Prof. Patrick A. Ndakidemi_____ _____

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Dr. Ernest R. Mbega_____ _____

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Dr. Kelvin M. Mtei _____ _____

Signature and Name of Supervisor Date

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by The Nelson Mandela African Institution of Science and Technology a dissertation entitled: "Characterization and effectiveness of indigenous rhizobia nodulating common bean (*Phaseolus vurgalis* L.) in Hai district, Northern Tanzania".

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
CR	Congo Red
DNA	Deoxyribonucleic acid
NCBI	National Center for Biotechnology Information
NM-AIST	Nelson Mandela African Institution of Science and Technology
PCR	Polymerase Chain Reaction
RNA	ribonucleic acid
TN	Total Nitrogen
YEMA	Yeast Extract Mannitol Agar
YEMB	Yeast Extract Mannitol Broth

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Rhizobium is a gram negative soil bacterium capable of infecting legume roots, inducing the formation of nodules and fixing nitrogen through the process known as Biological Nitrogen Fixation (BNF) in the soil (Eskin, 2012; Mohammadi and Sohrabi, 2012; Namkeleja *et al.*, 2016; Poonia, 2011). The BNF takes place when rhizobia form a symbiotic relationship with legume plants as a results, they trap and fix atmospheric nitrogen into a form such as ammonia (Laguerre *et al.*, 1996; Namkeleja *et al.*, 2016; Pawar *et al.*, 2014; Poonia, 2011). This form of mutualistic symbiotic relationship has been described as the most important biological mechanism for providing nitrogen to the plants as alternative to expensive synthetic nitrogen fertilizers in agriculture (Freiberg *et al.*, 1997). The Biological Nitrogen Fixation has grown attention in recent years in Africa because it is an environmental friendly farm input as it prevents groundwater pollution by nitrates and its less expensive (Berrada and Fikri-Benbrahim, 2014; Bhaganagare *et al.*, 2013). It is a useful technology in agricultural systems in order to overcome the problems associated with depletion of soil fertility as it manages soil acidity and salinity, which is accumulated due to frequent application of synthetic nitrogen fertilizers (Loganathan *et al.*, 2010; Nyoki and Ndakidemi, 2014; Tairo and Ndakidemi, 2013). Moreover, the use of inorganic nitrogen fertilizers usually get lost by volatilization, de-nitrification and leaching of nitrate into ground water and report shows that only 30% to 50% is used by the crop (Graham and Vance, 2003).

Nitrogen is the most limiting nutrient for plant growth and crop yield (Mnalku, *et al.*, 2009). Low soil N is a major constraint to increased yields on farmers' fields. BNF can alleviate soil infertility and increase crop yields (Bekunda *et al.*, 2010). Some African countries such as Rwanda, Malawi, Kenya, Egypt and Zimbabwe have started to exploit legume N_2 fixation as an alternative to N fertilizer (Bala *et al.*, 2011; Mugabe, 1994; Otieno *et al.*, 2009; Woomer *et al.*, 2014). However, symbiotic N_2 fixation can only be achieved in the presence of efficient rhizobial strains in the soil (whether native) or introduced via inoculation (George *et al.*, 2007).

The inoculation of legumes with effective rhizobia can improve plant growth and grain yield (George *et al.*, 2007). Inoculation with effective and persistent rhizobia can replace the

application of N fertilizer (Otieno *et al.*, 2009). However, poor nodulation and lack of response to inoculation have frequently been reported in field experiments involving common bean (Hungria *et al*., 2000; Mostasso *et al*., 2002). In Kenya, lack of response to rhizobial inoculation has also been reported for common bean (Musandu and Ogendo, 2002) due to the number of factors including incompatibility between the host plant and bacteria, and great sensitivity of the legume-rhizobia symbiosis to environmental stress (Hungria *et al*., 2000; Kaschuk *et al*., 2006). Poor viability of inoculants strains, higher concentration of soil mineral N, and presence of highly competitive native rhizobia that restrict occupancy by inoculants stains (George *et al.*, 2007) have also been cited as the constraints to symbiotic N_2-fixation.

Rhizobial strains selection as was done in Brazil, has proven the potential contribution of highly efficient and competitive rhizobia in agriculture (Hungria *et al*., 2000). Thus a program of continuous strain selection could help in reversing low bean yields while addressing the issue of soil N depletion. *Rhizobium tropici* has for example been identified as an effective strain with greater symbiotic stability even under stressful conditions (Hungria *et al*., 2000). Essentially *R. tropici* has replaced *R. leguminosarum* bv. phaseoli which was used as the commercial inoculant strain in Brazil in the early 19th century and later reported to have lost its symbiotic properties in several field and glasshouse experiments (Hungria *et al*., 2000). It is therefore important to continue to identify more efficient, competitive and genetically stable rhizobial strains for replacing existing inoculants strains that are in the process of losing their symbiotic ability and/or efficiency. One of the aims of this study was to evaluate the symbiotic efficiency of rhizobia isolated from root nodules of common bean farmers' fields, by measuring photosynthate production and biomass accumulation.

Common bean is a promiscuous host that is nodulated by a variety of rhizobia (Kaschuk *et al.*, 2006) that makes it an interesting model for the studies of rhizobial species. Rhizobia isolated from root nodules are generally characterized phenotypically, physiologically and/or molecularly in order to determine identity and diversity (Laguerre *et al.*, 1994; Martens *et al.*, 2008). The use of molecular techniques to optimize conditions for symbiosis of plants and bacteria might contribute to an increasing exploitation of BNF (Bala *et al.*, 2011; Mugabe, 1994). DNA-based methods have become increasingly used to characterize rhizobia for more than 25 years (Andrews and Andrews, 2017). In particular, phylogenetic analyses of sequences of the 16S ribosomal RNA(rRNA) gene, a range of "housekeeping" genes and

genes involved in the symbiosis have been developed as a "standard approach" (Martens *et al.*, 2007; Peix *et al.*, 2015).

Characterization of rhizobia is increasingly becoming complex as strain identity has to be revised periodically due to the discovery of new genera and species (Lindström *et al.*, 2015; Martens *et al.*, 2008). It had been recommended by The Ad Hoc Committee for re-evaluation of species definition on the use of 16S rRNA, housekeeping gene, symbiotic gene and DNA-DNA hybridization as the molecular criteria for species delineation (Pongslip, 2012; Stackebrandt *et al.*, 2002). In this study characterization of common bean-nodulating rhizobial isolates was done using 16S rRNA and housekeeping genes (gyrB).

To practically explore the BNF technology in agriculture, inoculation of legumes with characterised rhizobia inoculants is common worldwide (Bala *et al.*, 2011). However, such technology is not well established in most African countries including Tanzania (Bala *et al.*, 2011; Mugabe, 1994). There are number of uncharacterised indigenous rhizobia that are existing in the Tanzanian soils (Namkeleja, 2017; Ndakidemi *et al.*, 2014).

Phenotypic and biochemical characterization used in characterizing rhizobia, basing on morphological features such as colony morphology and biochemical responses of the bacterium are not accurate as they vary depending on the media used and the conditions of the growth (Amijee and Giller, 1998; Giller, 2001). Therefore, molecular techniques are usually recommended and are more reliable for characterising rhizobia, as they are capable of distinguishing genera, species, and even strain level and they are easy and quick to use, as well (Macrae, 2000). This study was conducted to isolate rhizobia from root nodules of the indigenous *Phaseolus* bean, then to authenticate, characterise and perform sequence analysis to identify indigenous rhizobia strain that nodulate *Phaseolus* bean as well as to determine the effectiveness of the identified strain in nodulating the *Phaseolus* bean as a means to advance BNF technology, which is useful to enhance availability of N in the soil.

1.2 Problem statement and justification

The role and characteristics based on cell growth, colony morphology, and biochemical responses of indigenous rhizobia for nitrogen fixation in common bean has been established in Tanzania (Amijee and Giller, 1998; Mugabe, 1994; Namkeleja, 2017). However, there is little information on the molecular characteristics of the responsible indigenous rhizobia and

their effectiveness in nodulating the crop (Bala *et al.*, 2011; Mugabe, 1994; Woomer *et al.*, 2014).

Considering the adaptation of native rhizobia to local soil conditions, it is important to characterize the indigenous strains and to identify the most effective ones for use in inoculants production. Thus the aim of this study was to characterize the native rhizobia isolated from root nodules of common bean growing on different soils form Hai District in Northern Tanzania.

Housekeeping (*gyrB*) and 16S rRNA genes were used in characterising the rhizobia isolates. The 16S rRNA has been used successfully in similar studies. For instants in study for phylogeny analysis on elite rhizobia strain used in Brazilian commercial inoculants (Menna *et al.*, 2006), Valverde *et al.* (2006) in finding closely related species that nodulate common bean in Portugal and a study by Gisèle Laguerre *et al.* (1994) to establish genetic relationships between and within the genera and species of rhizobia nodulating *Phaseolus* bean in France. Moreover, the 16S rRNA gene sequence analysis has a greater power for measuring the degree of relationships between organisms above the species level (Martens *et al.*, 2008; Martens *et al.*, 2007). The gene is found in all living organisms which allow the comparison of the phylogenetic relationships among different organisms and allow construction of the tree of life and it is highly conserved, thus they do not undergo mutation easily (Martens *et al.*, 2007; Willems and Collins, 1993). However, to be able to characterise the rhizobia at species level, housekeeping gene (*gyrB*) was used as phylogenic markers since it distinguishes rhizobia of very closely species.

The indigenous rhizobia strain identified in this study provides a means towards making the inoculants available and accessible to smallholder farmers. This was done considering the high diversity of indigenous rhizobia species in Tanzanian soils.

1.3 Objectives of the study

1.3.1 General objective

To characterise indigenous rhizobia based on 16S rRNA and housekeeping gene (*gyrB*), and evaluate their effectiveness in forming nodules and yield of common bean varieties in Northern Tanzania.

1.3.2 Specific objectives

The specific objectives were:

(i) To identify indigenous rhizobia isolates based on 16SrRNA and housekeeping gene (*gyrB*) and morphological characteristics of bean growing in different soil of Hai District in northern Tanzania.

(ii) To evaluate effectiveness of rhizobia isolates from selected soils of Hai District on common beans growth.

1.4 Research questions

(i) What are the morphological and genetic characteristics of indigenous rhizobia isolates extracted from bean growing in different soil environments in Hai District in Northern Tanzania based on 16SrRNA and housekeeping gene (*gyrB*)?

(ii) How effective are the isolated indigenous rhizobia in nodulating common bean and influencing growth?

1.5 Significance of the study

These The findings of this study provides knowledge to the Tanzanian farmers and African at large on soil fertility management through the provision and application of indigenous rhizobia inoculants so as to enhance BNF of bean plants in Tanzania. The study also provides effective and competitive indigenous rhizobia strains that would be genetically characterized and may be commercialized for inoculants production in Tanzania.

CHAPTER TWO

LITERATURE REVIEW

2.1 Common bean production

Common beans (*Phaseolus vurgalis L.*) are among the most diverse plants in both tropics and temperate areas (Sprent and James, 2007) and can be grown in different agro-ecological zones (Hendawey and Younes, 2013). They are angiosperm plants belonging to the family Leguminosae/Fabaceae (Doyle and Luckow, 2003; Forest and Chase, 2009; Tran and Nguyen, 2009). Due to their ability to fix nitrogen in association with rhizobia, bean plants can grow in very low fertile soils (Freitas *et al.*, 2004; Wong, 2003). Although bean plants are grown for the purpose of providing the traditional diets throughout the world, they provide numerous benefits to soil as well as to the crops through intercropping (Graham and Vance, 2003; Ndakidemi *et al.*, 2011; Nhamo *et al.*, 2003; Stajkovic *et al.*, 2011). Low income of the small-scale farmers in Africa who are the major bean producers, made them to rarely apply fertilizers during bean production, therefore, the crop is largely dependent on fixed nitrogen from native nitrogen fixers (Dakora and Keya, 1997). Relying on native nitrogen fixers without prior information on its efficiency and compatibility with host legume lead to low or failure of crop production, since in most cases, native nitrogen fixers are competitive to inoculants but not efficient strain and possibly incompatible to the host plant (Wilkinson and Parker, 1996). The interaction between plants and soil bacteria occurs much in the rhizosphere (Marschner *et al.*, 2011). The identification of native strains capable of fixing nitrogen with specific *Phaseolus* bean will provide an inexpensive solution for enhancing common bean production by the majority of small scale farmers in Tanzania.

2.2 Common bean production in Tanzania

Common bean (*Phaseolus vulgaris* L.) is among the most important grain legume crops grown in many parts of the eastern and central African countries (Amijee and Giller, 1998; Aserse *et al.*, 2012; Ndakidemi *et al.*, 2006; Ribeiro *et al.*, 2013). It is widely grown by small holder farmers as it provides a substantial source of dietary protein and carbohydrate to supplement a main diet of maize and cassava (Okigbo, 1977; Ribeiro *et al.*, 2013).

In Tanzania, including Hai district, farmers grow beans mainly for local consumption as it is one of the staple foods in many parts of the country (Karel *et al.*, 1980) as well as for cash income (Hillocks *et al.*, 2006). Beans are the main grain legume crop grown in Tanzania,

where they are often intercropped with maize, as well as grown as sole crop (Hillocks *et al.*, 2006). Bean crop require substantial amount of rainfall for better production, in dry areas supplementary irrigation is needed to obtain optimum yield (Katungi *et al.*, 2009). The areas with reliable rainfall and cooler temperatures such as mid to high altitude areas of the country are therefore the major areas of production (Wortmann, 1998). In Tanzania, the most suitable areas for bean cultivation are in the northern zone particularly Arusha and Kilimanjaro Regions, the Great Lakes region in the west, Kigoma and Kagera regions and in the Southern Highlands, Iringa and Mbeya regions (Katungi *et al.*, 2009). Tanzania is second largest producer of dry beans in sub Saharan Africa after Kenya and among the top twenty largest producers in the world (Hillocks *et al.*, 2006). Average bean yields in Tanzania are around 500 kg/ha although the potential yield under reliable rain fed conditions is 1500–3000 kg/ha, using improved varieties and proper crop and land husbandry (Hillocks *et al.*, 2006; Ndakidemi *et al.*, 2006). Low yield obtained by most smallholder farmers in Africa is mainly caused by poor seed quality, poor performance of the local landraces, since mostly are susceptible to pests and diseases, low soil fertility, drought and poor crop management, such as late weeding as well as low supply of nitrogen to support a good seed yield with high protein content (Giller *et al.*, 1998; Mwandemele and Nchimbi, 1990; Ndakidemi *et al.*, 2006; Okalebo *et al.*, 2007; Pereira *et al.*, 2006). Under such conditions the ability of *Phaseolus* to fix atmospheric nitrogen is likely to be an important factor determining yields. This is due to the fact that bean crop is mainly grown by the resource-poor small scale farmers (Graham, 1981; Ndakidemi *et al.*, 2006) who are not able to purchase expensive inorganic fertilizers.

At present, there are much emphasizes on the application 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; Nyoki and Ndakidemi, 2014; Tairo and Ndakidemi, 2013). Biological Nitrogen Fixation by rhizobia is considered as less expensive and environmentally friendly alternative to improve crop yield in comparison to chemical nitrogen fertilizers (Gaurav *et al.*, 2009; Ghimire, 2002; Haru and Ethiopia, 2012; Meade *et al.*, 1985; Mfilinge *et al.*, 2015; Nyoki and Ndakidemi, 2013). Inoculation of legumes with characterized rhizobia inoculants is the most used BNF technology in agriculture as evidenced in previous studies (Bull *et al.*, 2002; Chisholm *et al.*, 2006; Deshwal and Chaubey, 2014; Lindström *et al.*, 2010) but in most African countries including Tanzania, this technology is not well established (Bala *et al.*, 2011; Mugabe, 1994).

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). Therefore, the need for isolation and molecular characterization of indigenous rhizobia is very important to determine the right strain that nodulate *Phaseolus* bean, will help to identify the suitable rhizobia strains for inoculants production aiming to enhancing crop yield.

2.3 Biological nitrogen fixation in Common Bean

Biological Nitrogen FFixation is a major source of N for African small holder farmers who use little or no fertilizer, especially for Bean production (Smaling *et al.*, 2008) because it is neither available nor affordable (Bohlool *et al.*, 1992). Rinnofner *et al.*, (2008) described BNF in the context of legume catch crops, as an additional benefit. Studies (Alves *et al.*, 2003; Araújo *et al.*, 2006; Hungria *et al.*, 2006; Zotarelli *et al.*, 1998) revealed that rhizobia contribute up to 80% of the above-ground N accumulation in soybean through N fixation. Another study carried out in Uganda shows that perennial crops contributes 22% of nitrogen through BNF, and 44% for annual crops (Nkonya *et al.*, 2008). It is estimated that about 11.1 million metric tons of nitrogen is fixed annually through BNF in developing countries (Hardarson *et al.*, 2001). About twice of this amount of mineral fertilizers will be required to be supplied in the legume crops to achieve the same crop yield level (Hardarson *et al.*, 2001). Biological Nitrogen Fixation contributes about 100 million tons of nitrogen for terrestrial ecosystems, 30 to 300 million tons for marine ecosystems and 20 million tons from chemical fixation due to atmospheric phenomena (Rakash and Rana, 2013). Full exploitation of BNF could reduce expenditure on importation of Nitrogen fertilizers in most African countries (Mugabe, 1994). It is estimated that *Rhizobium* alone could provide more than 50% of the fertilizer required for crop production in most of the marginal lands of Kenya, Zimbabwe and Tanzania (Mugabe, 1994).

The symbiotic process is triggered by limited nitrogen of the host plant which has to select its specific rhizobia from number of bacteria in the rhizosphere (Leidi and Rodriguez-Navarro, 2000; Maróti and Kondorosi, 2014; Ndakidemi and Dakora, 2003). To archive this the host plant secretes flavonoids signal molecules from the root which act as chemo-attractants, as well as the inducers of *Rhizobium* nodulation gene (Downie, 2014; Nadal and Paszkowski, 2013; Wang *et al.*, 2012). The bacteria signal molecules called Nod factors are produced following the expression of nod genes by flavonoids signals, and they trigger the infection

signals, the infection process and nodule formation (Downie, 2014; Nadal and Paszkowski, 2013; Wang *et al.*, 2012).

Atmospheric N_2 is reduced to ammonium within root nodules, by nitrogenase for plant uptake (Zahran, 1999). Thus in the legume-rhizobia symbiosis, the host plant supplies the microsymbiont with photosynthate in exchange for N compounds from BNF (Glick, 2003).

Biological nitrogen fixation is a renewable, and environmentally safe sustainable source of N for agriculture that can replace the use of chemical fertilizer (Rahmani *et al.*, 2011). However, the amount of nitrogen fixed by common bean plants is very low and is reported to range from 5 to 29 kg ha^{-1} in Kenyan soils (Ngome, 2009). Limited skills, low quality inoculants, acid soils and high soil temperatures, among others are the most serious problems affecting nodulation and N fixation in tropical cropping systems. Studies on molecular characterization of rhizobia would provide useful information on rhizobia species that nodulate specific bean crops and provision of inoculants that would improve legume production and hence their contribution to soil fertility would be realized.

2.4 Diversity of rhizobia strains nodulating common bean

Common bean is one of the most promiscuous hosts in its symbiotic association with rhizobia (Pinto *et al.*, 2007). The legume is reported to be nodulated by different genera and species of root nodule bacteria, which include *Rhizobium. tropici* bv. *phaseoli, R. etli* bv. *phaseoli, R. leguminosarum* bv. *phaseoli, Sinorhizobiummeliloti,* S. *fredii, R. freirei, R.phaseoli, R. gallicum* bv. *phaseoli*and *R. giardinii* bv. *Phaseoli* (Anyango *et al*., 1995; Dall'Agnol *et al*., 2013). *Rizobium etli* is the dominant group in both the Mesoamerica and Andrea centers of genetic diversification (Aguilar *et al.*, 2006). *R. etli* has however been isolated from other parts of the world probably due to seed from trade and human migration (Aguilar *et al.*, 2006). In Europe, it is believed that a viable cell of *R. etli* was introduced with common bean seeds, followed by transfer of symbiotic plasmid to strain of local *R. leguminosarum* bv. *phaseoli* and *R. gallicum* bv. *Phaseoli* (Pinto *et al.*, 2007). *Rhizobium tropici* was originally isolated from common bean nodule in Colombia and found to be equally abundant in Brazilian soils (Pinto *et al.*, 2007). So far, Brazilian soils are the source of the greatest majority of the *R. tropici* bv. *phaseoli* strains isolated (Hungria *et al.*, 2006; Pinto *et al.*, 2007).

In Africa, *R. tropici* bv. *phaseoli* was reported to nodulate common bean in Kenya, Ethiopia, Senegal and Gambia (Anyango *et al.*, 1995; Diouf *et al.*, 2000; Van berkum *et al.*, 1996). *Rhizobium etli* was also isolated in Senegal and Gambia (Diouf *et al.*, 2000) as well as *S. meliloti* and *S. fredii* in Tunisia (Mnasri *et al.*, 2007). *Rhizobium leguminosarum* was commonly found in Europe, however, its presence has been reported in soils of Colombia and Tunisia. *Rhizobium gallicum* and *R. giardinii* are other fast growing rhizobia nodulating common bean in Europe, Central America (Mexico) as well as Africa (Aserse *et al.*, 2012; Shamseldin and Werner, 2005; Silva *et al.*, 2005). It is notable that neither *R. gallicum* nor *R. giardinii* has been isolated in the Americas, the center of origin (Martinez-Romero, 2003). *R. phaseoli* which is less abundant and closely related to *R. etli* has been isolated in the center of origin of common bean and in Ethiopia (Aserse *et al.*, 2012; López-Guerrero *et al.*, 2012). Recently, Dall'Agnol *et al.* (2013) also identified *R. freirei* in Brazil as another group of common bean rhizobia which are very effective in fixing N_2. *Burkoholderia phymatum* has also been reported to nodulate common bean in Moroccan and Amazonian soils (Coutinho *et al.*, 2013; Ferreira *et al.*, 2012). Clearly, the diversity of root-nodule bacteria nodulating common bean is large, and the associated microsymbionts are limited in their global distribution. Rhizobial diversity plays an important role in developing long-term strategies for increasing the contribution of biological N_2 fixation to agricultural productivity. Genetic tools are largely used for systematic analysis for rhizobial diversity.

2.5 Isolation of rhizobia

Isolation of rhizobia provides a means in discovering strong strain for nitrogen fixation in various legumes. There are still number of rhizobia which are not yet identified exceeds that identified (Giller, 2001) and effectiveness in nitrogen fixation by soil rhizobia population vary between species (Singleton and Tavares, 1986). Isolation of rhizobia strain from nonspecific (promiscuous) legumes such as common bean (*Phaseolus vulgaris* L.) provides a wide chance of identifying new effective strain for such legumes (Aserse *et al.*, 2012; Valverde *et al.*, 2006). It is important to isolate rhizobia strains that nodulate *Phaseolus vurgalis* because it is the most important legume crop cultivated in most African countries and its yield potential is not yet realized.

2.6 Molecular characterization of rhizobia

Molecular techniques for characterization of rhizobia provide precise information on closely related rhizobia strains and facilitate the discovery of several new rhizobial phylogenetic that

were formerly unknown (Macrae, 2000). The molecular techniques for rhizobial characterization were introduced and practiced since classification based on phonotypical and physiological characters did not reflect true evolutionary relationship among rhizobial species (Giller, 2001). With molecular techniques, different rhizobia strains can be detected compared with the phenotypic techniques. Thus, the application of molecular techniques is more reliable for characterizing rhizobia as it is not influenced by environmental factors. Molecular techniques are easy and quick to use, as well as capable of distinguishing genera, species, and even strain level.

The application of such molecular techniques in characterizing elite rhizobia strains facilitates the identification of accurate species of rhizobia that are effective for nodulating and fixing nitrogen as well as performing other functions in *Phaseolus* bean, hence simplifies inoculants production. In Tanzania, there is little information on the molecular characteristics of the responsible indigenous rhizobia and their effectiveness in nodulating the *Phaseoulus* bean. Considering the adaptation of native rhizobia to local soil conditions, it is important to characterize the indigenous strains and to identify the most effective ones for use in inoculants production.

2.6.1 Sequencing analysis for 16S rRNA gene

The 16S rRNA is the mostly used gene for bacterial identification as it can stretch up to 1500 base pairs (bp) that include conserved and variable regions that offer useful information in taxonomic studies. The gene coding for it is referred to as 16S rRNA and used for reconstruction of phylogenies. The 16S rRNA gene sequencing is a precise and quick method of identifying wide variety of aerobic and anaerobic bacteria (Petti *et al.*, 2005) and it has a greater power for measuring the degree of relationships between organisms above the species level (Martens *et al.*, 2008; Woese *et al.*, 1990). Moreover, the 16S rRNA gene is highly conserved, thus the function of the gene does not change, which indicates that random sequence variations can occur. However, the 16S rRNA gene sequence analysis often lacks resolving power at and below the species level (Aserse *et al.*, 2012; Martens *et al.*, 2008; Stackebrandt and Goebel, 1994). The weakness in the use of 16S rRNA in the diversity studies is caused by the presence of multiple copies in the genome of some bacteria, susceptibility to genetic recombination, and horizontal gene transfer (Aserse *et al.*, 2012). There are reports of bacteria, which represent different species but have identical or nearly identical 16S rRNA gene sequence (Li *et al.*, 2009; Martens *et al.*, 2008; 2007). To overcome

this drawback, protein-coding genes have been proposed as alternative phylogenetic markers to discriminate between closely related species.

2.6.2 Housekeeping gene

Housekeeping gene sequences provide reliable alternative information which is used to assess bacterial relationships at species level (Martens *et al.*, 2007). Analysis of multiple protein-encoding housekeeping genes is a widely applied tool for the investigation of taxonomic relationship among rhizobia (Adékambi and Drancourt, 2004; Christensen *et al.*, 2004; Holmes *et al.*, 2004; Naser *et al.*, 2005; Thompson *et al.*, 2005; Wertz *et al.*, 2003). This is because the use of housekeeping genes has higher degree of divergence which allows species discrimination (Martens *et al.*, 2007). In comparison with 16S rRNA genes, the higher degree of sequence divergence of housekeeping genes are superior for identification purposes, since the more-conserved rRNA gene sequences do not always allow species discrimination.

Martens *et al.* (2008) have confirmed the superiority of multilocus sequence analysis of housekeeping genes over DNA-DNA hybridization. In that study, 10 housekeeping genes (*atpD, dnaK, gap, glnA, gltA, gyrB, pnp, recA, rpoB* and *thrC*) were used which demonstrated clear species boundaries and higher discrimination potential for all the housekeeping genes. Sometimes, a strain might show variable grouping that is caused by the type of gene. This variation can be attributed to the differing evolutionary histories of the genes, or to horizontal gene transfer which sometimes affects the sequence structure of the protein-coding gene (Rivas *et al.*, 2009). In this study both 16S rRNA and protein-coding housekeeping gene (*gyrB*) were used to assess the taxonomic relationship among rhizobia nodulating *Phaseolus* bean in Hai district, Nothern Tanzania, in order to get the accurate rhizobia strains for inoculants production in common bean.

2.7 Symbiotic effectiveness of common bean rhizobia

Enhancement on symbiotic N supply from N_2 fixation with efficient rhizobial strains plays an important role in agricultural and economic sustainability of common bean in the tropics (Kaschuk *et al.*, 2006). As discussed earlier, common bean is nodulated by a wide range of soil rhizobia. However, effectiveness in N_2 fixation is not necessarily guaranteed as nodulation sometimes leads to less effective symbioses (Argaw and Mnalku, 2017; Minalku *et al.*, 2009). Poor nodulation and low N_2 fixation have frequently been reported in field experiments (Hungria *et al.*, 2003; Hungria and Vargas, 2000; Mostasso *et al.*, 2002).

Therefore, understanding strain effectiveness is essential for inoculants production. However, in studies where inoculants have been used, the response to inoculation has been variable due to host plant genetics and its nodulation promiscuity (Rahmani *et al.*, 2011). Physical and biological factors are among other factors that can hinder survival of the rhizobia. Mariangela Hungria and Vargas (2000) found sensitivity of common bean-rhizobia symbiosis to environmental stresses, which led to low N_2 fixation. Increased legume production requires the identification of native strains that are highly adaptable, effective and specific to the agro-ecological zone.

2.8 Factors affecting nodulation and Biological Nitrogen Fixation in field grown legumes

Poor environmental conditions and plant state cause rhizobia not to express their full potential in fixing nitrogen (Balasubramanian and Sinha, 1976; Elsheikh and Wood, 1990). The functional state of legume plant and the optimum environmental conditions supporting the macro and micro-symbionts have great influence on the process of nitrogen fixation (LaRue and Patterson, 1981). The populations of nitrogen fixing rhizobia in the soil vary in their tolerance to major environmental factors (Graham, 1992). Environmental stresses can affect the functions of the host plant and symbiotic rhizobia (Hungria and Vargas, 2000). The most threatening environmental conditions for the functions of nitrogen fixing rhizobia are marginal lands with low rainfall, acidic soils with poor water holding capacity, nutrient stress and temperature extremes (Zahran, 1999).

2.8.1 Temperature

The soil maximum temperature in the tropics usually exceed 40^0C at 5 cm and 50^0C at 1 cm depth (Eaglesham and Ayanaba, 1984; Hafeez *et al.*, 1988; Lal, 1993), this temperature range can limit nodulation as have effects on the root nodule structure, function and root hair infection (Day *et al.*, 1978; Eaglesham and Ayanaba, 1984; Graham, 1992). Different legumes can tolerate different range of temperature, for example, the optimum temperature for nodule functioning in common beans (*Phaseolus vulgaris*) is between 25˚C - 30˚C (Alexandre and Oliveira, 2013) and for soybean and cowpea is 35˚C - 40˚C (Michiels *et al.*, 1994). High temperature also affect the exchange of molecular signals between rhizobia and the host plants, hence reduces rhizobial survival and establishment in tropical soils (Hungria, 1994). The studies showed that the release of nod-gene inducers from soybean and common bean roots was decreased at 39°C (Hungria, 1994; Hungria and Stacey, 1997). High temperature probably affects much the root infection process component, since the sensitivity

located at the nodulation sites (Barrios *et al.*, 1963; Frings, 1976; Lie, 1981; Pankhurst and Gibson, 1973). Moreover, high temperature inhibits root-hair formation, hence reduce the number of sites for nodulation (Frings, 1976; Jones and Tisdale, 1921), adherence of bacteria to the root hairs (Frings, 1976), root-hair penetration and infection-thread formation (Barrios *et al.*, 1963; Frings, 1976; Joffe, 1961; Pankhurst and Gibson, 1973). It also affects nodule initiation, rhizobial release from the infection thread, and bacteroid development (Pankhurst and Gibson, 1973; Roughley, 1970; Vincent, 1980). Even if nodules are formed, high temperatures may affect nodule function (Dart and Mercer, 1965; Lindemann and Ham, 1979; Piha and Munns, 1987).

It has been reported that in culture media, rhizobia grow at optimum temperature ranging from 25˚C to 31˚C whilst rhizobia isolated from hot and dry Sahel Savannah environment grow well at 40˚C (Zahran, 2001). The recommended temperature favourable for root hair development and large number of infection in legumes is between 15 and 20˚C. In view of the above, researches on the rhizobia population in relation to temperature across different zones in Africa would lead to identification of temperature tolerance rhizobia species that nodulate legume of different temperature conditions. Therefore, isolation and characterization of native rhizobia species from different temperature range in Africa is essential for obtaining temperature tolerant rhizobia species for improvements of legume yield.

2.8.2 Drought

Both rhizobia and legume can exist in soils with low moisture levels with the lowest population densities reported in most desiccated environments (Zahran, 1999). Drought compromises plant and rhizobial growth, and is a main cause of nodulation failure and low N_2 fixation (Ledgard and Steele, 1992). Water stress affects rhizobial growth and their survival; hence reduce their population structure in soil, nodule formation and longevity, nodule function and synthesis of leg haemoglobin. Moreover, severe stress may lead to irreversible ceases of N_2fixation (Sprent, 1971). Drought implies that less water is available for the transport of nitrogenous compounds from nodules. Studies has shown that under drought conditions nitrogenase activity decreases, since there is decrease in nodule cortical permeability which limit oxygen supply to the bacterial zone, restricting respiration and course simultaneous decrease in nitrogenase activities (Durand *et al.*, 1987; Sprent, 1976; Walsh, 1995). Nitrogenase activities can also be constrained by low leghaemoglobin content (Serraj *et al.*, 1999).

It was suggested that, drought causes poor success of soybean inoculation in soils with a high indigenous population of *R. japonicum* (Hunt *et al.*, 1981). Furthermore, the decrease in the soil moisture from 5.5% to 3.5% significantly decreased the number of infection threads formed inside root hairs and completely inhibited the nodulation of *T. subterraneum* (Worrall and Roughley, 1976). For successful legume-rhizobia interaction, conducive rhizospheric environment is very important; although the magnitude of drought effects and the rate of inhibition of the symbiosis generally depend on the stage of growth and development, as well as the severity of the stress. For example, mild water stress reduces only the number of nodules formed on roots of soybean, while moderate and severe water stress reduces both the number and size of nodules (Williams and de Mallorca, 1984). It was reported that (Pena-Cabriales and Castellanos, 1993) there was more detrimental effect to nodulation and nitrogen fixation when water stress imposed during vegetative growth than that imposed during the reproduction stage. A number of tropical legumes, like Alfalfa (*Medicago sativa)* (Aparicio-Tejo and Sánchez-Díaz, 1982; Wahab and Zahran, 1983), groundnuts (*Arachis hypogaea)*(Simpson and Daft, 1991), Garden pea (*Pisumsativum*) (Wahab and Zahran, 1979; Guerin *et al.*, 1990; Zahran and Sprent, 1986), soybean (*Glycine max*) (Devries *et al.*, 1989; Kirda *et al.*, 1989; Weisz *et al.*, 1985), *Vigna*sp. (Pararajasingham and Knievel, 1990; Venkateswarlu *et al.*, 1989), exhibit a reduction in nitrogen fixation when subject to drought condition.

Drought has effect on N_2 fixation since nodule initiation, growth, and activity are all more sensitive to water stress than are general root and shoot metabolism (Albrecht *et al.*, 1984; Zahran and Sprent, 1986). Most dry lowlands in Africa are characterized with low moisture content and high annual temperature range. Therefore, successful isolation of rhizobia from such environment will definitely result in obtaining good rhizobia candidates for establishing successful symbioses in drought environments useful for production of legumes.

2.8.3 Soil pH

Various soil pH ranges constrains symbiotic N_2 fixation in both tropical and temperate soils (Munns, 1986), limiting *Rhizobium* survival and persistence in soils and reducing nodulation (Brockwell *et al.*, 1991; Graham *et al.*, 1982; Ibekwe *et al.*, 1997). Nodulation intensity, N_2 fixation and plant growth can be affected by soil acidity and toxicity from manganese and aluminium as well as calcium deficiency (Eaglesham and Ayanaba, 1984), and extremely cation exchange capacity (CEC) 6.0 - 12.0 cmol/Kg of soil (for poor soils) (Simon *et al.*,

2014; Zephania *et al.*, 2015). Effective nitrogen fixation by rhizobia to most leguminous plants occurs on neutral or slightly acidic soils (Bordeleau and Prévost, 1994). Legumes and their rhizobia respond to various acidic conditions (Brockwell *et al.*, 1995; Vargas and Graham, 1988), some species, like *Lucaena leucocephala* are extremely sensitive to acidity, while others, such as *Lotus tenuis*, tolerate relatively low soil pH (Correa and Barneix, 1997). Others can tolerate both conditions like common beans (*Phaseolus vulgaris*) can be nodulated by both rhizobia from low pH and high pH soils as it is reported in the study conducted in Kenya (Bordeleau and Prévost, 1994; Graham, 1992). The fast-growing strains of rhizobia are considered to be less tolerant to acid pH than slowly growing strains of *Bradyrhizobium* (Graham *et al.*, 1994). However, some strains of the fast-growing rhizobia, for example, *R. loti* and *R. tropici*, are highly acid tolerant (Cooper *et al.*, 1985; Cunningham and MuNNS, 1984; Graham *et al.*, 1994). Current research reports support the existence of acid-tolerant fast-growing strains, since both fast- and slow-growing strains of *Vigna unguiculata* which are tolerant to pH values as low as 4.0 have been isolated (Mpepereki *et al.*, 1997). The rhizobial growth is considered to be at optimum pH between 6.0 to 7.0 (Jordan, 1984), although some few rhizobia species can grow well at pH less than 5.0 (Graham *et al.*, 1994).

Acidity affects nodulation and N_2 fixation during early stages in the infection process, including the exchange of molecular signals between symbiotic partners and attachment to the roots (Zahran, 1999). Studies by Hungria and Stacey (1997) reported that, release of nod-gene inducers by soybean and common bean roots was less at pH 4.5 than at pH 5.8. Furthermore, Mckay and Djordjevic (1993) have reported that low pH can affect the production and excretion of nodulation factors in strains of *R. leguminosarum* bv. and *R. trifolii*. Low pH also affects other stages of nodule establishment and function, as is the growth of the host plant (Graham, 1981; Munns, 1986). Researchers have reported that, at pH less than 5.0 most legume species fail to nodulate because cannot withstand acidic condition (Andrew, 1978). Studies on relationship between soil pH and rhizobial population and function is very important in Africa, as soil characteristics vary with altitude and rhizobia diversity. Isolation and characterization of rhizobia from various locations with wide range of pH is a roadway to acquire effective native rhizobia that tolerates extreme pH.

2.8.4 Salt and osmotic tresses

The factors such as soil properties, climatic conditions and plant growth stage cause variation on response of legumes to salinity (Bernstein *et al.*, 1974; Maas and Hoffman, 1977). The

interaction between legume – rhizobia and nodule formation can be affected at the initial stage by salt and osmotic stresses (Ballen and Graham, 2002; Qureshi *et al.*, 2010; Sulieman and Tran, 2013). Rhizobia can tolerate salinity from 4.5 to 5.2 dsm^{-1} but root hair formation on legume cannot since it is more sensitive to salt than rhizobia cells (Saxena and Rewari, 1992). The legume response to salinity varies greatly depending on factors such as climatic conditions, soil properties, and the stage of growth (Cordovilla *et al.*, 1995; del Pilar Cordovilla *et al.*, 1995). The *Rhizobium leguminosarum* nodulating common beans (*Phaseolus vulgaris*) can tolerate up to 350mM NaCl concentration in broth culture while those for *Vigna unguiculata* can tolerate up to 450mM NaCl concentration (Zahran, 2001). Some rhizobia species can tolerate moderate salinity soils and fix nitrogen effectively e.g., *Pisum sativum*. (Hafeez *et al.*, 1988), while other legumes, like *Vicia faba*, *Phaseolus vulgaris*, and *Glycine max*, are more salt tolerant and can sustain nitrogen fixation under saline conditions(Wahab and Zahran, 1981).

The legume-*Rhizobium* symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia (Velagaleti *et al.*, 1990; Zahran, 1991; Zahran and Sprent, 1986). Salt stress inhibits the early stages of *Rhizobium*-legume symbioses. Several studies have been done to examine the effects of salt stress on nodulation and nitrogen fixation of legumes (Delgado *et al.*, 1994; Ikeda *et al.*, 1992; Nair *et al.*, 1993; Velagaleti *et al.*, 1990; Wahab and Zahran, 1979, 1981). The reduction of N_2-fixing activity by salt stress is generally attributed to a reduction in respiration of the nodules (Delgado *et al.*, 1994; Ikeda *et al.*, 1992; Walsh, 1995) and a reduction in cytosolic protein production, specifically leghemoglobin, by nodules (Delgado *et al.*, 1993; Delgado *et al.*, 1994). The salt-induced distortions in nodule structure could also be reasons for the decline in the N_2 fixation rate by legumes subject to salt stress (Sprent and Zahran, 1988; Zahran and Abu-Gharbia, 1995; Zahran and Sprent, 1986). Reduction in photosynthetic activity might also affect N_2 fixation by legumes under salt stress.

Isolation of efficient salt tolerance native rhizobia strain from saline soils can be a way to establish a successful rhizobia-legume symbiosis for saline environments (Bouhmouch *et al.*, 2005; Hartel and Alexander, 1986). Hence, isolation, molecular characterization and determining the population density in relation to salt and osmotic stress for native rhizobia nodulating common beans, cowpeas, soybeans and peas across favourable to unfavourable rhizobia growth conditions across Africa is a means for improvement of legume production

CHAPTER THREE

MATERIALS AND METHODS

3.1 Description of study area

This study was conducted at Hai District, Kilimanjaro Region in Northern Tanzania, which lies between latitudes 2° 25' S and 4° 15' S and between Longitudes 36° 25' 30" E and 38° 10' 45" E. The area experiences bimodal rainy seasons and the average rainfall varies from 500 mm per year in low altitude to 2000 mm per year in high altitude. The average temperature ranges from 15°C to 30°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 1351 m and above, upper zone from 1200 m – 1351 m, middle zone from 900 m – 1200 m and the lower zone which is below 900m above the sea level. This study was done in only three zones such as upper, middle and lower zone where common bean are grown.

3.2 Sample collection

3.2.1 Soil sample collection

Soil samples were collected from the farmer's field in three agro-ecological zones which are lower, middle and upper zone. In each zone, 5 villages and 2 fields in each village were randomly selected for soil sample collection which made a total of 30 samples. The selection based in the fields on which common beans have been grown for more than 3 years. 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 collected, were recorded by using GPS in WGS 84 systems. From each zone rhizospheric soil samples were collected at a depth of 0-20 cm, using spade.

3.2.2 Nodule sample collection

Common bean nodules were collected from the farmer's field in three agro ecological zones of Hai District, lower, middle and upper zones. In each zone, 5 villages and 2 fields (named A and B) in each village were randomly selected for nodules collection which makes a total of 30 samples. The samples were collected from fields on which common beans have been grown for more than 3 years.

Plants of common bean genotypes at the flowering stage were carefully dug up with their root nodule intact from a 1 m^2 area from farmers' fields to obtain nodules (Somasegaran and Hoben, 2012). One plant was sampled from each field and soil carefully removed from the roots to avoid nodule loss. The coordinates of each sampling point were recorded by using GPS in WGS 84 systems. The roots with nodules were cut at the level crown of each plant and immediately kept in icebox. Common bean nodules were transferred to the Laboratory at Nelson Mandela African Institution of Science and Technology (NM-AIST) for determination of indigenous characteristics and effectiveness experiment.

3.3 Soil chemical properties

Soil samples were air dried and sieved through a 2 mm sieve and analysed for pH, total nitrogen (TN), available phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), Sodium (Na), Iron (Fe), Zink (Zn), organic matter content and soil texture, following standard protocols.

3.4 Isolation of rhizobia and culturing

The roots with nodules were washed under running tap water to remove adhering soil particles, and 5 nodules from each sample were detached for isolation (Somasegaran and Hoben, 2012). The 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 in 3% sodium hypochlorite for 5 minutes, followed by repeated rinsing in sterilized water for 5 times to get rid of the sterilizing agents, as described by (Somasegaran and Hoben, 2012). Surface-sterilized nodules were crushed in a sterile petridish with a drop of sterilized double distilled water. A loopful of the nodule suspension was picked and streaked on yeast extract mannitol agar (YEMA) containing Congo-red (Table 1). The plates were incubated at 28°C for 3-7 days in an inverted position to avoid contamination by condensation of water. The composition of YEMA is indicated in Table 1. Bacterial growth on plates was observed every day after incubation. Re-streaking of isolates was done to obtain pure single separate rhizobial colonies. Pure single colonies of the rhizobia isolates were maintained 4°C to slow their growth for later use.

Table 1: YEMA w/Congo red constituents (Vicent, 1970)

Chemical	Amount(g)/L
K_2HPO4	0.5
MgSO4.7H2O	0.2
NaCl	0.1
Yeast Extract Powder	0.5
Mannitol	10.0
Agar Bacterological	15
Distilled water	1L
Congo Red	0.025
pH	6.8 - 7

3.5 Authentication of rhizobia isolates

The nodulation ability of isolates was tested by inoculating the common bean host plants with cultured isolates in the greenhouse under strict microbiologically controlled conditions following Koch's postulates (Bala *et al.*, 2010; Hassen *et al.*, 2014). Seeds were surface-sterilized following the procedure of Somasegaran and Hoben (2012) and planted into sterilized plastic pots containing sterile sand in the green house and supplied with N-free nutrient solution (Somasegaran and Hoben, 1985). The compositions of nutrient solutions are mentioned in Table 2 as described by Broughton and Dilworth (1971).

Four replicate pots per isolate were used, and plants were irrigated three times a week using sterile N-free nutrient solution. Four un-inoculated pots were included as control. After six weeks, the plants were harvested and observed for the presence of root nodules on plants. Strains which managed to nodulate *Phaseolus* bean grown in sterile media were proved to be rhizobia and those failed to nodulate considered as non-rhizobia for *Phaseolus* bean.

Table 2: Composition of N-free Nutrient Solution (Broughton and Dilworth, 1971)

Stock Solutions	Element	Form	MW	g/l	M
1	Ca	$CaCl_2•2H_2O$	147.03	294.1	2
2	P	KH_2PO_4	136.09	136.1	1
3	Fe	Fe-citrate	355.04	6.7	0.02
	Mg	$MgSo_4•7H_2O$	246.5	123.3	0.5
	K	K_2SO_4	174.06	87	0.5
	Mn	$MnSO_4•H_2O$	169.02	0.338	0.002
4	B	H_3BO_3	61.84	0.247	0.004
	Zn	$ZnSO_4•7H_2O$	287.56	0.288	0.001
	Cu	$CuSO_4•5H_2O$	249.69	0.1	0.0004
	Co	$CoSO_4•7H_2O$	281.12	0.056	0.0002
	Mo	$Na_2MoO_2•2H_2O$	241.98	0.048	0.0002

3.6 Characterization of rhizobia isolates

3.6.1 Morphological characterization of chizobial Isolates.

A colon of cultured rhizobia isolate was streaked on the YMA plates containing 0.025 g/L Congo red, incubated at 28℃ for 5 days, and assessed for colony morphology. The colonies were assessed for growth, colour (white/milky and transparency), shape, appearance, texture, absorption of Congo red, colony elevation (convex, raised, flattened,) and colony margin. Colony colour was determined by observing colony colour during growth. Colony shape was determined by observing the shape (round flat, round domed, round conical or oval) of the rhizobial colonies on YMA plates.

Rhizobial colonies were characterized as fast growers (if they took ≤5 days for colonies to visually appear) or slow growers if they took ≥7 days for colonies to visually appear (Somasegaran and Hoben, 1994; Vincent, 1970).

3.6.2 Molecular characterization of authenticated rhizobia isolates

(i) Rhizobial DNA isolation

Rhizobial DNA was extracted using *Quick*-DNA™ Fungal/Bacterial Miniprep Kit, according to the manufacturer instructions. The integrity of isolated DNA was examined on 1% (w/v) agarose gel containing ethidium bromide, and 1 x TBE were employed in electrophoresis at 80 V for 50 min, using DNA ladder, to confirm DNA purity. Gels were then stained with gel loading dye.

The pure DNA was stored at -20℃ for further analysis.

(ii) PCR amplification of 16S rRNA region

The 16S-rRNA of bacterial genomic DNA was amplified in 25 µL reaction mixture containing 10 µl (5x) Green GoTaq® Flexi Buffer (Promega), 1 µl Taq polymerase (5U) (Promega), 0.5 µl (10 uM) of each forward and reverse (Weisburg *et al.*, 1991), primer and1 µL (40-50 ng) DNA as template. DNA amplifications were performed by Thermal cycle (T100 BIORAD, USA) with respective primers and standard temperature profile (Table 3). The amplified products were examined on horizontal electrophoresis in 1% agarose gels stained with EZ-Vision Blue light Dye in TBE buffer. The DNA ladder of 1kb was used to estimate the band sizes, and electrophoresis was carried out at 80 V for 50 min with standard gels.

(iii) PCR amplification of housekeeping gene *gyr*B

The PCR amplification of *gyr*B genes of rhizobial genome was done, as described for 16S rRNA. The primers used and thermal cycle conditions are listed in Table 3. The same procedures used for 16S-rRNA were followed to examine the PCR-amplified product on agarose gel.

Table 3: Primers and their temperatures used for PCR amplification

Primer	Sequence	Temperature
16S rRNA For.[1]	For:-5'AGA GTT TGA TCC TGG CTC AG3'	2 min 95°C, 35 X (1.5 min 95°C, 30 s
16S rRNA Rev.[1]	Rev:-5'AAG GAG GTG ATC CAG CC3	56°C), 1.5 min 72°C, 15 min 72°C,
		10°C
gyrB For.[2]	For:-5'TTC GAC CAG AAY TCC TAY AAG	10 min 95°C, 30 X (30 s 94°C, 30 s
gyrB Rev.[2]	G3'	58°C, 1 min 75°C) 15 min 72°C
	Rev:-5'AGC TTG TCC TTS GTC TGC G3')	

[1]Weisburg *et al.* (1991); [2]Marek-Kozaczuk *et al.* (2013)

3.7 Evaluation of symbiotic effectiveness of rhizobial isolates

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 seeds of *Phaseolus* bean 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.

3.7.1 Preparation of sterile media

The river sand was collected and cleaned by removing all debris, 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 to cool. After cooling, the sterile soil was put in 2 kg plastic pots ready for growing the *Phaseolus* bean.

3.7.2 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 conical flask containing 60 ml of Yeast Extract Mannitol Broth (YEMB). The inoculated flask 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 ml of YEMB (≈ 5 x 10^8 cells/ml) containing rhizobia were applied in 2 kg plastic pots containing sterile sand and seeds of *Phaseolus* bean.

3.7.3 Seed sterilization and planting

Seeds of *Phaseolus* bean with the same size were sterilized by rinsing with 95% alcohol for 10 seconds, followed by 3% Sodium hypochlorite for 4 minutes, then sterilants 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 24 hours, then sown in pots containing sterilized sand. Then some pots were inoculated with the isolated *rhizobium* 10mls strains, while some was inoculated with standard rhizobia inoculants "CIAT 899" as positive control, some were non-inoculated but supplied with N_2 and other were left non- inoculated as negative control.

3.7.4 Experimental layout and treatments

Four replicate pots per isolate were inoculated with 10 ml (≈ 5x 10^8 cells/ml) of YEMB containing isolated *rhizobium* strains**,** four pots un-inoculated but supplied with nitrogen were included as control while four were inoculated with standard rhizobia inoculants "CIAT 899" as positive control and other four were left non- inoculated as negative control (Fig. 1). Plants were irrigated three times a week using sterile N-free nutrient solution (Somasegaran and Hoben, 1985). The compositions of nutrient solutions are mentioned in Table 2 as described by Broughton and Dilworth (1971). After 5 weeks, growth parameters such as number of nodules, nodule size, stem girth, number of leaves, shoot length and biomass (dry weight) were measured and compared as described by Bala *et al.* (2010) and Woomer *et al.* (2013).

3.8 Data collection

3.8.1 Nodulation assessment

At 42 days after emergence (flowering stage), plants were carefully uprooted from the sand and separated into shoots, roots and nodules after washing roots in tap water. The detached nodules were counted.

3.8.2 Stem girth and shoot length

Measuring of stem girth and shoot length were conducted in the fifth week after planting. Stem girth was measured by using digital Vernier calliper in millimetre (mm); shoot length was measured by using a tape measure in centimetre (cm).

3.8.3 Plant dry biomass yield (nodules, roots and shoot dry mass)

Shoots, roots and nodules were separately oven-dried at 60℃ for 48 hr and weighed to determine shoot, root and nodule dry matter. Dry biomass was measured in gram (g) by using weighing balance after drying in microwave at 60°C for 48 hours. Nodules were directly counted after removing soils in the roots.

3.8.4 Colour ranking of plant leaves

Plant colour ranking was done by looking colour of the *Phaseolus* bean leaves and ranking them in 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).

3.8.5 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 hundred milligram (100 mg) of green leaf slices of *Phaseolus* bean from each treatment was dissolved in 7 ml of Dimethyl Sulphoxide (DMSO) placed in 15 ml vials and then incubating at 4°C for 72 hours. Then the extract was diluted by adding 3 ml of DMSO hence making it 10 ml. Then 300 µl of the leaf extract were transferred to disposable 96 well plates 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 645 nm and 663 nm wavelength. Then the total chlorophyll content was calculated by using equation suggested by Arnon (1949):

$$\textbf{Chlt } (gl^{-1}) = 0.0202D_{645} + 0.00802D_{663}$$

Where **D** is the density value measured at the respective wavelength as measured in UV spectrophotometer.

3.8.6 Relative symbiotic effectiveness of rhizobia isolates (SEi)

Relative Symbiotic effectiveness (SEi) was calculated using dry matter yield of shoot, where each inoculated plant was compared with N-fed control. The formula used was described by Cardoso *et al.* (2012).

$$SEi = \frac{Xi - Xu}{Xn - Xu} \times 100$$

Where i, u and n = dry matter of plants inoculated with the rhizobial isolates, dry matter of un-inoculated control and dry matter of N-fed plants, respectively.

3.9 Data 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. 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 version 10, software.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Soil chemical analysis of the study sites

The results of soil analysis components such as N, P, K, Na, Ca, Mg, K, Mn, Fe, Zn, and pH showed that, the soils in Hai district had deficit in soil N and P in some soils in different villages, while exchangeable cations (Ca, and Mg) are in normal levels, except for Na, which was inadequate. Micronutrients (Cu, Mn, Fe, Zn) appeared limiting in some soils. The level of soil phosphorus was excessively high at Usari village (RS25) (50.29 mg/Kg soil) and lowest at Mungushi village (RS1) (12.17 mg/Kg soil) (Table 4). Generally soils at different locations showed minor differences in their content of major nutrients, with in some villages showing more fertile than the other.

Table 4: Analysis of the soil chemical properties of the study area

Location	Village/Farm	Sample	pH	N	Av. P	K^+	Ca^{2+}	Mg^{2+}	Na	Cu^+	Mn	Fe	Zn
			1:1.25 H_2O	(%)	(mg/kg soil)	Exch. Bases(me/100g)				(ppm)	(ppm)	(ppm)	(ppm)
Middle zone (900-1350M)	Mungushi	RS1	7.6	0.112	21.17	3.36	17.42	22.62	0.09	1.33	24.07	13.09	5.23
	Mungushi	RS2	7.3	0.020	12.97	15.22	22.30	12.71	1.34	0.54	3.56	27.52	1.42
	Kware	RS3	6.5	0.120	15.77	9.97	26.26	12.91	0.75	0.62	4.40	26.94	1.41
	Kware	NRS4	6.8	0.053	13.47	10.50	30.83	15.16	0.80	0.35	6.02	8.77	1.16
	Amani	RS5	6.9	0.067	13.37	1.99	20.57	22.73	0.38	0.56	5.92	19.70	0.81
	Amani	RS6	7.2	0.081	28.63	4.41	33.72	12.99	0.66	0.48	1.21	17.02	0.53
	Shirinjoro	NS7	6.3	0.101	28.23	14.69	23.01	17.39	1.14	7.46	25.75	22.99	4.65
	Shirinjoro	RS8	6.2	0.084	18.30	1.89	19.68	22.83	0.27	1.06	25.37	21.28	2.32
	Kwasadala	RS9	5.8	0.014	13.70	1.57	18.08	17.25	0.11	1.19	25.94	25.06	2.37
	Kwasadala	RS10	5.9	0.050	15.40	1.68	15.02	16.76	0.09	0.90	24.93	24.57	2.95
Lower zone (> 900)	Rundugai	RS11	8.3	0.067	15.81	17.32	28.52	22.50	2.15	0.57	17.45	32.94	1.01
	Rundugai	RS12	8.1	0.067	20.46	14.17	23.06	21.24	1.43	0.15	8.41	7.03	0.61
	Kwatito	RS13	7.9	0.042	21.89	18.89	37.90	21.66	6.26	0.12	3.04	2.07	0.33
	Kwatito	RS14	8.0	0.017	20.62	15.22	31.19	21.11	4.47	0.19	5.92	1.67	0.41
	Chekimaji	RS15	7.4	0.006	30.29	16.27	33.14	20.84	4.47	0.15	4.55	1.98	0.41
	Chekimaji	RS16	7.1	0.025	33.98	3.04	13.37	23.56	0.14	0.38	20.56	6.70	1.46
	Longoi	RS17	6.5	0.031	39.11	3.78	17.06	22.87	0.14	0.44	19.58	2.68	1.91
	Longoi	RS18	6.3	0.031	24.29	3.67	14.17	23.52	0.11	0.31	20.02	4.23	1.57
	Mbatakero	RS19	6.4	0.014	20.67	3.46	13.91	22.07	0.16	0.33	21.54	15.89	2.18
	Mbatakero	RS20	6.2	0.056	18.64	3.04	18.30	22.94	0.11	0.26	22.54	10.53	3.29
Upper zone (1351- 1800M)	Mulama	RS21	5.8	0.039	20.64	2.94	10.84	22.64	0.07	0.33	24.73	20.98	1.37
	Mulama	RS22	5.8	0.050	22.32	2.10	11.28	15.04	0.09	11.44	26.78	32.27	5.19
	Mudio	RS23	6.5	0.078	18.93	2.94	12.97	18.86	0.36	11.27	25.83	30.14	4.38
	Mudio	RS24	6.4	0.048	15.30	2.41	12.40	15.99	0.09	11.52	26.67	30.84	4.53
	Usari	RS25	6.7	0.059	50.29	1.99	13.37	16.41	0.18	9.07	26.58	31.02	4.11
	Usari	RS26	6.4	0.067	17.00	1.36	13.86	14.65	0.09	2.09	26.49	34.53	4.76
	Olori	RS27	5.9	0.095	20.92	2.62	19.77	20.69	0.16	10.18	25.98	18.24	5.03
	Olori	RS28	5.7	0.109	19.06	1.89	22.12	22.94	0.18	11.09	26.26	27.13	6.45
	Nshara	RS29	6.5	0.090	19.15	2.20	30.21	23.16	0.11	11.08	24.50	18.12	5.61
	Nshara	RS30	6.3	0.092	19.19	1.68	18.17	21.51	0.09	9.30	26.43	31.88	5.01
	Critical levels		**5.5**	**0.25**	**20**	**0.22**	**4.0**	**2**	**< 1**	**0.4**	**< 5**	**5**	**0.4**

RS=Rhizospheric Soil, Number after RS represent village and farm in which nodules were collected. N=Nitrogen, Av. P=Available Phosphorus, K=Potassium, Ca=Calcium, Mg=Magnesium, Na=Sodium, Cu= copper, Mn= Manganese, Fe= Iron, Zn= Zink.

4.2 Characterization of isolated rhizobia strains nodulating common bean in Hai district northern Tanzania

4.2.1 Morphological characteristics of rhizobia nodulating common bean in Hai district

Before the advent of molecular techniques, morphological, cultural and biochemical characterizations were used to assess rhizobial diversity. The validity of this approach is based on growth difference in culture media especially when adjusted to create stress that mimics low or high concentration of nutrients, pH and salinity.

A total of 30 bacterial strains were isolated from the nodules of *Phaseolus* bean grown in different framers' fields of 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) with Congo red after inoculation (Fig. 1a), and tuned creamy white and Milky white in colour when grown on Yeast Manitol Broth (Fig. 1b). The rhizobial isolates also failed to absorb Congo Red when incubated in the dark at 28°C for 5 days. The colonies were round in shape with entire margins. The characteristics of the isolated strains were presented in the Table 5.

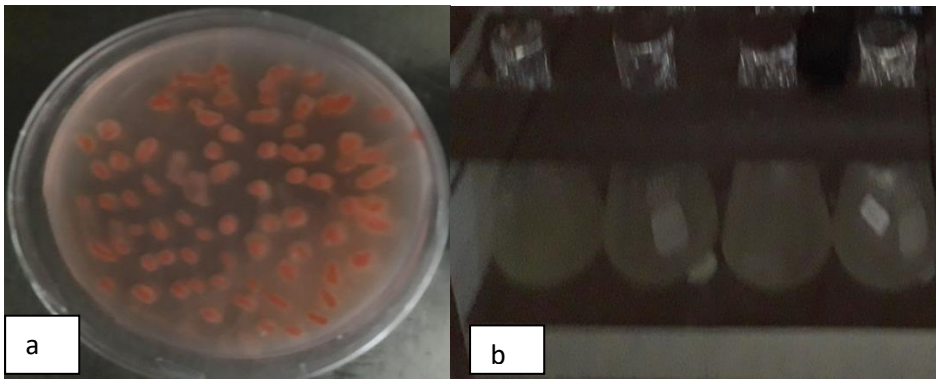

Figure 1: Pure colonies of the indigenous rhizobia nodulating *Phaseolus* bean grown on; (a) Yeast Manitol Agar with Congo Red, (b)Yeast Monitol Broth.

Table 5: Characteristics of isolated indigenous rhizobia strain grown on YEMA containing Congo red

Village name	Field	Isolates	Characteristics							
			Colony shape	Colony colour	Colony appearance	Colony Transparency	Colony Texture	Absorption of Congo red	Colony elevation	Colony Margin
Mungushi	A	IR 1	Circular	Milky white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Mungushi	B	IR 2	Circular	Cream white	Dull	Translucent	Firm dry	Not absorbed	Convex	Entire
Kware	A	IR 3	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kware	B	IR 4	Circular	Milky white	shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Amani	A	IR 5	Oval	Cream yellow	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Amani	B	IR 6	Oval	Cram white	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Shirinjoro	A	IR 7	Oval	Cream yellow	Dull	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Shirinjoro	B	IR 8	Oval	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kwasadala	A	IR 9	Circular	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kwasadala	B	IR 10	Oval	Milky white	Shiny	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Rundugai	A	IR 11	Oval	Cream yellow	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Rundugai	B	IR 12	Circular	Milky white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kwatito	A	IR 13	Oval	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kwatito	B	IR 14	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Chekimaji	A	IR 15	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Chekimaji	B	IR 16	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Longoi	A	IR 17	Oval	Cream white	Shiny	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Longoi	B	IR 18	Circular	Cream yellow	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Mbatakero	A	IR 19	Circular	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Mbatakero	B	IR 20	Circular	Cream yellow	Dull	Opaque	Firm dry	Not absorbed	Convex	Entire
Mulama	A	IR 21	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Mulama	B	IR 22	Oval	Milky white	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Mudio	A	IR 23	Circular	Cream white	Shiny	Opaque	Firm dry	Not absorbed	Convex	Entire
Mudio	B	IR 24	Oval	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Usari	A	IR 25	Circular	Milky white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Usari	B	IR 26	Circular	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Olori	A	IR 27	Circular	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Olori	B	IR 28	Oval	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Nshara	A	IR 29	Oval	Cream white	shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Nshara	B	IR 30	Oval	Milky white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire

Note: IR stands for Indigenous *Rhizobium*

4.2.2 Molecular characterization of isolated rhizobia nodulating common bean in Hai district northern Tanzania

(i) PCR-amplification of 16S-rRNA gene

The PCR amplification of full length 16S rRNA gene of rhizobial genome yielded a single band of about 1500 bp as an amplified product with isolates IR4, IR5, IR7, IR8, IR9, IR10, IR11, IR12, IR13, IR14, IR15, IR16, IR17, IR20, IR22, IR 24, IR25, IR29 and IR30 of tested 30 isolates from Kware, Amani, Shirinjoro, Kwasadala of middle lands, Rundugai, Kwatito, Chekimaji, Longoi, Mbatakero, of lower lands, and Mulama, Mudio, Usari, and Nshara of upper lands respectively of the tested 30 isolates as shown in Fig. 2 and Table 6.

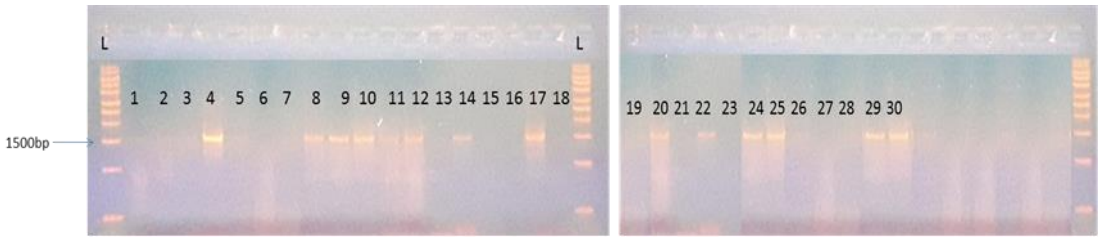

Figure 2: PCR – Amplified Products of 16S rRNA using 1kb DNA ladder

Number 1 to 30 represents the Indigenous Rhizobia isolated from different soils in Hai District, northern Tanzania named: IR1=Mungushi (farm A), IR2=Mungushi (farm B), IR3=Kware (farm A), IR4= Kware (farm B), IR5=Amani (fam A), IR6=Amani (farm B), IR7=Shirinjoro (farm A), IR8= Shirinjoro (farm B), IR9= Kwasadala (farm A), IR10= Kwasadala (farm B), IR11= Rundugai (farm A), IR12=Rundugai (farm B), IR13=Kwatito (farm A), IR14=Kwatito (farm B), IR15= Chekimaji (farm A), IR16=Checkimaji (farm B), IR17=Longoi (farm A), IR18=Longoi (farm B), IR19= Mbatakero (farm A), IR20=Mbatakero (farm B), IR21 Mulama (farm A), IR22=Mulama farm B), IR23=Mudio (farm A), IR24 =Mudio (farm B), IR25=Usari (farm A), IR26=Usari (farm B), IR27=Olori (farm A), IR28=Olori (farm B), IR29=Nshara (farm A), IR30 = Nshara (farm B).

(ii) PCR amplification of housekeeping (gyrB) gene

The PCR amplification of full length Housekeeping *gyrB* gene of rhizobial genome yielded a single band of about 750 bp as an amplified product with isolates IR10, IR11, IR15, IR16, IR21, IR23, IR24, IR25 and IR26 of tested 30 isolates from Kwasadala of middle lands, Rundugai and Chekimaji of lower lands, Mulama, mudio, and usari of upper lands (Fig. 3).

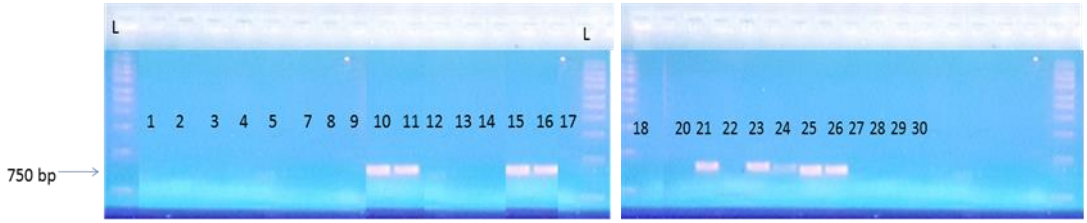

Figure 3: PCR – amplification product of housekeeping gyrB gene. M = 1kb ladder

Number 1 to 30 represents the Indigenous Rhizobia isolated from different soils in Hai District, northern Tanzania named: IR1=Mungushi (farm A), IR2=Mungushi (farm B), IR3=Kware (farm A), IR4= Kware (farm B), IR5=Amani (fam A), IR6=Amani (farm B), IR7=Shirinjoro (farm A), IR8= Shirinjoro (farm B), IR9= Kwasadala (farm A), IR10= Kwasadala (farm B), IR11= Rundugai (farm A), IR12=Rundugai (farm B), IR13=Kwatito (farm A), IR14=Kwatito (farm B), IR15= Chekimaji (farm A), IR16=Checkimaji (farm B), IR17=Longoi (farm A), IR18=Longoi (farm B), IR19= Mbatakero (farm A), IR20=Mbatakero (farm B), IR21 Mulama (farm A), IR22=Mulama farm B), IR23=Mudio (farm A), IR24 =Mudio (farm B), IR25=Usari (farm A), IR26=Usari (farm B), IR27=Olori (farm A), IR28=Olori (farm B), IR29=Nshara (farm A), IR30 = Nshara (farm B).

Table 6: DNA Amplification using 16S rRNA and *gyrB* Primers

Source/Village	Field	Isolate	16SrRNA	gyrB
Mungushi	A	IR 1	-	-
Mungushi	B	IR 2	-	-
Kware	A	IR 3	-	-
Kware	B	IR 4	+	-
Amani	A	IR 5	+	-
Amani	B	IR 6	-	-
Shirinjoro	A	IR 7	+	-
Shirinjoro	B	IR 8	+	-
Kwasadala	A	IR 9	+	-
Kwasadala	B	IR 10	+	+
Rundugai	A	IR 11	+	+
Rundugai	B	IR 12	+	-
Kwatito	A	IR 13	+	-
Kwatito	B	IR 14	+	-
Chekimaji	A	IR 15	+	+
Chekimaji	B	IR 16	+	+
Longoi	A	IR 17	+	-
Longoi	B	IR 18	-	-
Mbatakero	A	IR 19	-	-
Mbatakero	B	IR 20	+	-
Mulama	A	IR 21	-	+
Mulama	B	IR 22	+	-
Mudio	A	IR 23	-	+
Mudio	B	IR 24	+	-
Usari	A	IR 25	+	+
Usari	B	IR 26	-	+
Olori	A	IR 27	-	-
Olori	B	IR 28	-	-
Nshara	A	IR 29	+	-
Nshara	B	IR 30	+	-

Note: + = Amplification, - = no amplification

4.3 Symbiotic effectiveness of isolated rhizobia strains on *Phaseolus* bean

4.3.1 Plant nodulations

All 30 rhizobial isolates inoculated on common bean plants induced different levels of nodulation (Table 6). Nodule mean number per plant ranged from 30 for IR 20 to 151 for IR5. The isolates that elicited greater nodulation (nodule mean number per plant) on common bean (Table 6) included IR5 (151 nodules per plant), IR27 (107 nodules), IR10 (98 nodules), IR3 (91 nodules), IR6 (90 nodules), IR 19 (89 nodules), IR2, IR8, IR14 (88 Nodules) and NR24 (87 nodules per plant). The remaining isolates were intermediate in their nodulation of the common bean. The *Phaseolus* bean inoculated with commercial inoculants CIAT 899 showed the least number of nodules (21nodules plant^{-1}), while un-inoculated pots showed 0 numbers of nodules (Fig. 4).

Figure 4: Roots of *Phaseolus* bean of different treatments. A - Roots of non-inoculated *Phaseolus* bean, B-Roots of *Phaseolus* bean inoculated with Commercial inoculants CIAT 899 and C- roots of *Phaseolus* bean inoculated with the indigenous rhizobia strain.

4.3.2 Nodules dry biomass

Nodules dry Biomass also differed between and among rhizobial isolates, and ranged from 0.086 g.plant^{-1} to 0.209 g.plant^{-1} (Table 6). Isolate IR 9 produced the highest nodule dry matter followed by isolates IR14, IR27, IR16 and IR17, IR6, IR11 and IR13, IR19 followed by IR1 and IR12. In contrast isolates IR 20 and IR7 produced the least nodule dry mass (Table 6). The *Phaseolus* bean inoculated by Commercial strains CIAT 899 showed the nodule biomass of 0.088gplant^{-1}.

4.3.3 Shoot dry mass

Shoot dry biomass accumulated also differed between and among the rhizobial strains. Isolate IR 12 produced the highest shoot biomass (1.48 g.plant^{-1}) followed by IR10 and IR3 (1.46

g.plant^{-1}), IR1 (1.44 g.plant^{-1}), IR2 (1.43 g.plant^{-1}), IR28 (1.4 g.plant^{-1}), IR6 (1.36 g.plant^{-1}) and IR8 (1.2 g.plant^{-1}). In contrast, isolate IR20 produced the least shoot biomass (0.78 g.plant^{-1}), followed by IR23 and IR30 (0.83 g.plant^{-1}). Commercial strain, CIAT 899 produced 1.11g.Plant-1 shoot biomass which is the average compared to the isolated native Rhizobia. About 14 isolates produced shoot dry matter higher than the N_2 fed plants (Table 7). The non-inoculated *Phaseolus* bean produced the lowest shoot biomass (0.05g.Plant^{-1}) compared to the isolated native strains, CIAT 899 and N-fed plants.

4.3.4 Root biomass

Rhizobial isolate IR11 produced the most (3.84 g.plant^{-1}) root biomass, followed by IR6 (3.07 g.plant $^{-1}$), IR2 (2.89 g.plant $^{-1}$), IR9 (2.39 g.plant^{-1}), IR12 2.36 g.plant^{-1}), IR4 (2.30g.plant^{-1}), and IR 26 (2.26 g.plant^{-1}). About 14 produced more root biomass than the N_2 fed plants (Table 4.4). In contrast, isolates IR20 and IR23 produced the least root biomass (1.01 and 0.02 g.plant^{-1}, respectively) which was even lower than that of the un-inoculated control (0.59g.plant^{-1}) and 14 isolated produced root biomass compared to commercial strain CIAT 899 (1.36g.Plant^{-1})

4.3.5 Colour of the plant leaves

Phenotypic observation of leaves colour was done, *Phaseolus* bean inoculated with IR7, IR18, IR22, IR1 and IR4 showed deep green colour as the colour of N-fed plants (Table 7), the rest were light green as shown on the table 7. *Phaseolus* bean inoculated with commercial rhizobia CIAT 899 were showing green colour and un-inoculated plants were pale-green (Fig. 5).

Figure 5: Leaf colouring and growth of *Phaseolus* bean when tested for symbiotic effectiveness.

A- Non inoculated *Phaseolus* bean, B - *Phaseolus* bean inoculated with commercial inoculants CIAT 899, C – *Phaseolus* bean non-inoculated but supplied with N, D - *Phaseolus* bean inoculated with one of the isolates of indigenous rhizobia strain.

4.3.6 Chlorophyll content

Rhizobia isolate NR 10 produced high chlorophyll content as that of N-fed plants (0.0342 $\mu gChlg^{-1}$), followed by IR 12 (0.0339 $\mu gChlg^{-1}$), IR 3 (0.0338 $\mu gChlg^{-1}$), IR 1 (0.0335 $\mu gChlg^{-1}$) and IR 23 (0.0314 $\mu gChlg^{-1}$). The commercial strain showed lower chlorophyll content compare to the isolated native rhizobia, whereas the un-inoculated negative control *Phaseolus* bean showed the lowest chlorophyll content among all plants (Table 6).

4.3.7 Relative symbiotic effectiveness of the rhizobial isolates

Relative symbiotic effectiveness was calculated using shoot dry mass which is best measure of the symbiotic effectiveness. The results manifest 30 variations in all rhizobia isolates (Fig. 6). The results showed that, there were 21 isolates which were highly effective (≥100% relative effectiveness), 8 Isolates were effective (70% ≤ relative effectiveness ≤100%) and 1 isolates was ineffective ≤ 70% as evaluated by (Rejili *et al.*, 2012) (Fig. 6). 16 isolate were effective than commercial standard rhizobia CIAT 899.

4.4 Influence of the isolated indigenous rhizobia strains on different symbiotic effectiveness parameters.

Isolated rhizobia strains showed significant influence on various growth parameters. They have shown a significant influence on the number of nodules per plant and root dry biomass at $p<0.01$. Shoot dry biomass, shoot length and nodule dry biomass at $p<0.001$, and stem girth and leaf chlorophyll content at $p < 0.05$. Amongst the plant growth parameters which showed highly significant differences due to inoculation of the isolated strains in *Phaseolus* bean are the number of nodules, Chlorophyll content, shoot dry biomass and shoot length. There is a high significance difference ($p < 0.001$) in chlorophyll content, root dry mass and shoot dry mass between inoculated and non-inoculated *Phaseolus* bean.

Table 7: Influence of Isolated rhizobia on nodule number, nodule dry weight, colour of the leaves and chlorophyll content

			Parameter Measured			
Village	**Field**	**Treatment**	**Nodule number**	**Nodule dry Biomass (g)**	**Colour of plant leaves**	**Chlorophyll content (μgChlg^{-1})**
		-ve control	0.00±0.00^{h}	0.000±0.0000^{h}	1.94±0.20^{h}	0.0072±0.00046^{f}
		N-fed	0.00±0.00^{h}	0.000±0.0000^{h}	4.50±0.29^{abcde}	0.0342±0.00223^{a}
		CIAT 899	21.75±3.33^{g}	0.088±0.0085^{g}	3.00±0.05^{g}	0.0110±0.00207^{f}
Mungushi	A	IR1	80.00±5.02^{def}	0.193±0.0036^{bcd}	4.68±0.24^{abcd}	0.0335±0.00047^{a}
Mungushi	B	IR2	88.75±5.07^{cd}	0.189±0.0052^{cde}	3.07±0.90^{g}	0.0260±0.00314^{bcde}
Kware	A	IR3	91.75±7.47^{bcd}	0.190±0.0063^{bcd}	4.16±0.29^{abcde}	0.0338±0.00019^{a}
Kware	B	IR4	78.00±6.96^{def}	0.181±0.0028^{cde}	4.55±0.21^{abcde}	0.0304±0.00274^{abc}
Amani	A	IR5	151.75±13.66^{a}	0.170±0.0032^{def}	4.28±0.32^{abcde}	0.0292±0.00215^{abc}
Amani	B	IR6	90.00±1.29^{cd}	0.197±0.0037^{bcd}	4.04±0.17^{bcde}	0.0306±0.00218^{ab}
Shirinjoro	A	IR7	75.75±5.01^{def}	0.169±0.0030^{defg}	4.81±0.11^{a}	0.0269±0.00101^{bcde}
Shirinjoro	B	IR8	88.75±1.65^{cd}	0.191±0.0151^{ab}	4.29±0.22^{abcde}	0.0303±0.00093^{abc}
Kwasadala	A	IR9	84.00±2.97^{cde}	0.209±0.0163^{a}	4.10±0.17^{abcde}	0.0279±0.00149^{abcd}
Kwasadala	B	IR10	98.00±6.82^{bc}	0.187±0.0039^{bcd}	4.30±0.24a^{bcde}	0.0342±0.00066^{a}
Rundugai	A	IR11	76.75±3.42^{def}	0.197±0.0049^{abc}	4.14±0.14^{abcde}	0.0209±0.00265^{e}
Rundugai	B	IR12	83.00±3.79^{cde}	0.193±0.0049^{abcd}	4.23±0.20^{abcde}	0.0339±0.00241^{ab}
Kwatito	A	IR13	78.75±4.07^{def}	0.197±0.0053^{abc}	4.14±0.18^{abcde}	0.0207±0.00169^{e}
Kwatito	B	IR14	88.25±5.48^{cd}	0.203±0.0069^{ab}	4.24±0.33^{abcde}	0.0260±0.00224^{bcde}
Chekimaji	A	IR15	79.25±6.29^{def}	0.194±0.0136^{abc}	4.04±0.21^{bcde}	0.0288±0.00138^{abcd}
Chekimaji	B	IR16	66.25±3.64^{f}	0.199±0.0059^{abc}	3.97±0.04^{def}	0.0281±0.00288^{abcd}
Longoi	A	IR17	77.75±4.59^{def}	0.199±0.0084^{abc}	4.01±0.13^{cde}	0.0268±0.00265^{bcde}
Longoi	B	IR18	85.00±3.92^{cde}	0.189±0.0032^{bcd}	4.74±0.16^{ab}	0.0241±0.00290^{cde}
Mbatakero	A	IR19	89.75±12.34^{cd}	0.194±0.0080^{abcd}	4.00±0.21^{def}	0.0252±0.00235^{bcde}
Mbatakero	B	IR20	30.25±2.75^{g}	0.086±0.0031^{gh}	4.36±0.24^{bcde}	0.0283±0.00158^{abcd}
Mulama	A	IR21	80.50±4.33^{def}	0.180±0.0049^{cde}	3.29±0.22f^{g}	0.0285±0.00345^{abcd}
Mulama	B	IR22	83.25±3.82^{cde}	0.182±0.0027^{cde}	4.72±0.26^{abc}	0.0314±0.00292^{ab}
Mudio	A	IR23	70.50±5.66^{ef}	0.166±0.0043^{fgh}	3.97±0.07^{ef}	0.0226±0.00153d^{e}
Mudio	B	IR24	87.00±5.82^{cd}	0.186±0.0040^{cdef}	3.86±0.14^{ef}	0.0280±0.00474^{abcd}
Usari	A	IR25	80.00±4.56^{def}	0.181±0.0044^{cdef}	3.12±0,20^{g}	0.0241±0.00256^{cde}
Usari	B	IR26	79.75±9.24^{de}f	0.183±0.0106^{abcd}	3.95±0.14^{ef}	0.0304±0.00213^{abc}
Olori	A	IR27	107.25±12.26^{b}	0.200±0.0034^{ab}	4.15±0.28^{abcde}	0.0301±0.00140^{abc}
Olori	B	IR28	89.25±2.95^{cd}	0.193±0.0033^{abcd}	4.02±0.07^{cde}	0.0291±0.00387^{abc}
Nshara	A	IR29	79.50±2,78^{def}	0.178±0.0042^{defg}	4.04±0.04^{bcde}	0.0284±0.00175^{abcd}
Nshara	B	IR30	65.75±2.78^{f}	0.186±0.0051^{cdef}	4.28±0.25^{abcde}	0.0298±0.00257^{abc}
		F Statistics	**23.3****	**59.63*****	**5.2*****	**6.3 *****

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

Table 8: Influence of isolated rhizobia on shoot length, stem girt, shoot and root dry weight

			Parameter measured			
Village	**Field**	**Treatment**	**Shoot length (cm)**	**Stem girth (mm)**	**Shoot Dry Biomass (g)**	**Root Dry Biomass (g)**
		-ve control	13.50±0.65^{i}	1.39±0.12^{i}	0.05±0.01^{f}	0.59±0.06^{f}
		N-fed	60.00±1.29^{ab}	4.72±0.15^{a}	0.99±0.08^{bcde}	1.28±0.01^{def}
		CIAT 899	34.50±2.47^{fgh}	2.91±0.34^{g}	1.11±0.01^{bcde}	1.36±0.10^{ef}
Mungushi	A	IR1	41.50±6.03^{defgh}	3.48±0.26^{cdefgh}	1.44±0.13^{ab}	1.24±0.34^{def}
Mungushi	B	IR2	45.50±3.69^{cdef}	4.20±0.17^{abc}	1.43±0.18^{b}	2.89±1.08^{abc}
Kware	A	IR3	35.00±6.56^{efgh}	2.96±0.58^{fgh}	1.46±0.24^{b}	1.72±0.58^{bcde}
Kware	B	IR4	39.25±5.17^{defgh}	3.59±0.23^{bcdefg}	1.13±0.12^{bcde}	2.00±0.32^{bcde}
Amani	A	IR5	37.00±4.45^{defgh}	3.60±0.32^{bcdefg}	0.69±0.23^{e}	1.16±0.59^{def}
Amani	B	IR6	54.50±2.40^{abc}	3.47±0.25^{cdefg}	1.36±0.17^{bcd}	3.07±1.33^{ab}
Shirinjoro	A	IR7	32.00±4.60^{gh}	2.88±0.17^{h}	0.85±0.16^{cde}	1.50±0.27^{cdef}
Shirinjoro	B	IR8	44.25±3.68^{cdefgh}	3.45±0.37^{cdefgh}	1.20±0.38^{bcde}	2.02±0.80^{bcde}
Kwasadala	A	IR9	39.75±5.66^{defgh}	4.01±0.03^{abcde}	0.81±0.24^{de}	2.39±0.82^{abcd}
Kwasadala	B	IR10	44.25±8.82^{cdefgh}	4.19±0.27^{abcd}	1.46±0.22^{ab}	1.61±0.78^{bcde}
Rundugai	A	IR11	39.25±3.82^{defgh}	3.34±0.01^{efgh}	1.11±0.27^{bcde}	3.84±0.80^{a}
Rundugai	B	IR12	47.75±4.13^{bcde}	3.38±0.25^{efgh}	1.48±0.52^{a}	2.36±0.50^{bcd}
Kwatito	A	IR13	48.00±6.65^{bcd}	3.92±0.31^{bcde}	1.12±0.11^{bcde}	1.20±0.22^{def}
Kwatito	B	IR14	42.25±4.07^{cdefgh}	3.84±0.09^{bcde}	1.12±0.12^{bcde}	2.14±0.49^{bcd}
Chekimaji	A	IR15	34.50±5.42^{fgh}	3.67±0.28^{bcdefg}	1.00±0.34^{bcde}	1.13±0.30^{def}
Chekimaji	B	IR16	37.00±6.24^{defgh}	3.38±0.33^{efgh}	0.93±0.24^{bcde}	1.23±0.44d^{ef}
Longoi	A	IR17	42.50±6.46^{cdefgh}	4.00±0.40^{abcde}	0.92±0.11^{bcde}	1.27±0.24d^{ef}
Longoi	B	IR18	45.25±6.05^{cdef}	4.31±0.2918^{def}	0.98±0.21^{bcde}	1.25±0.33^{def}
Mbatakero	A	IR19	33.75±3.50^{fgh}	3.62±0.46^{bcdefgh}	0.91±0.28^{bcde}	1.17±0.45^{def}
Mbatakero	B	IR20	31.50±4,05^{h}	3.50±0.13^{cdefgh}	0.78±0.12^{e}	1.01±0.26^{def}
Mulama	A	IR21	63.50±0.65^{a}	3.51±0.33^{cdefgh}	1.01±0.17^{bcde}	1.32±0.14^{def}
Mulama	B	IR22	44.50±5.38^{cdefg}	3.92±0.14^{bcde}	0.99±0.06^{bcde}	1.40±0.30^{def}
Mudio	A	IR23	33.25±4.09^{fgh}	3.82±0.22^{bcde}	0.83±0.12.^{cde}	1.02±0.22^{def}
Mudio	B	IR24	62.50±1.85^{a}	4.32±0.19^{ab}	1.14±0.11^{bcde}	2.30±0.40^{bcd}
Usari	A	IR25	45.75±6.79^{cdef}	3.71±0.31^{bcdef}	1.20±0.23^{bcde}	1.36±0.26^{def}
Usari	B	IR26	33.75±3.35^{fgh}	3.46±0.25^{scdefg}	1.15±0.07^{bcde}	2.26±0.40^{bcd}
Olori	A	IR27	41.25±4.37^{defgh}	3.70±0.26^{bcdef}	0.96±0.23^{bcde}	1.43±0.44^{cdef}
Olori	B	IR28	49.25±2.66^{bcd}	3.83±0.16^{bcde}	1.40±0.17^{bc}	2.06±0.55^{bcd}
Nshara	A	IR29	41.75±5.85^{cdefgh}	3.43±0.27^{defgh}	0.83±0.05^{cde}	1.62±0.21^{bcde}
Nshara	B	IR30	39.25±3.59^{defgh}	3.34±0.13^{efgh}	1.08±0.04^{bcde}	1.79±0.50^{bcde}
		F Statistics	**4.5*****	**4.4****	**2.9 *****	**1.9****

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

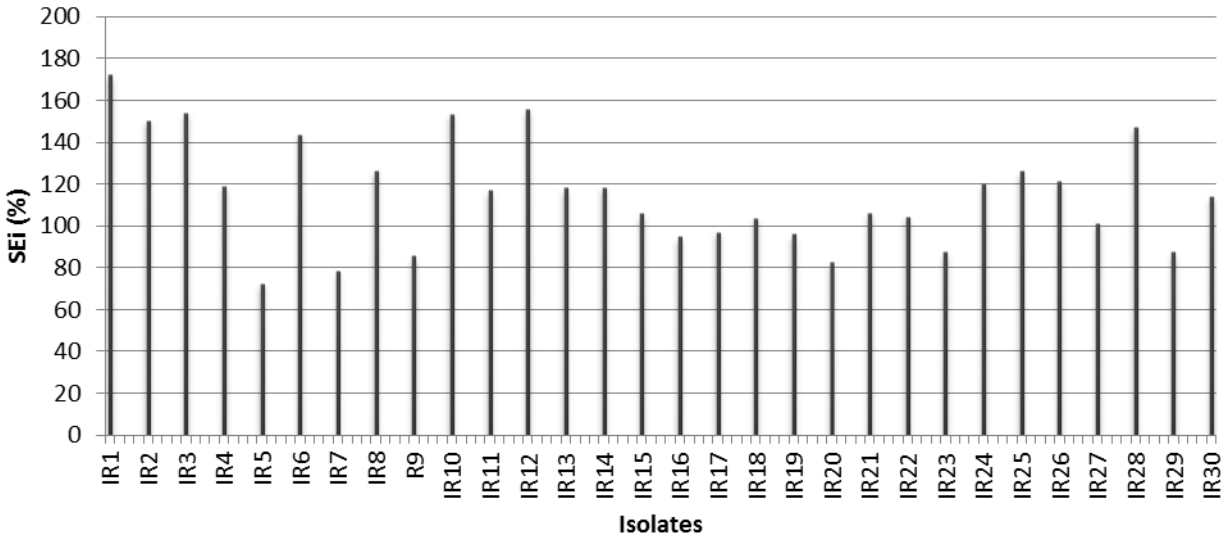

Figure 6 : Relative symbiotic effectiveness of the rhizobial isolates

4.5 Discussion

The chemical properties of the rhizospheric soils from the locations where the nodule sample were taken showed low levels of nitrogen contents (0.006 to 0.12%) but high levels of K. The pH values slightly acidic ranging from 5.8 to 8.3 which is within the optimum pH range for crop production (6.0 - 8.2) (Rodrigues *et al.*, 2006). It was also found to be favourable for *Phaseolus* infective strains of rhizobia (Yaman and Cinsoy, 1996). Evaluating symbiotic effectiveness of indigenous rhizobia was done purposely as an effort towards tackling the problem of nitrogen deficiency in the soils of smallholder farmers in Africa. 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 and Chakrabartty, 1993; Halder *et al*., 1990; RodriguezNavarro *et al*., 2000; Son *et al*., 2006). Also Rhizobium have the ability to convert atmospheric nitrogen gas (N_2) into ammonia (NH3), a form which can be utilized by the plants, hence solving a problem of nitrogen deficiency (Dall'Agnol *et al*., 2014; Giller, 2001; Lindström and Mousavi, 2010; Lindström *et al*., 2010; Loganathan *et al*., 2014a).

The categories of bacterial growth are consistent with characterization of growth of rhizobia (Somasegaran and Hoben, 1985). The rhizobial isolates incubated in the dark at 28°C for 5 days, failed to absorb Congo red, which is a typical characteristic of the family *Rhizobiaceae* (Fentahun *et al.*, 2013; Somasegaran and Hoben, 1985; Woomer *et al.*, 2011). Morphologically, all the isolates were round in shape with smooth texture with similarity to those described by (Pinto *et al.*, 2007), variation in colony colour was noted, with most of the isolate showing whitish or creamy in colour which agrees with the report that common bean rhizobia exhibit a whitish or creamy colony colour (Fentahun *et al.*, 2013), which is also the characteristics of *Bradyrhizobia Spp*. (Howieson and Dilworth, 2016). The characteristics of the isolated rhizobia strains presented resemble to the standard characteristics of rhizobia, and hence proved that the isolated strains were rhizobia. Furthermore, the indigenous rhizobia isolated from *Phaseolus* bean on the studies conducted in Tanzania and Kenya revealed the colony characteristics which were the same as those found in this study (Muthini *et al.*, 2014; Namkeleja, 2017; Simon *et al.*, 2014). These findings gave hopeful indicator towards production of inoculants for *Phaseoulus* bean indigenous to Tanzania since most of the isolated strains showed ability to induce nodulation on the roots of Phaseolus bean.

The PCR results show that, out of 30 rhizobia isolate, 18 were amplified with 16S rRNA, 9 were amplified with *gyrB*, 3 were amplified with both 16s rRNA and *gyrB* and 8 were not amplified with any of the of the selected primers. The failure to amplify by selected primers could be due to due to presence of multiple copies in the genome of some bacteria, susceptibility to genetic recombination, and horizontal gene transfer (Aserse *et al.*, 2012), in addition, 16S rRNA is limited in discriminating the characteristics at and below the species level (Aserse *et al.*, 2012; Martens *et al.*, 2008; Stackebrandt and Goebel, 1994). Furthermore, the reports of other studies show that, there are bacteria which represent different species but have identical or nearly identical 16S rRNA gene sequence (Li *et al.*, 2009; Martens *et al.*, 2008; 2007). This explains the reason for some bacteria to amplify with 16S rRNA gene only but not to housekeeping *gyrB* gene. The housekeeping gene *gyrB* has higher degree of divergence which allows species discrimination (Martens *et al.*, 2007), hence in this study PCR application with *gyrB* occurred in few rhizobial isolates and few were amplified by both genes. These implying that, there is presence of the Rhizobia species in the different soils of Hai District, northern Tanzania, since most of the isolates in all three varying altitudes amplified with primers. Therefore, there is a need for further sequencing

analysis to identify these species of rhizobia that could be used for inoculants production for improving common bean production in Tanzania.

The symbiotic effectiveness 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), root dry mass (gm), nodule dry mass (gm), colour rank of the plant leaves, stem girth (mm) and chlorophyll content per gram of leaf (µgChlg-1) as compared with the commercial/standard strain (CIAT 899). The findings from the study (Muthini *et al.*, 2014; Rodriguez-Navarro *et al.*, 2000; Simon *et al.*, 2014) agree with the findings from this study that some of the indigenous rhizobia isolates has higher ability of forming effective nodules in the roots of *Phaseolus* bean than commercial strains. Moreover, These results are contrary to those described in the study done by Mungai and Karubiu (2017) who reported that inoculation with native rhizobia isolated from two farms in Njoro, Kenya, were not effective in nodulation in common bean. 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, root dry mass and plant leaves colour as opposed to Sharma and Kumawat (2011) who demonstrated that nodule number is not an appropriate measure of effectiveness in rhizobia–legume symbiosis.

Phaseolus bean supplied with N, showed higher performance on stem girth, shoot length, plant leave colour and chlorophyll contents but were not effective on shoot and root dry mass. 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 the study both shoot dry mass and chlorophyll content, the highest value was obtained in plants inoculated with indigenous strain IR1, IR3, IR10 and IR12 and the lowest was obtained in non-inoculated plants (negative control), which 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.

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 $P < 0.001$. 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. Generally, the isolates from most villages in all agro-ecological zones showed a greater performance on symbiotic effectiveness. This means that, rhizobia can perform efficiently in different altitudes considering the availability of favourable environmental condition and soil conditions.

These results suggest that, these promising strains could be used as biofertilizers for field applications. However, more trials need to be conducted to determine the effectiveness of the stimulation on nodulation, biomass, and nitrogen fixation related to yields of several beans cultivars in different climatic conditions.

CHAPTER FIVE

CONCLUSION AND RECCOMENDATIONS

5.1 Conclusion

In this study, soil analysis showed lower levels of nitrogen and available phosphorus in all agro ecological zones where indigenous rhizobia were obtained. Hence rhizobia inoculants are highly needed in this area for nitrogen fixation and phosphorus solubilization, to enhance bean production.

Morphological and molecular techniques were used to discriminate rhizobial isolates of common bean from Hai district, Northern. Results showed the presence of fast growing rhizobia in the soil, when grown on Yeast Extract Manitol Agar (YEMA) containing Congo red, since they failed to absorb Congo red. Furthermore, all of them were round and milky to creamy colour which is the characteristics of nitrogen fixing rhizobia. The characteristic of the fast-grower proved to be consistent with genus *Rhizobium* and *Bradyrhizobium Spp.* This indicating that there are different species of rhizobia in Tanzanian soils that are needed to be identified through further molecular techniques.

PCR analysis of both 16S rRNA and housekeeping gene (*gyr*B), showed the amplification of 10 isolates by 16S rRNA gene only, 5 housekeeping gene (*gyr*B) only, and 3 isolates by both genes. This implies the presence of diversity among the collected isolates, since the genes have different levels of discriminating the characteristics of bacteria. Hence further studies on molecular techniques on sequencing and phylogenetic analyses can be employed to identify rhizobia strains that could be used for inoculants production, in order to get effective nitrogen fixing rhizobia specific for nodulating *Phaseolus*, for improving bean production. *Rhizobium* isolated suggests the needs to perform more molecular analyses to elucidate the potential presence of novel *Rhizobium* species in Tanzania.

Most of the isolates were effective in nodulating the *Phaseolus* bean and showed great performance in other growth parameters compared to the Standard /Commercial Strain, CIAT 899. Strains IR1 from Mungushi village, IR3 from Kware village, IR10 from Kwasadala village in middle agro ecological zone and IR12 from Rundugai in lower agro ecological zone, showed an outstanding performance in symbiotic effectiveness than the standard/commercial strain CIAT 899. They came up as the best strain by influencing nodulation in the roots of the *Phaseolus* bean than CIAT 899 which was used as control. This

implies 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. 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 *Phaseolus* bean.

Most of the isolates which amplified with the primers in PCR analysis were identified as rhizobia species in morphological characterization when grown on YEMA w/Congo red and YMB. They also showed high relative symbiotic effectiveness (≥100%) when inoculated into *Phaselous* bean grown in the screen house in pot experiment, as compared to standard/ commercial strain CIAT 899 and N-fed plants. Therefore, there is a need to perform sequences of these rhizobia species to find out which kinds of species are present in Northern Tanzania.

5.2 Recommendations

The legume-rhizobia symbiosis provides a window for trapping the abundant N_2 in the atmosphere for plant uptake. The success of the legume-rhizobia symbiosis in enhancing soil fertility and crop yields depends on the efficiency of the rhizobial microsymbiont among other factors.

Common bean is an important food grain legume in most parts of the world including Tanzania. This study was aimed at assessing the identity of indigenous rhizobia nodulating common bean in Northern Tanzania, a strategy that could enhance soil fertility and crop yield for food and nutritional security through the identification of elite indigenous strains native to the country.

Towards ensuring food security, sustainable use of natural resources is a critical means to enhance agricultural productivity. Thus, biological nitrogen fixation can be used as an efficient natural means to fix N_2 for plant uptake, a process that plays an important role in land remediation. The use of inoculants as bio-fertilizers to improve soil N economy through BNF has proven to be economically beneficial and necessary in all soils deficient of nitrogen. While rhizobia appear to be widely distributed as the legume hosts themselves, the number of highly effective rhizobia strains may be limited in some environments.

Therefore, strains which showed high potential in N_2-fixation in pot experiments could be used for further investigation for their efficiency under field conditions for use in inoculants

production. However, their competitiveness in the presence of other commercial and indigenous strains needs to be evaluated. The findings of this study indicate that, it is possible to reduce the use of nitrogen mineral fertilization or completely avoid it in common bean production through inoculation with highly effective strains.

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RESEARCH OUTPUTS

Output 1: Research paper

Morphological Assessment and Effectiveness of Indigenous Rhizobia Nodulating Common Beans (*Phaseolus Vulgaris* L.) in Hai District, Northern Tanzania

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Abstract

Thirty *Rhizobium* strains were isolated from root nodules collected from *Phaseolus vulgaris* L. from farmer's fields in Northern Tanzania. Morphological characterization showed that all the isolates were fast grower having taken 2 – 5 days to grow in Yeast Extract Mannitol Agar w/ Congo Red and failed to absorb Congo Red, and displayed whitish/creamy colour on Yeast Extract Mannitol Broth which are purely characteristics of rhizobia. In testing the effectiveness, the treatments were laid down in a randomized complete block design in four replications in the screen house experiment. The beans were inoculated with the rhizobia isolates and compared with commercial rhizobia CIAT 899, N fertilized (20 kg Nha^{-1}) and un-inoculated control treatments. All of the isolated rhizobia strains showed significant influence on the number of nodules per plant at $p<0.01$, seventeen isolates showed significance influence on shoot dry biomass at $p<0.01$, and on root dry biomass at $p<0.001$ as compared with N-fed plants and commercial strain CIAT 899. The un-inoculated Phaseolus bean did not induce nodules. Such results implied indigenous rhizobia are effective as compared to the commercial rhizobial strain CIAT 899, thus they can be used for nodulating Phaseolus bean for improving soil fertility through nitrogen fixation in Tanzania.

Key words: Symbiotic Effectiveness, Nitrogen Fixation, *Rhizobial* Inoculant

Introduction

Rhizobium is gram negative soil bacteria which inhabits root nodules of most leguminous crops and fix nitrogen through a symbiotic relationship [1]. Rhizobia trap and fix nitrogen into a form such as ammonia after establishing symbiotic association with legume plant through the process of Biological Nitrogen Fixation (BNF) [2-4]. The BNF provides alternative source of nitrogen to synthetic nitrogen fertilizers [5,6], which is less expensive and conducive to environment, as it does not produce nitrates that course pollution to the ground water [7,8]. The BNF is very important in agriculture as it helps to overcome the problems associated with depletion of soil fertility since it manages soil acidity and salinity [9]. The BNF is a primary source of nitrogen for small-scale farmers who use less or no fertilizer, especially for legumes production [10]. Evaluation on yield of legumes inoculated under field conditions in northern Tanzania showed that, *Bradyrhizobium* and *rhizobium* were very efficient in supplying nitrogen to common beans and soybeans respectively [11].

Nitrogen is the most limiting nutrient for plant growth and crop yield [12]. Low soil N is a major constraint to increased yields on farmers' fields. BNF can alleviate soil infertility and increase crop yields [13]. Some African countries such as Rwanda, Malawi, Kenya, Egypt and Zimbabwe have started to exploit legume N_2 fixation as an alternative to N fertilizer [14]. However, symbiotic N_2 fixation can only be achieved in the presence of efficient rhizobial strains in the soil, whether native or introduced via inoculation [15].

The inoculation of legumes with effective rhizobia can improve plant growth and grain yield [15]. Inoculation with effective and persistent rhizobia can replace the application of N fertilizer [14]. However, poor nodulation and lack of response to inoculation have frequently been reported in field experiments involving common bean [16,17]. In Kenya, lack of response to rhizobial inoculation has also been reported for common bean [18] due to the number of factors including incompatibility between the host plant and bacteria, and great sensitivity of the legume-rhizobia symbiosis to environmental stress [16,19]. Poor viability of inoculants strains, higher concentration of soil mineral N, and presence of highly competitive native rhizobia that restrict occupancy by inoculants stains [15] have also been cited as the constraints to symbiotic N_2-fixation.

Rhizobial strains selection as was done in Brazil has proven the potential contribution of highly efficient and competitive rhizobia in agriculture [16]. Thus a program of continuous strain selection could help in reversing low bean yields while addressing the issue of soil N

depletion. It is therefore important to continue to identify more efficient, competitive and genetically stable rhizobial strains for replacing existing inoculants strains that are in the process of losing their symbiotic ability and/or efficiency. The aim of this study was to evaluate the symbiotic efficiency of rhizobia isolated from root nodules of common beans obtained from farmers' fields of Hai District in Northern Tanzania, by measuring biomass accumulation, chlorophyll content along with characters.

Material and methods

Nodules sample collection

Nodule samples were collected from common bean grown in different farmer's fields with varying altitude: highlands with altitude above 1200m, middle land with altitude between 900-1200m and lowland with altitude below 900m above sea level in Hai District, Kilimanjaro Region in Northern Tanzania, which lies between latitudes 2° 25' and 4° 15' and between Longitudes 36° 25' 30" and 38° 10' 45". At each location, 5 villages and 2 fields in each village were randomly selected for nodules collection which makes a total of 30 samples.

Soil Chemical Properties

Rhizospheric soil samples were collected in the area where nodules were obtained. The samples were air dried and sieved through a 2mm sieve and analysed for pH, total nitrogen (TN), available phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), Sodium (Na), Iron (Fe), Copper (Cu), Manganese (Mn) and Zink (Zn), following standard protocols.

Isolation of Rhizobia and Culturing

The roots with nodules were washed under running tap water to remove adhering soil particles, and 5 nodules from each sample were detached for isolation [22]. The nodules were surface-sterilized by immersed in 95% ethanol for 10 seconds then in 3% sodium hypochlorite for 5 minutes, followed by repeated rinsing in sterilized water for 5 times [22]**.** Surface-sterilized nodules were crushed in a sterile petridish with a drop of sterilized double distilled water. A loopful of the nodule suspension was picked and streaked on yeast extract mannitol agar (YEMA) containing Congo-red. The plates were incubated at 28°C for 3-7 days in an inverted position to avoid contamination by condensation of water. Bacterial growth on plates was observed every day after incubation. Re-streaking of isolates was done to obtain pure single separate rhizobial colonies.

Authentication of rhizobial isolates

The nodulation ability of isolates was tested by inoculating the common bean host plants with cultured isolates in the greenhouse under strict microbiologically controlled conditions following Koch's postulates [20,21]. Seeds were surface-sterilized following the procedure [22] and planted into sterilized plastic pots containing sterile sand in the green house and supplied with N-free nutrient solution [23]**.** The composition of nutrient solutions was as shown in Table 2 [24].

Four replicate pots per isolate were used, and plants were irrigated three times a week using sterile N-free nutrient solution. Four un-inoculated pots were included as negative control. After six weeks, the plants were harvested and observed for the presence of root nodules on plants. Strains which managed to nodulate *Phaseolus* bean grown in sterile media were proved to be rhizobia and those that failed to nodulate were considered as non-rhizobia for *Phaseolus* bean.

Morphological Identification of Rhizobia isolates.

A colon of cultured rhizobia isolate was streaked on the YEMA plates containing 0.025 g/L Congo red, incubated at **28℃** for 5 days, and assessed for colony morphology. The colonies were assessed for growth, colour **(**white/milky and transparency**),** shape, appearance, texture, absorption of Congo red, colony elevation **(**convex, raised, flattened,) and colony margin. Colony colour was determined by observing colony colour during growth**.** Colony shape was determined by observing the shape (round flat, round domed, round conical or oval) of the rhizobial colonies on YEMA plates.

PCR Identification of Authenticated rhizobia Isolates

Rhizobial DNA isolation and PCR amplification of 16S rRNA region

Rhizobial DNA was extracted using *Quick*-DNA™ Fungal/Bacterial Miniprep Kit, according to the manufacturer instructions. The integrity of isolated DNA was examined on 1% (w/v) agarose gel containing EZ-Vision Blue Light Dye, and 1x TBE were employed in electrophoresis at 80V for 50 min, using DNA ladder of 1kb, to confirm DNA purity. Gels were then stained with gel loading dye. The pure DNA was stored at -20℃ for further analysis.

PCR amplification of 16S rRNA region

The 16S-rRNA of bacterial genomic DNA was amplified in 25µL reaction mixture containing 10µl (5x) Green GoTaq® Flexi Buffer (Promega), 1µl Taq polymerase (5U) (Promega), 0.5 µl (10uM) of each forward and reverse [25], primer and1 µL (40-50 ng) DNA as template. DNA amplifications were performed by Thermal cycle (T100 BIORAD, USA) with respective primers and standard temperature profile (Table 1).The amplified products were examined on horizontal electrophoresis in 1% agarose gels stained with EZ-Vision Blue light Dye in TBE buffer. The DNA ladder of 1kb was used to estimate the band sizes, and electrophoresis was carried out at 80V for 50min with standard gels.

PCR amplification of housekeeping gene *gyr*B

The PCR amplification of *gyr*B genes of rhizobial genome was done, as described for 16S rRNA. The primers used and thermal cycle conditions are listed in Table 1. The same procedures used for 16S-rRNA were followed to examine the PCR-amplified product on agarose gel.

Table 1: Primers and their temperatures used for PCR amplification

Primer	Sequence	Temperature
16S rRNA For.[1]	For:-5'AGA GTT TGA TCC TGG CTC AG3'	2 min 95^0C, 35 X (1.5 min 95^0C, 30 s
16S rRNA Rev.[1]	Rev:-5'AAG GAG GTG ATC CAG CC3	56^0C), 1.5 min 72^0C, 15 min 72^0C,
		10^0C
gyrB For.[2]	For:-5'TTC GAC CAG AAY TCC TAY AAG	10 min 95^0C, 30 X (30 s 94^0C, 30 s
gyrB Rev.[2]	G3'	58^0C, 1 min 75^0C) 15 min 72^0C
	Rev:-5'AGC TTG TCC TTS GTC TGC G3')	

[1][25]; [2][26]

Evaluation of symbiotic effectiveness of rhizobial isolates

Symbiotic effectiveness of the rhizobia strains was evaluated through greenhouse experiments at the Nelson Mandela African Institution of Science and Technology, Arusha Tanzania.

Preparation of sterile media

The river sand was collected and cleaned by removing all debris, 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 to cool then was put in 2 kg plastic pots ready for growing the *Phaseolus* bean.

Preparation of isolated strains for inoculation

The authenticated strains of indigenous rhizobia were re-grown on plates containing Yeast Extract Mannitol Agar (YEMA) with Congo red (CR) and incubated at 28°C. After three days, a single colony of rhizobia strains was taken from the plates and inoculated in conical flask containing 60ml of Yeast Extract Mannitol Broth (YEMB). The inoculated flask of YEMB were covered with autoclaved foil paper and kept in a shaker incubator for 72hours at 28°C and 120 rpm (rotation per minute).

Seed sterilization and planting

Seed of *Phaseolus* bean with the same size were sterilized by rinsing with 95% alcohol for 10seconds, followed by 3% Sodium hypochlorite for 4minutes, then sterilants drained off. After that seeds were rinsed with eight changes of sterile water. Then seed were submerged in sterile water 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 changes of sterile water and left at room temperature for 24hours [22], then sown in pots containing sterilized sand in the screen house.

Experimental layout and treatments

Four replicate pots per isolate were inoculated with 10ml (≈ $5x10^8$cells/ml) of YEMB containing isolated *rhizobium* strains, four pots un-inoculated but supplied with nitrogen were included as control while four were inoculated with standard rhizobia inoculants "CIAT 899" as positive control and other four were left non- inoculated as negative control. Plants were irrigated three times a week using sterile N-free nutrient solution [23]**.** The compositions of nutrient solutions are mentioned in Table 2 [24].

Table2: Composition of N-free Nutrient Solution [[24]

Stock Solutions	Element	Form	Molecular Weight	g/l	Moles
1	Ca	$CaCl_2 \cdot 2H_2O$	147.03	294.1	2
2	P	KH_2PO_4	136.09	136.1	1
3	Fe	Fe-citrate	355.04	6.7	0.02
	Mg	$MgSo_4 \cdot 7H_2O$	246.5	123.3	0.5
	K	K_2SO_4	174.06	87	0.5
	Mn	$MnSO_4 \cdot H_2O$	169.02	0.338	0.002
4	B	H_3BO_3	61.84	0.247	0.004
	Zn	$ZnSO_4 \cdot 7H_2O$	287.56	0.288	0.001
	Cu	$CuSO_4 \cdot 5H_2O$	249.69	0.1	0.0004
	Co	$CoSO_4 \cdot 7H_2O$	281.12	0.056	0.0002
	Mo	$Na_2MoO_2 \cdot 2H_2O$	241.98	0.048	0.0002

Colour ranking of plant leaves

Plant colour ranking was done by looking colour of the *Phaseolus* bean leaves and ranking them in scale of 1 to 5 whereby 1 = severely chlorotic leaves, 2 = pale green, 3 = green, 4 = light green and 5 = deep green healthy leaves [30].

Nodule count and measuring stem girth, shoot length, root and shoot dry mass

Nodule count, stem girth, shoot length and dry mass were recorded in the fifth week after planting. Stem girth was measured by using digital Vernier caliper in millimeter (mm); shoot length was measured by using a tape measure in centimeter (cm). Dry biomass of the nodules, roots and shoot were measured in milligram (mg) by using weighing balance after drying them in microwave at 60◦C for 48 hours. Nodules were directly counted after removing soils in the roots.

Chlorophyll content determination

Chlorophyll content was determined by following procedures explained [27] and referring previous studies [28,29]. A one hundred milligram (100mg) of green leaf slices of *Phaseolus* bean from each treatment was dissolved in 7mls of Dimethyl Sulphoxide (DMSO) placed in 15mls falcon tubes and then incubating at 4◦C for 72hours. Then the extract was diluted by adding 3mls of DMSO hence making it 10mls. Then 300µl of the leaf extract were transferred to disposable 96 well plates 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):

$$\textbf{Chlt (gl-1)} = \mathbf{0.0202D_{645} + 0.00802D_{663}}$$

where **D** is the density value measured at the respective wavelength as measured in UV spectrophotometer.

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. Treatment means of the growth parameters such as shoot length, stem girth, number of nodules per plant, leaf chlorophyll contents, and shoot dry and nodules biomass were compared by using Fisher LSD as the post hoc significance test. All above analysis was done by using STATISTICA software.

Results

Soil Chemical Analysis

The chemical properties of rhizospheric soils from the locations where the nodule sample were taken showed low levels of nitrogen contents (0.006% to 0.12%) but high levels of K. The pH values were slightly acidic and alkaline ranging from 5.8 to 8.3. The level of soil available phosphorus was excessively high at Usari village (RS25) (50.29mg/Kg soil) and lowest at Mungushi village (RS1) (12.17 mg/Kg soil) (Table 3). Generally soils at different locations showed minor differences in their content of major nutrients, with some villages showing to be more fertile than the others.

Table3. Analysis of the soil chemical properties of the study areas

Location	Village/Farm	Samp le	N (%)	P (mg/kg)	K^+	Ca^{2+}	Mg^{2+}	Na	Cu^+ (ppm)	Mn (ppm)	Fe (ppm)	Zn (ppm)
					(Exchangeable Bases(me/100g)							
Middle lands Between 900 and 1300 m	Mungushi	RS1	0.112	21.17	3.36	17.42	22.62	0.09	1.33	24.07	13.09	5.23
	Mungushi	RS2	0.020	12.97	15.22	22.30	12.71	1.34	0.54	3.56	27.52	1.42
	Kware	RS3	0.120	15.77	9.97	26.26	12.91	0.75	0.62	4.40	26.94	1.41
	Kware	RS4	0.053	13.47	10.50	30.83	15.16	0.80	0.35	6.02	8.77	1.16
	Amani	RS5	0.067	13.37	1.99	20.57	22.73	0.38	0.56	5.92	19.70	0.81
	Amani	RS6	0.081	28.63	4.41	33.72	12.99	0.66	0.48	1.21	17.02	0.53
	Shirinjoro	RS7	0.101	28.23	14.69	23.01	17.39	1.14	7.46	25.75	22.99	4.65
	Shirinjoro	RS8	0.084	18.30	1.89	19.68	22.83	0.27	1.06	25.37	21.28	2.32
	Kwasadala	RS9	0.014	13.70	1.57	18.08	17.25	0.11	1.19	25.94	25.06	2.37
	Kwasadala	RS10	0.050	15.40	1.68	15.02	16.76	0.09	0.90	24.93	24.57	2.95
Lowlands Below 900m	Rundugai	RS11	0.067	15.81	17.32	28.52	22.50	2.15	0.57	17.45	32.94	1.01
	Rundugai	RS12	0.067	20.46	14.17	23.06	21.24	1.43	0.15	8.41	7.03	0.61
	Kwatito	RS13	0.042	21.89	18.89	37.90	21.66	6.26	0.12	3.04	2.07	0.33
	Kwatito	RS14	0.017	20.62	15.22	31.19	21.11	4.47	0.19	5.92	1.67	0.41
	Chekimaji	RS15	0.006	30.29	16.27	33.14	20.84	4.47	0.15	4.55	1.98	0.41
	Chekimaji	RS16	0.025	33.98	3.04	13.37	23.56	0.14	0.38	20.56	6.70	1.46
	Longoi	RS17	0.031	39.11	3.78	17.06	22.87	0.14	0.44	19.58	2.68	1.91
	Longoi	RS18	0.031	24.29	3.67	14.17	23.52	0.11	0.31	20.02	4.23	1.57
	Mbatakero	RS19	0.014	20.67	3.46	13.91	22.07	0.16	0.33	21.54	15.89	2.18
	Mbatakero	RS20	0.056	18.64	3.04	18.30	22.94	0.11	0.26	22.54	10.53	3.29
Highlands Above 1300m	Mulama	RS21	0.039	20.64	2.94	10.84	22.64	0.07	0.33	24.73	20.98	1.37
	Mulama	RS22	0.050	22.32	2.10	11.28	15.04	0.09	11.44	26.78	32.27	5.19
	Mudio	RS23	0.078	18.93	2.94	12.97	18.86	0.36	11.27	25.83	30.14	4.38
	Mudio	RS24	0.048	15.30	2.41	12.40	15.99	0.09	11.52	26.67	30.84	4.53
	Usari	RS25	0.059	50.29	1.99	13.37	16.41	0.18	9.07	26.58	31.02	4.11
	Usari	RS26	0.067	17.00	1.36	13.86	14.65	0.09	2.09	26.49	34.53	4.76
	Olori	RS27	0.095	20.92	2.62	19.77	20.69	0.16	10.18	25.98	18.24	5.03
	Olori	RS28	0.109	19.06	1.89	22.12	22.94	0.18	11.09	26.26	27.13	6.45
	Nshara	RS29	0.090	19.15	2.20	30.21	23.16	0.11	11.08	24.50	18.12	5.61
Critical levels		**5.5**	**0.25**	**20**	**0.22**	**4.0**	**2**	**< 1**	**0.4**	**< 5**	**5**	**0.4**

RS=Rhizospheric Soil, Number after RS represent village and farm in which nodules were collected. N=Nitrogen, Av. P=Available Phosphorus, K=Potassium, Ca=Calcium, Mg=Magnesium, Na=Sodium, Cu= copper, Mn= Manganese, Fe= Iron, Zn= Zink.

Morphological identification of rhizobia isolates

A total of 30 bacterial strains were isolated from the nodules of *Phaseolus* bean grown in different framers' fields of Hai District in Kilimanjaro Region Tanzania. All isolated strains were fast grower having taken 2 – 5days to grow in Yeast Extract Mannitol Agar (YEMA) with Congo red after inoculation.), and tuned creamy white and Milky white in colour when grown on Yeast Mannitol Broth (Table 4). The rhizobial Isolates also failed to absorb Congo Red when incubated in the dark at 28^0C for 5 days. The colonies were round in shape with entire margins (Table 4).

Table 4. Characteristics of isolated indigenous rhizobia strain grown on YEMA containing Congo red and YEMB

Village name	Field	Isolates	Characteristics							
			Colony shape	Colony colour	Colony appearance	Colony Transparency	Colony Texture	Absorption of Congo red	Colony elevation	Colony Margin
Mungushi	A	IR 1	Circular	Milky white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Mungushi	B	IR 2	Circular	Cream white	Dull	Translucent	Firm dry	Not absorbed	Convex	Entire
Kware	A	IR 3	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kware	B	IR 4	Circular	Milky white	shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Amani	A	IR 5	Oval	Cream yellow	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Amani	B	IR 6	Oval	Cram white	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Shirinjoro	A	IR 7	Oval	Cream yellow	Dull	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Shirinjoro	B	IR 8	Oval	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kwasadala	A	IR 9	Circular	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kwasadala	B	IR 10	Oval	Milky white	Shiny	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Rundugai	A	IR 11	Oval	Cream yellow	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Rundugai	B	IR 12	Circular	Milky white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kwatito	A	IR 13	Oval	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Kwatito	B	IR 14	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Chekimaji	A	IR 15	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Chekimaji	B	IR 16	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Longoi	A	IR 17	Oval	Cream white	Shiny	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Longoi	B	IR 18	Circular	Cream yellow	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Mbatakero	A	IR 19	Circular	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Mbatakero	B	IR 20	Circular	Cream yellow	Dull	Opaque	Firm dry	Not absorbed	Convex	Entire
Mulama	A	IR 21	Circular	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Mulama	B	IR 22	Oval	Milky white	Dull	Opaque	Smooth viscous	Not absorbed	Convex	Entire
Mudio	A	IR 23	Circular	Cream white	Shiny	Opaque	Firm dry	Not absorbed	Convex	Entire
Mudio	B	IR 24	Oval	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Usari	A	IR 25	Circular	Milky white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Usari	B	IR 26	Circular	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Olori	A	IR 27	Circular	Cream yellow	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Olori	B	IR 28	Oval	Cream white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Nshara	A	IR 29	Oval	Cream white	shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire
Nshara	B	IR 30	Oval	Milky white	Shiny	Translucent	Smooth viscous	Not absorbed	Convex	Entire

Note: IR stands for Indigenous Rhizobium

PCR identification of isolated indigenous rhizobia

PCR-amplification of 16S-rRNA gene

The PCR amplification of full length 16S rRNA gene of rhizobial genome yielded a single band of about 1500bp as an amplified product with isolates IR4, INR5, IR7, IR8, NR9, IR10, IR11, IR12, IR13, IR14, IR15, IR16, IR17, IR20, IR22, IR 24, IR25, IR 29 and IR30 of tested 30 isolates from Kware, Amani, Shirinjoro, Kwasadala of middle lands, Rundugai, Kwatito, Chekimaji, Longoi , Mbatakero, of lower lands, and Mulama, Mudio, Usari, and Nshara of upper lands respectively. (Fig. 1)(Table5).

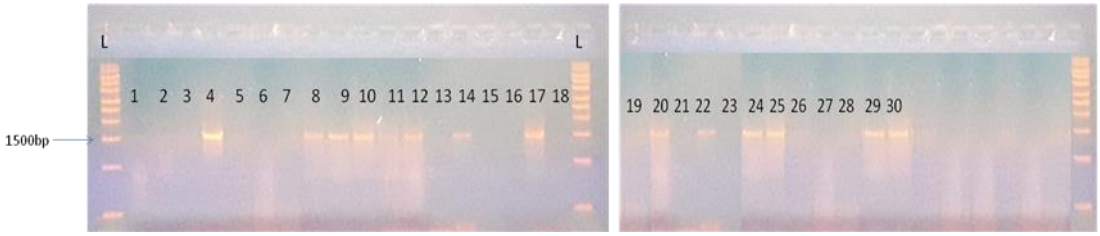

Figure1: PCR – Amplified Products of 16S rRNA. M= 1kb ladder
Number 1 to 30 represents the Indigenous Rhizobia isolated from different soils in Hai District, northern Tanzania named: IR1=Mungushi (farm A), IR2=Mungushi (farm B), IR3=Kware (farm A), IR4= Kware (farm B), IR5=Amani (fam A), IR6=Amani (farm B), IR7=Shirinjoro (farm A), IR8= Shirinjoro (farm B), IR9= Kwasadala (farm A), IR10= Kwasadala (farm B), IR11= Rundugai (farm A), IR12=Rundugai (farm B), IR13=Kwatito (farm A), IR14=Kwatito (farm B), IR15= Chekimaji (farm A), IR16=Checkimaji (farm B), IR17=Longoi (farm A), IR18=Longoi (farm B), IR19= Mbatakero (farm A), IR20=Mbatakero (farm B), IR21 Mulama (farm A), IR22=Mulama farm B), IR23=Mudio (farm A), IR24 =Mudio (farm B), IR25=Usari (farm A), IR26=Usari (farm B), IR27=Olori (farm A), IR28=Olori (farm B), IR29=Nshara (farm A), IR30 = Nshara (farm B).

PCR amplification of housekeeping (*gyr*B) gene

The PCR amplification of full length Housekeeping *gyrB* gene of rhizobial genome yielded a single band of about 750bp as an amplified product with isolates IR10, IR11, IR15, IR16, IR21, IR23, IR24, IR25, and IR 26 of tested 30 isolates from Kwasadala of middle lands, Rundugai and Chekimaji of lower lands, Mulama, Mudio, and Usari of upper lands (Fig. 2)(Table5).

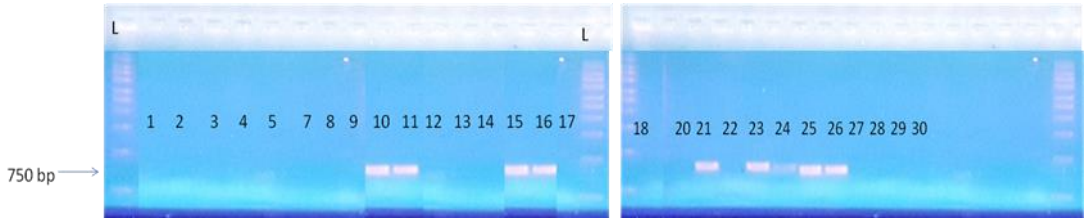

Figure2: PCR – amplification product of housekeeping gyrB gene. M = 1kb ladder
Number 1 to 30 represents the Indigenous Rhizobia isolated from different soils in Hai District, northern Tanzania named: IR1=Mungushi (farm A), IR2=Mungushi (farm B), IR3=Kware (farm A), IR4= Kware (farm B), IR5=Amani (fam A), IR6=Amani (farm B), IR7=Shirinjoro (farm A), IR8= Shirinjoro (farm B), IR9= Kwasadala (farm A), IR10= Kwasadala (farm B), IR11= Rundugai (farm A), IR12=Rundugai (farm B), IR13=Kwatito (farm A), IR14=Kwatito (farm B), IR15= Chekimaji (farm A), IR16=Checkimaji (farm B), IR17=Longoi (farm A), IR18=Longoi (farm B), IR19= Mbatakero (farm A), IR20=Mbatakero (farm B), IR21 Mulama (farm A), IR22=Mulama farm B), IR23=Mudio (farm A), IR24 =Mudio (farm B), IR25=Usari (farm A), IR26=Usari (farm B), IR27=Olori (farm A), IR28=Olori (farm B), IR29=Nshara (farm A), IR30 = Nshara (farm B).

Table 5: DNA Amplification using 16S rRNA and *gyrB* Primers

Source/Village	Field	Isolate	16SrRNA	gyrB
Mungushi	A	IR 1	-	-
Mungushi	B	IR 2	-	-
Kware	A	IR 3	-	-
Kware	B	IR 4	+	-
Amani	A	IR 5	-	-
Amani	B	IR 6	-	-
Shirinjoro	A	IR 7	-	-
Shirinjoro	B	IR 8	+	-
Kwasadala	A	IR 9	+	-
Kwasadala	B	IR 10	+	+
Rundugai	A	IR 11	+	+
Rundugai	B	IR 12	+	-
Kwatito	A	IR 13	-	-
Kwatito	B	IR 14	+	-
Chekimaji	A	IR 15	-	+
Chekimaji	B	IR 16	-	+
Longoi	A	IR 17	+	-
Longoi	B	IR 18	-	-
Mbatakero	A	IR 19	-	-
Mbatakero	B	IR 20	+	-
Mulama	A	IR 21	-	+
Mulama	B	IR 22	-	-
Mudio	A	IR 23	-	+
Mudio	B	IR 24	+	-
Usari	A	IR 25	-	+
Usari	B	IR 26	-	+
Olori	A	IR 27	-	-
Olori	B	IR 28	-	-
Nshara	A	IR 29	+	-
Nshara	B	IR 30	+	-

Note: + = Amplification, - = no amplification

Symbiotic Effectiveness of the Isolated Indigenous Rhizobia Strains
Plant Nodulations

All 30 rhizobial isolates inoculated on common bean plants induced different levels of nodulation (Table 6). Nodule mean number per plant ranged from 30 for NR20 to 151 for NR5. The isolates that showed greater nodulation (nodule mean number per plant) on common bean (Table 5) included IR5 (151 nodules per plant), IR27 (107 nodules), IR10 (98 nodules), IR3 (91 nodules), IR6 (90 nodules), IR19 (89 nodules), IR2, IR8, IR14 (88 Nodules) and IR24 (87 nodules per plant). The remaining isolates were intermediate in their nodulation of the common bean. The *Phaseolus* bean inoculated with commercial inoculants CIAT 899 showed the least number of nodules (21nodules plant^{-1}), while un-inoculated pots showed 0 numbers of nodules (Table 5).

Nodules Dry Biomass

Nodules dry Biomass also differed between and among rhizobial isolates, and ranged from 0.086g.plant^{-1} to 0.20g.plant^{-1} (Table 6). Isolate NR9 produced the highest nodule dry matter followed by isolates IR14, IR27, IR16 and IR17, IR6, IR11 and IR13, IR19 followed by IR1 and IR12. In contrast, isolates IR 20 and IR7 produced the least nodule dry mass (Table 6). The *Phaseolus* bean inoculated by Commercial strains CIAT 899 showed the nodule biomass of 0.088gplant^{-1}.

Colour of the Plant Leaves

Phenotypic observation of leaves colour was done. The *Phaseolus* bean inoculated with IR7, IR18, IR22, IR1 and IR4 showed deep green colour as the colour of N-fed plants (Table 6). The rest had light green as shown on the Table 5. *Phaseolus* bean inoculated with commercial rhizobia CIAT 899 were showing green colour and un-inoculated plants were pale-green.

Chlorophyll Content

Rhizobia isolate IR10 produced high chlorophyll content as that of N-fed plants (0.0342 µgChlg^{-1}), followed by IR12 (0.0339 µgChlg^{-1}), IR3 (0.0338 µgChlg^{-1}), IR1 (0.0335 µgChlg^{-1}) and IR23 (0.0314 µgChlg^{-1}). The commercial inoculants CIAT 899 showed lower chlorophyll content compare with the isolated native rhizobia, whereas the un-inoculated negative control *Phaseolus* bean showed the lowest chlorophyll content among all plants (Table 6).

Table 6: Influence of Isolated rhizobia on nodule number, nodule dry weight, colour of the leaves and chlorophyll content

Village	Field	Treatment	Parameter Measured			
			Nodule number	**Nodule dry Biomass (g)**	**Colour of plant leaves**	**Chlorophyll content (µgChlg^{-1})**
na		Un-inoculated	0.00±0.00^{h}	0.000±0.0000^{h}	1.94±0.20^{h}	0.0072±0.00046^{f}
na		N-fed	0.00±0.00^{h}	0.000±0.0000^{h}	4.50±0.29^{abcde}	0.0342±0.00223^{a}
na		CIAT 899	21.75±3.33^{g}	0.088±0.0085^{g}	3.00±0.05^{g}	0.0110±0.00207^{f}
Mungushi	A	IR1	80.00±5.02^{def}	0.193±0.0036^{bcd}	4.68±0.24^{abcd}	0.0335±0.00047^{a}
Mungushi	B	IR2	88.75±5.07^{cd}	0.189±0.0052^{cde}	3.07±0.90^{g}	0.0260±0.00314^{bcde}
Kware	A	IR3	91.75±7.47^{bcd}	0.190±0.0063^{bcd}	4.16±0.29^{abcde}	0.0338±0.00019^{a}
Kware	B	IR4	78.00±6.96^{def}	0.181±0.0028^{cde}	4.55±0.21^{abcde}	0.0304±0.00274^{abc}
Amani	A	IR5	151.75±13.66^{a}	0.170±0.0032^{def}	4.28±0.32^{abcde}	0.0292±0.00215^{abc}
Amani	B	IR6	90.00±1.29^{cd}	0.197±0.0037^{bcd}	4.04±0.17^{bcde}	0.0306±0.00218^{ab}
Shirinjoro	A	IR7	75.75±5.01^{def}	0.169±0.0030^{defg}	4.81±0.11^{a}	0.0269±0.00101^{bcde}
Shirinjoro	B	IR8	88.75±1.65^{cd}	0.191±0.0151^{ab}	4.29±0.22^{abcde}	0.0303±0.00093^{abc}
Kwasadala	A	IR9	84.00±2.97^{cde}	0.209±0.0163^{a}	4.10±0.17^{abcde}	0.0279±0.00149^{abcd}
Kwasadala	B	IR10	98.00±6.82^{bc}	0.187±0.0039^{bcd}	4.30±0.24a^{bcde}	0.0342±0.00066^{a}
Rundugai	A	IR11	76.75±3.42^{def}	0.197±0.0049^{abc}	4.14±0.14^{abcde}	0.0209±0.00265^{e}
Rundugai	B	IR12	83.00±3.79^{cde}	0.193±0.0049^{abcd}	4.23±0.20^{abcde}	0.0339±0.00241^{ab}
Kwatito	A	IR13	78.75±4.07^{def}	0.197±0.0053^{abc}	4.14±0.18^{abcde}	0.0207±0.00169^{e}
Kwatito	B	IR14	88.25±5.48^{cd}	0.203±0.0069^{ab}	4.24±0.33^{abcde}	0.0260±0.00224^{bcde}
Chekimaji	A	IR15	79.25±6.29^{def}	0.194±0.0136^{abc}	4.04±0.21^{bcde}	0.0288±0.00138^{abcd}
Chekimaji	B	IR16	66.25±3.64^{f}	0.199±0.0059^{abc}	3.97±0.04^{def}	0.0281±0.00288^{abcd}
Longoi	A	IR17	77.75±4.59^{def}	0.199±0.0084^{abc}	4.01±0.13^{cde}	0.0268±0.00265^{bcde}
Longoi	B	IR18	85.00±3.92^{cde}	0.189±0.0032^{bcd}	4.74±0.16^{ab}	0.0241±0.00290^{cde}
Mbatakero	A	IR19	89.75±12.34^{cd}	0.194±0.0080^{abcd}	4.00±0.21^{def}	0.0252±0.00235^{bcde}
Mbatakero	B	IR20	30.25±2.75^{g}	0.086±0.0031^{gh}	4.36±0.24^{bcde}	0.0283±0.00158^{abcd}
Mulama	A	IR21	80.50±4.33^{def}	0.180±0.0049^{cde}	3.29±0.22f^{g}	0.0285±0.00345^{abcd}
Mulama	B	IR22	83.25±3.82^{cde}	0.182±0.0027^{cde}	4.72±0.26^{abc}	0.0314±0.00292^{ab}
Mudio	A	IR23	70.50±5.66^{ef}	0.166±0.0043^{fgh}	3.97±0.07^{ef}	0.0226±0.00153d^{e}
Mudio	B	IR24	87.00±5.82^{cd}	0.186±0.0040^{cdef}	3.86±0.14^{ef}	0.0280±0.00474^{abcd}
Usari	A	IR25	80.00±4.56^{def}	0.181±0.0044^{cdef}	3.12±0,20^{g}	0.0241±0.00256^{cde}
Usari	B	IR26	79.75±9.24^{de}f	0.183±0.0106^{abcd}	3.95±0.14^{ef}	0.0304±0.00213^{abc}
Olori	A	IR27	107.25±12.26^{b}	0.200±0.0034^{ab}	4.15±0.28^{abcde}	0.0301±0.00140^{abc}
Olori	B	IR28	89.25±2.95^{cd}	0.193±0.0033^{abcd}	4.02±0.07^{cde}	0.0291±0.00387^{abc}
Nshara	A	IR29	79.50±2,78^{def}	0.178±0.0042^{defg}	4.04±0.04^{bcde}	0.0284±0.00175^{abcd}
Nshara	B	IR30	65.75±2.78^{f}	0.186±0.0051^{cdef}	4.28±0.25^{abcde}	0.0298±0.00257^{abc}
		F Statistics	**23.3****	**59.63*****	**5.2*****	**6.3 *****

*Values represent Mean ± SE, values with dissimilar letter(s) in a column are significant by Fisher LSD at P=0.05.IR=Indigenous Rhizobium. na=not applicable

Shoot Dry Mass

Shoot dry biomass accumulated also differed between and among the rhizobial strains. Isolate NR 12 produced the highest shoot biomass (1.48 g.plant^{-1}) followed by IR10 and IR3 (1.46 g.plant^{-1}), IR1 (1.44 g.plant^{-1}), IR2 (1.43 g.plant^{-1}), IR28 (1.4 g.plant^{-1}), IR6 (1.36 g.plant^{-1}) and IR8 (1.2 g.plant^{-1}). In contrast, isolate IR20 produced the least shoot biomass (0.78 g.plant^{-1}), followed by IR23 and IR30 (0.83 g.plant^{-1}). Commercial strain, CIAT 899 produced 1.11g.Plant^{-1} shoot biomass which is the average compared with the isolated native Rhizobia. About 14 isolates produced shoot dry matter higher than the N_2 fed plants (Table 7). The non-inoculated *Phaseolus* bean produced the lowest shoot biomass (0.05g.Plant^{-1}) compared with the isolated native strains, CIAT 899 and N-fed plants.

Root Biomass

Rhizobial isolate IR11 produced highest (3.84 g.plant^{-1}) root biomass, followed by NR6 (3.07 g.plant $^{-1}$), IR2 (2.89 g.plant $^{-1}$), IR9 (2.39 g.plant^{-1}), IR12 2.36 g.plant^{-1}), IR4 (2.30g.plant^{-1}), and IR26 (2.26 g.plant^{-1}). About 14 isolates produced more root biomass than the N_2 fed plants (Table 6). In contrast, isolates IR20 and IR23 produced the least root biomass (1.01 and 0.02 g.plant^{-1}, respectively) which was even lower than the un-inoculated control (0.59g.plant^{-1}). The 14 isolated native strains produced more root biomass compared with commercial strain CIAT 899 (1.36g.Plant^{-1})(Table7).

Table 7**: Influence of isolated rhizobia on shoot length, stem girt, shoot and root dry weight**

Village	Field	Treatment	Parameter measured			
			Shoot length (cm)	**Stem girth (mm)**	**Shoot Dry Biomass (g)**	**Root Dry Biomass (g)**
na		Un-inoculated	13.50±0.65^{i}	1.39±0.12^{i}	0.05±0.01^{f}	0.59±0.06^{f}
na		N-fed	60.00±1.29^{ab}	4.72±0.15^{a}	0.99±0.08^{bcde}	1.28±0.01^{def}
na		CIAT 899	34.50±2.47^{fgh}	2.91±0.34^{g}	1.11±0.01^{bcde}	1.36±0.10^{ef}
Mungushi	A	IR1	41.50±6.03^{defgh}	3.48±0.26^{cdefgh}	1.44±0.13^{ab}	1.24±0.34^{def}
Mungushi	B	IR2	45.50±3.69^{cdef}	4.20±0.17^{abc}	1.43±0.18^{b}	2.89±1.08^{abc}
Kware	A	IR3	35.00±6.56^{efgh}	2.96±0.58^{fgh}	1.46±0.24^{b}	1.72±0.58^{bcde}
Kware	B	IR4	39.25±5.17^{defgh}	3.59±0.23^{bcdefg}	1.13±0.12^{bcde}	2.00±0.32^{bcde}
Amani	A	IR5	37.00±4.45^{defgh}	3.60±0.32^{bcdefg}	0.69±0.23^{e}	1.16±0.59^{def}
Amani	B	IR6	54.50±2.40^{abc}	3.47±0.25^{cdefg}	1.36±0.17^{bcd}	3.07±1.33^{ab}
Shirinjoro	A	IR7	32.00±4.60^{gh}	2.88±0.17^{h}	0.85±0.16^{cde}	1.50±0.27^{cdef}
Shirinjoro	B	IR8	44.25±3.68^{cdefgh}	3.45±0.37^{cdefgh}	1.20±0.38^{bcde}	2.02±0.80^{bcde}
Kwasadala	A	IR9	39.75±5.66^{defgh}	4.01±0.03^{abcde}	0.81±0.24^{de}	2.39±0.82^{abcd}
Kwasadala	B	IR10	44.25±8.82^{cdefgh}	4.19±0.27^{abcd}	1.46±0.22^{ab}	1.61±0.78^{bcde}
Rundugai	A	IR11	39.25±3.82^{defgh}	3.34±0.01^{efgh}	1.11±0.27^{bcde}	3.84±0.80^{a}
Rundugai	B	IR12	47.75±4.13^{bcde}	3.38±0.25^{efgh}	1.48±0.52^{a}	2.36±0.50^{bcd}
Kwatito	A	IR13	48.00±6.65^{bcd}	3.92±0.31^{bcde}	1.12±0.11^{bcde}	1.20±0.22^{def}
Kwatito	B	IR14	42.25±4.07^{cdefgh}	3.84±0.09^{bcde}	1.12±0.12^{bcde}	2.14±0.49^{bcd}
Chekimaji	A	IR15	34.50±5.42^{fgh}	3.67±0.28^{bcdefg}	1.00±0.34^{bcde}	1.13±0.30^{def}
Chekimaji	B	IR16	37.00±6.24^{defgh}	3.38±0.33^{efgh}	0.93±0.24^{bcde}	1.23±0.44d^{ef}
Longoi	A	IR17	42.50±6.46^{cdefgh}	4.00±0.40^{abcde}	0.92±0.11^{bcde}	1.27±0.24d^{ef}
Longoi	B	IR18	45.25±6.05^{cdef}	4.31±0.2918^{def}	0.98±0.21^{bcde}	1.25±0.33^{def}
Mbatakero	A	IR19	33.75±3.50^{fgh}	3.62±0.46^{bcdefgh}	0.91±0.28^{bcde}	1.17±0.45^{def}
Mbatakero	B	IR20	31.50±4,05^{h}	3.50±0.13^{cdefgh}	0.78±0.12^{e}	1.01±0.26^{def}
Mulama	A	IR21	63.50±0.65^{a}	3.51±0.33^{cdefgh}	1.01±0.17^{bcde}	1.32±0.14^{def}
Mulama	B	IR22	44.50±5.38^{cdefg}	3.92±0.14^{bcde}	0.99±0.06^{bcde}	1.40±0.30^{def}
Mudio	A	IR23	33.25±4.09^{fgh}	3.82±0.22^{bcde}	0.83±0.12.^{cde}	1.02±0.22^{def}
Mudio	B	IR24	62.50±1.85^{a}	4.32±0.19^{ab}	1.14±0.11^{bcde}	2.30±0.40^{bcd}
Usari	A	IR25	45.75±6.79^{cdef}	3.71±0.31^{bcdef}	1.20±0.23^{bcde}	1.36±0.26^{def}
Usari	B	IR26	33.75±3.35^{fgh}	3.46±0.25^{scdefg}	1.15±0.07^{bcde}	2.26±0.40^{bcd}
Olori	A	IR27	41.25±4.37^{defgh}	3.70±0.26^{bcdef}	0.96±0.23^{bcde}	1.43±0.44^{cdef}
Olori	B	IR28	49.25±2.66^{bcd}	3.83±0.16^{bcde}	1.40±0.17^{bc}	2.06±0.55^{bcd}
Nshara	A	IR29	41.75±5.85^{cdefgh}	3.43±0.27^{defgh}	0.83±0.05^{cde}	1.62±0.21^{bcde}
Nshara	B	IR30	39.25±3.59^{defgh}	3.34±0.13^{efgh}	1.08±0.04^{bcde}	1.79±0.50^{bcde}
		F Statistics	**4.5*****	**4.4****	**2.9 *****	**1.9****

*Values represent Mean±SE, values with dissimilar letter(s) in a column are significant by Fisher LSD at P=0.05.IR=Indigenous Rhizobium.na=not applicable

Discussion

The results of soil analysis components showed pH range of 6.0 - 8.2 which is within the optimum pH range for crop production [31]. It was also found to be favourable for *Phaseolus* infective strains of Rhizobia [32]. The soils in Hai district had deficit in soil N and P in some soils in different villages, while exchangeable cations (Ca, and Mg) were in normal levels, except for Na, which was inadequate. Micronutrients (Cu, Mn, Fe, Zn) appeared limiting in some soils. 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 [33-36]. Besides Rhizobium has the ability to convert atmospheric nitrogen gas (N_2) into ammonia (NH_3), a form which can be utilized by the plants, hence solving a problem of nitrogen deficiency [37-41].

The categories of bacterial growth are consistent with characterization of growth of rhizobia [23]. The rhizobial isolates incubated in the dark at 28°C for 5 days, failed to absorb Congo red, which is a typical characteristic of the family *Rhizobiaceae* [42,23,43]. Morphologically, all the isolates were round in shape with smooth texture which are the similar characteristics as to those described in previous studies [44]. The variation in colony colour was noted, with most of the isolate showing whitish or creamy in colour when grown on Yeast Manitol Broth at 28°C for 72 hours, which agrees with the report that common bean rhizobia exhibit a whitish or creamy colony colour [42]. The characteristics of the isolated rhizobia strains presented in Table 4 below resemble the standard characteristics of rhizobia, and hence proved that the isolated strains were rhizobia. Furthermore, the indigenous rhizobia isolated from *Phaseolus* bean on the studies conducted in Tanzania and Kenya revealed the colony characteristics which were the same as those found in this study [45,46,30]. These findings gave hopeful indicator towards production of inoculants for *Phaseolus* bean native to Tanzania since most of the isolated strains showed ability to induce nodulation on the roots of *Phaseolus* bean.

The PCR results show that, out of 30 rhizobia isolate, 10 were amplified with 16S rRNA, 5 were amplified with *gyrB*, 3 were amplified with both 16s rRNA and *gyrB* and 12 were not amplified with any of the of the selected primers. The failure to amplify by selected primers could be due to due to presence of multiple copies in the genome of some bacteria, susceptibility to genetic recombination, and horizontal gene transfer [47], in addition, 16S

rRNA is limited in discriminating the characteristics at and below the species level [47-49]. Furthermore, the reports of other studies shows that, there are bacteria which represent different species but have identical or nearly identical 16S rRNA gene sequence [50,48,51]. This explain the reason for some bacteria to amplify with 16S rRNA gene only but not to housekeeping *gyrB* gene. The housekeeping gene *gyrB* has higher degree of divergence which allows species discrimination [51], hence in this study PCR application with *gyrB* occurred in few rhizobial isolates and few were amplified by both genes. These implying that, there is presence of the Rhizobia species in the different soils of Hai District, northern Tanzania, since most of the isolates in all three varying altitudes amplified with primers. Therefore, there is a need for further sequencing analysis to identify these species of rhizobia that could be used for inoculants production for improving common bean production in Tanzania

Isolated rhizobia strains showed significant influence on number of nodules per plant, nodule dry biomass, leaf colour and chlorophyll content (Table6). Significant difference were also observed in shoot dry biomass, shoot length, stem girth, and root dry biomass (Table7).

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), root dry mass (gm), nodule dry mass (gm), colour rank of the plant leaves, stem girth (mm) and chlorophyll content per gram of leaf (µgChlg-1) as compared with the commercial/standard strain (CIAT 899). The findings from the study [45,35,30] agree with the findings from this study that some of the indigenous rhizobia isolates has higher ability of forming effective nodules in the roots of *Phaseolus* bean than commercial strains. Moreover, these results are contrary to those described in another study [52] who reported that inoculation with native rhizobia isolated from two farms in Kenya, were not effective in nodulation in common bean. 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, root dry mass and plant leaves colour as opposed to the study that demonstrated that nodule number is not an appropriate measure of effectiveness in rhizobia–legume symbiosis [53].

Phaseolus bean supplied with N, showed higher performance on stem girth, shoot length, plant leave colour and chlorophyll contents but were not effective on shoot and root dry mass. 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 [54,55,53]. Therefore, the shoot dry mass and chlorophyll content is the main parameters which determine the effectiveness of the inoculants.

From this study, highest shoot dry mass and chlorophyll content were obtained in plants inoculated with indigenous strain IRI, IR3, IR10 and IR12 and the lowest was obtained in non-inoculated plants (negative control), which corresponds with previous studies [45,30]. This implies that there is high possibility of having indigenous Rhizobial strains which are more effective in fixing nitrogen than the current standard strain CIAT 899.

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 native isolated rhizobial strains to fix nitrogen was shown by significant differences in the colour of inoculated plant and non-inoculated plants. 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 more ability of fixing nitrogen than the standard strain CIAT899 currently in use in northern Tanzania.

Evaluating symbiotic effectiveness of indigenous rhizobia was done purposely as an effort towards tackling the problem of nitrogen deficiency in the soils of smallholder farmers in Africa.

The legume-rhizobia symbiosis provides a window for trapping the abundant N_2 in the atmosphere for plant uptake.

Conclusion

Towards ensuring food security, sustainable use of natural resources is a critical means to enhance agricultural productivity. The use of inoculants as bio-fertilizers to improve soil N economy through BNF has proven to be economically beneficial and necessary in all soils deficient of nitrogen. The total of 30 strains of indigenous rhizobia strain was isolated and proved to be rhizobia in an authentication experiment and Morphological observations. The characteristic of the fast-grower proved to be consistent with genus *Rhizobium* obtained from the 16S rRNA and housekeeping gene (*gyr*B) PCR analysis. The result of PCR amplification of 16S rRNA showed the presence of diversity among the collected isolates. *Rhizobium*

isolated suggests the needs to perform more molecular analyses to elucidate the potential presence of novel *Rhizobium* species in Tanzania.

Most of the isolates were effective in nodulating the *Phaseolus* bean and showed great performance in other growth parameters compared to the Standard /Commercial Strain, CIAT 899. Strains NR1 from Mungushi village, IR3 from Kware village, IR10 from Kwasadala village in middle lands and IR12 from Rundugai in lowlands, showed an outstanding performance in symbiotic effectiveness than the standard/commercial strain CIAT 899. This implies 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.

Therefore strains which showed high potential in N_2-fixation in pot experiments could be used for further investigation for their efficiency under field conditions for use in inoculants production. However, their competitiveness in the presence of other commercial and indigenous strains needs to be evaluated. The findings of this study indicate that, it is possible to reduce the use of nitrogen mineral fertilization or completely avoid it in common bean production through inoculation with highly effective strains.

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Output 2: Poster Presentation

Morphological Assessment and Effectiveness of Indigenous Rhizobia Nodulating Common Beans (*Phaseolus Vulgaris* L.) in Hai District, Northern Tanzania

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Introduction

Rhizobia is a soil bacterium that fix atmospheric nitrogen into ammonia (Poonia, 2011). Biological Nitrogen Fixation (BNF) provides alternative source of synthetic nitrogen fertilizers. (Freiberg. et al., 1997). BNF technology in agriculture includes, inoculation of legumes with characterised rhizobia inoculants (Bull et al., 2002). Molecular techniques are usually recommended for characterising rhizobia. The aim of this study was to evaluate the symbiotic efficiency of rhizobia isolated from root nodules of common beans obtained from farmers' fields of Hai District in Northern Tanzania, by measuring biomass accumulation, chlorophyll content along with characters.

Objectives

➢To identify indigenous rhizobia isolates based on 16SrRNA and housekeeping gene (*gyrB*) and morphological characteristics of bean growing in different soil of Hai District in northern Tanzania.

➢ To evaluate effectiveness of rhizobia isolates from selected soils of Hai District on common beans growth.

Methods

Sampling: A total of 30 roots of common bean containing nodule samples were obtained from farmers fields in 15 villages of Hai District, Kilimanjaro, Northern Tanzania.

Isolation and Authentication of Rhizobia

Isolation of rhizobia from root nodule, then grown on petridish containing YEMA w/Cong red and incubated at 28 °C, for 72hrs. A pure colon was then grown on YMB at 28 °C, for 72hrs, at 120rpm to obtain rhizobia suspension.

Authentication of rhizobial isolates was done by supplying 10 ml of YEMB (≈ 5 x 10^8 cells/ml) containing rhizobia suspension on sterilized bean seeds grown in plastic pots containing sterilized sand.

Morphological Characterization of Rhizobial Isolates:

The colonies were assessed for growth, colour, shape, appearance, texture, absorption of Congo red, colony elevation and colony margin

Molecular identification of Authenticated rhizobia Isolates

DNA was extraction using *Quick*-DNA™ Fungal/Bacterial Mini prep Kit The integrity of isolated DNA examined using Nano Drop and 1% (w/v) agarose gel containing EZ Vision, and 1 x TBE buffer in electrophoresis at 80 V for 50 min, using 1Kb DNA ladder, to confirm DNA purity. PCR amplification was done using two Primers; 16S rRNA and Housekeeping *gyrB*

Evaluation of symbiotic effectiveness of rhizobia isolates:

The experiment was conducted in the screen house at NM-AIST.

Experimental Layout:

Four replicate pots per isolate were inoculated with 10 ml (≈ 5x 10^8 cells/ml) of YEMB containing *rhizobium* strains. isolated

Four pots un-inoculated but supplied with nitrogen were included as control

Four were inoculated with standard rhizobia inoculants "CIAT 899" as positive control

Four were left non- inoculated as negative control

Plants were irrigated three times a week using sterile N-free nutrient solution

Data Collection and Analysis

Growth parameters including, nodule count, shoot, roots and nodule biomass, shoot length, stem girth and chlorophyll content were measured 5 weeks after inoculation.

Relative symbiotic effectiveness (Sei%) was calculated.

$$SEi = \frac{Xi - Xu}{Xn - Xu} \times 100$$

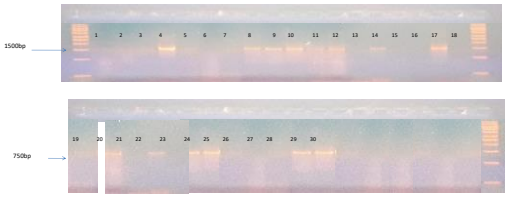
One way analysis of variance (ANOVA) was used for analysis of the data which seemed normally distributed. Treatment means of the growth parameters were compared by using Fisher LSD as the post hoc significance test, using STATISTICA 10. software.

Results and Discussion

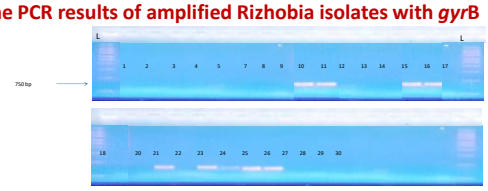
The rhizobial isolates incubated in the dark at 28˚C for 5 days, failed to absorb Congo red, which is a typical characteristic of the family *Rhizobiaceae*. Morphologically, all the isolates were round in shape with smooth texture.

Variation in colony colour was noted, with most of the isolate showing whitish or creamy in colour which agrees with the reports that common bean rhizobia exhibit a whitish or creamy colony colour, which is also the characteristics of *Bradyrhizobia Spp*.

The PCR results of amplified Rizhobia isolates with 16SrRNA



The PCR results of amplified Rizhobia isolates with *gyr*B



Some of the DNA of the ithe rhizobia isolates failed to amplify with selected primers, this could be due to presence of multiple copies in the genome of some bacteria, susceptibility to genetic recombination, and horizontal gene transfer (Aserse *et al.*, 2012), in addition, 16S rRNA is limited in discriminating the characteristics at and below the species level (Aserse *et al.*, 2012; Martens *et al.*, 2008) Furthermore, the reports of other studies show that, there are bacteria which represent different species but have identical or nearly identical 16S rRNA gene sequence Martens *et al.*, 2008; 2007). This explains the reason for some bacteria to amplify with 16S rRNA gene only but not to housekeeping *gyrB* gene. The housekeeping gene *gyrB* has higher degree of divergence which allows species discrimination (Martens *et al.*, 2007),.

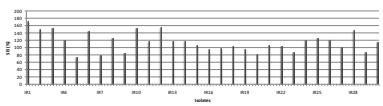
These implying that, there is presence of the Rhizobia species in soils of Hai District, northern Tanzania, since most of the isolates in all three varying altitudes amplified with primers

Influence of rhizobia isolates on symbiotic performance of the common bean plants

Most of the isolates were effective in nodulating the *Phaseolus* bean and showed great performance in other growth parameters compared to the Standard /Commercial Strain, CIAT 899.

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. Strains IR1 from Mungushi village, IR3 from Kware village, IR10 from Kwasadala village in middle agro ecological zone and IR12 from Rundugai in lower agro ecological zone, showed an outstanding performance in symbiotic effectiveness than the standard/commercial strain CIAT 899. (Fig Below)

Relative Symbiotic effectiveness (SEi%)



Conclusion

These findings gave hopeful indicator towards production of molecular characterized inoculants for *Phaseoulus* bean indigenous to Tanzania since most of the isolated strains showed ability on the performance of Phaseolus bean

Acknowledgement

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