CHARACTERIZATION AND EFFECTIVENESS DETERMINATION OF INDIGENOUS RHIZOBIA NODULATING LABLAB (*Lablab purpureus*) IN BABATI DISTRICT, 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

Arusha, Tanzania

August, 2021

ABSTRACT

The soil samples from fifteen villages which grow *Lablab* in Babati district were collected and used for planting *Lablab* in experimental pot to trap indigenous rhizobia. At a flowering stage, the plants were harvested and root nodules were collected for rhizobia isolation using Yeast Extract Mannitol Agar (YEMA). Under aseptic conditions, twenty-two isolates were obtained and characterized by morphological, biochemical and physiological characteristics. Furthermore, PCR amplification of 16S rDNA and symbiotic effectiveness were also evaluated. The results showed that, rhizobia were fast and slow growing in YEMA after 4 - 7 days of incubation. In YEMA with Congo red one isolates absorbed the color, while, physiologically all isolates were alkaline tolerant, however susceptible to acidity and sodium chloride above 3%. High diversity among isolates was observed in dendrogram at 70% similarity level by forming two groups with seven clusters. Only 18 isolates out of 22 were amplified. In testing symbiotic effectiveness, all 22 isolates induced nodules in *Lablab*, interestingly some were effective than commercial inoculants. Most of isolates recorded relative symbiotic effectiveness above 100, the highest was 193 for BR4. Isolates BR4, BR13, BR16, BR5 and BR18 were effective than other isolates and commercial strains. The results implied presence of diverse and effective indigenous rhizobia nodulating *Lablab* in Tanzania, also highlights their potential to be used for inoculating *Lablab* instead of commercial inoculants. To recommend characterization by sequencing to identify their genetic groups and field effectiveness evaluation is needed in order to identify more effective isolates for inoculants production.

DECLARATION

I, Eliah D. Malugu, 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 elsewhere for degree award in any other institution.

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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 determination of indigenous rhizobia nodulating Lablab (*Lablab purpureus*) in Babati district, Tanzania".

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ACKNOWLEDGMENT

I cannot thank enough our almighty God, Father in heaven for his steadfast love to me all the time in my studies, especially the guidance and energy He provided me during conducting my research and in the writeup. I am grateful to Center for Research, Agriculture advancement, Teaching Excellence and Sustainability (CREATES) for full sponsoring my studies and N2Africa Project for additional funds to facilitate this research through Nelson Mandela African Institution of Science and Technology (NM-AIST).

I direct my sincerely thanks are to my supervisors, Prof. Patrick A. Ndakidemi and Dr. Pavithravani B. Venkataramana for their tireless work in advising, encouragement, guidance and technical support on the whole study time. Hearty appreciations to the laboratory staff of NM-AIST microbiology and International Institute of Tropical Agriculture (IITA) Arusha for their technical assistance in time of laboratory works, their expertise made this research possible.

The deeply thanks to my mother Mrs. Martha Mgongo, my wife Angel Ernest and son Dunstan Mgongo for their patience in my absence, prayers and encouragements for all the time of my studies to the time of successful completion.

LIST OF ABBREVIATIONS AND SYMBOLS

ACIAR	Australian Center for International Agriculture Research
BNF	Biological Nitrogen Fixation
CIAT	International Center for Tropical Agriculture
CR	Congo red
DMSO	Dimethyl Sulphoxide
FAO	Food and Agriculture Organization of United Nation
GPA	Glucose Peptone Agar
IITA	International Institute of Tropical Agriculture
N	Nitrogen
NM-AIST	Nelson Mandela African Institution of Science and Technology
OD	Optical Density
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
TaCRI	Tanzania Coffee Research Institute
TARI	Tanzania Agricultural Research Institute
UV	Ultra Violet
YEMA	Yeast Extract Mannitol Agar
YMB	Yeast Extract Mannitol Broth

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CHAPTER ONE

INTRODUCTION

1.1 Background of the problem

In the resource poor and developing countries especially in sub-Saharan Africa, researchers have identified the potential of the neglected crops such as *Lablab* (*Lablab purpureus*) in reducing food insecurity and fostering social-economic development (Chivenge *et al*., 2015). *Lablab* has many uses, apart from use by humans as food, it has got many other uses; including being an alternative source of protein in animal feed (Maidala & Jarmari, 2016). It can serve many other purposes for instance green manure, as cover crop to reduce water runoff and hence erosion, as herbal medicines and in gardening or landscaping (Ahmed & Hasan, 2014; Gao *et al*., 2004). For decades *Lablab* has been mentioned among the neglected legumes in research and cultivation. But, *Lablab* is known for its drought tolerance when compared to other legumes such as common beans and cowpeas (Maass *et al*., 2010). With the current trends in climate changes, *Lablab* has a great potential for the future of African agriculture. As many other legumes, *Lablab* nodulate freely in soils and can fix atmospheric nitrogen (N) through a Biological Nitrogen Fixation (BNF) process (Simon *et al*., 2014), it is among legumes with high nitrogen fixing capacity hence can contribute enough nitrogen into agricultural systems. The nodulation and fixation of atmospheric nitrogen is facilitated by Legumes Nodulating Bacteria (LNB) (Mfilinge *et al*., 2014; Pervin *et al*., 2017), which are divided into several genera of which in general are referred to as Rhizobia (Benselama *et al*., 2018; Onyango *et al*., 2015).

Rhizobia are gram negative soil microorganisms which forms a symbiotic interaction with legumes. In symbiosis, legumes provide photosynthesis products to rhizobia, and in return rhizobia fix atmospheric nitrogen to plant in readily available form (Suzaki *et al*., 2015). In addition, Rhizobia have other important roles like promoting nutrient uptake in legumes (Ndakidemi *et al*., 2011), synthesis of phytohormones (Desbrosses & Stougaard, 2011; Ikenganyia *et al*., 2017) and reduce toxic effects of metals (Koskey *et al*., 2018). The rhizobia can either be inoculated to legumes as bio-fertilizers or be indigenous as they are naturally available in soils (Ballard *et al*., 2004; Weir *et al*., 2004). The effectiveness and contribution of several indigenous rhizobia to BNF and total plant performance has been found to outperform that of exotic strains (Simon *et al*., 2015). Indigenous rhizobia vary widely and

their diversity is affected by climate, land use systems and soil fertility status (Legesse & Assefa, 2014). In Tanzania, there is climate and soil properties variations from place to place, hence, there is possibility of having several types of indigenous rhizobia with different characteristics and symbiotic ability and efficiency.

The characterization and effectiveness determination of indigenous rhizobia to different legumes have been conducted in most regions of the world (Benselama *et al*., 2018), although in Tanzania, there is no study which has been done to characterize rhizobia associated with *Lablab*. Hence, by considering rhizobia-host relationship and their adaptation to different environments, characterization and effectiveness determination of local isolates becomes an important study so as to explore these microbial resources and optimize their use production for food and improved soil fertility. This study aims at isolating and identifying morphological, biochemical, physiological and PCR amplification of 16S rDNA of *Lablab* indigenous rhizobia. Moreover, it aims to determine the effectiveness of the indigenous nitrogen fixing rhizobia associated with *Lablab* in soils collected from Babati district, Tanzania.

1.2 Statement of the problem

Lablab is a legume native to African continent and has been cultivated in tropical Africa for centuries by local people (Maass *et al*., 2010; Sheahan, 2012). The crop is important in contributing to soil fertility and food security. Despite these vital roles, this crop is also mentioned as one of neglected and underutilized legume (Ewansiha *et al.*, 2016; Mabhaudhi *et al.*, 2017). This is because in most developing countries, agricultural policies have much been directed in enhancing production of few crops due to their economic importance in both domestic and global markets (Touré *et al*., 2012). This make other crops especially the native crops to be neglected by both public-private researchers and industry (Cullis & Kunert, 2017; Lessa *et al.*, 2016), making them being cultivated mainly for subsistence. For improving its production and promoting its uses, efforts must be dedicated in finding sustainable and cost-effective ways of production such as use of bio-fertilizers through rhizobia inoculation.

The use of rhizobia inoculants on other legume has been reported to significantly increase crop yield up to more than 100% (Ndakidemi *et al*., 2006). In Tanzania indigenous rhizobia of some leguminous crops such as *Phaseolus vulgaris* has been characterized and tested for their effectiveness (Namkeleja *et al.*, 2016). Although *Lablab* is grown by farmers in many parts of Tanzania, no information is available on native rhizobia associated with *Lablab*. It is due to

this information gap that this study aims to isolate, characterize and study the effectiveness of *Lablab* isolates for improving crops production and promote sustainable use of the soils.

1.3 Rationale of the study

Lablab has many purposes although its yields are limited. One of the methods which has showed to improve other legumes is use of rhizobia inoculant and more importantly are indigenous rhizobia. Therefore, this study aimed at investigating the indigenous rhizobia associated with *Lablab* so as to provide overview of their characteristics and potentiality in nitrogen fixation in step towards improving *Lablab* productivity and inoculants development.

1.4 Research objectives

1.4.1 General objective

To characterize and determine the effectiveness of indigenous rhizobia associated with *Lablab* in Babati district, Tanzania.

1.4.2 Specific objectives

(i) To isolate indigenous rhizobia from *Lablab* root nodules and characterize by morphological, biochemical, physiological and PCR amplification of 16S rDNA.

(ii) To evaluate the effectiveness of isolated indigenous rhizobia from *Lablab* root nodules for nitrogen fixation.

1.5 Research questions

(i) What are the morphological, biochemical, physiological and molecular characteristics of indigenous rhizobia isolated from *Lablab* root nodules?

(ii) How effective are the *Lablab* isolated rhizobia in fixing nitrogen compared to commercial strains?

1.6 Significance of the study

The findings from this study contribute to the following:

(i) Knowledge expansion of the possible diversity of the indigenous rhizobia associated with *Lablab* and their behavioral characteristics.

(ii) Identification of potential isolates that can significantly contribute in nitrogen fixation in *Lablab* productivity.

1.7 Delineation of the study

This study focused on characterizing and evaluating nitrogen fixation effectiveness of indigenous rhizobia associated with *Lablab* in fifteen villages of Babati district, Tanzania. Effectiveness evaluation was done in pot experiment that *Lablab* was grown up to early flowering stage.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Most of sub Saharan African soils have been depleted due to nutrients deficiency especially nitrogen and phosphorus (Drechsel *et al*., 2001). This has been attributed by population growth that has led to elevated land pressure, non-farm diversification and unsustainable agricultural practices hence, depletion of soil fertility (Jayne *et al.*, 2014). The reduction in soil fertility leads to reduced crop yield hence food insecurity. Inorganic fertilizers are commonly used to overcome the situation but are not accessible to most of African farmers, because it they are expensive (Laurette *et al*., 2015). Planting legumes either by crop rotation or intercropping with some cereal crops, has become an important and affordable way of improving soil condition and nutrients (Hadad, 1984). Legumes has been reported to facilitate in BNF, soil cover and as source of organic matter in the soils (Grönemeyer *et al*., 2017; Simon *et al*., 2014). The fixed N fixed can either be used by legumes themselves or nearby plants or can be accumulated in the soil for use by the subsequent plant to be grown (Nyoki & Ndakidemi, 2016). Therefore, by considering the advantages of legumes to soil fertility improvement, it is suggested that special attention is needed to facilitate researches especially for *Lablab.* This will contribute to its wide spread production and use in our communities for soil fertility improvement and ensuring of food security.

2.2 Biological nitrogen fixation

Biological Nitrogen Fixation (BNF) is the process by which nitrogen gas in the atmosphere is converted by legume nodulating bacteria into active form that can be used by plants *(Cardoso et al*., 2016; Sobti *et al*., 2015). This is facilitated by rhizobia which form a symbiotic association with leguminous plants (Ahmed & Hasan, 2014), where by rhizobia provides plants with N, and plants furnish rhizobia with the photosynthetic materials for energy to drive the process (Laranjo *et al*., 2014; Simon *et al*., 2014). This occur in the roots of legume, and the interface between soil and roots represents a highly dynamic environment for microbial populations and is often defined as the rhizosphere (Schmidt & Eickhorst, 2014), characterized by a great array of complex chemical and biological interactions (Lagos *et al*., 2015).

For legumes to nodulate, the appropriate rhizobia strains have to be present in the soil. This is due to the fact that rhizobia are specific to their hosts range (Koskey *et al*., 2018; Laranjo *et*

al., 2014). The compatibility between rhizobia and the host legumes is needed for successful nodulation and N fixation (Pandya *et al.,* 2013). If the incompatibility occurs between the two agents, the rhizobia will either fail to nodulate the legume or can nodulate but unable to fix nitrogen (Wang *et al.,* 2018). Hence, in order to achieve maximum BNF the characterization of local isolates will be an important tool so as to identify the presence of compatible isolates and their effectiveness in order to optimize their use for legumes production.

2.3 Indigenous rhizobia

Rhizobia which are naturally occurring in a particular soil are referred as indigenous to that geographical area (Chakraborty & Ramkrishna, 2005). The distribution of the hosts in the certain environment and their land use types, influences indigenous rhizobia distribution and its diversity (Mwangi *et al*., 2011). Being native to the environment they are considered superior than exotic as they are well adapted for symbiotic ability, environment and cropping system (Andrews & Andrews, 2017; Gao *et al*., 2004). The adaptation to conditions from where they are isolated may have potential for commercialization in the localities (Thilakarathna & Raizada, 2017).

For indigenous rhizobia to be more effective in soil the threshold level of population is supposed to be 100 cell g^{-1} of soil (Argaw, 2012; Ballard *et al*., 2004), at this level they shows no significant difference in nodulation and nitrogen fixation between the inoculated and un-inoculated plants (Singleton & Tavares, 1986; Thies *et al*., 1991). However, in soils with inefficient population, inoculation with exotic strains is inevitable to improve the legumes performance (Argaw & Tsigie, 2015; Namkeleja *et al.*, 2016; Ndusha *et al.*, 2017). Several studies conducted in Africa have identified African soils to be dominated by *Bradyrhizobium* species; this can be associated with unique soil and climatic conditions (Jaiswal & Dakora, 2019). In enhancing better understanding of populations and effectiveness of indigenous rhizobia, it is important to study more on the factors which might affect nodulation and nitrogen fixation. Likewise, it is crucial to recognize the ecology and characteristics of the native rhizobia in Tanzania. This understanding may lead to bioprospecting of indigenous candidate strains that promote sustainability.

2.4 Indigenous rhizobia nodulating *Lablab* (*Lablab purpureus*)

Lablab in among the nitrogen fixing crop that enhance soil fertility (Ewansiha *et al.,* 2016). Both indigenous and inoculant strains of rhizobia can form effective nodules with *Lablab*

(Massawe et al., 2017). Cross inoculation test with root nodules rhizobia isolated from tropical legumes *Lablab, Leucaena, Mimosa, Sesbania* and *Acacia* reported that, *Lablab* effectively nodulated with fast growing isolates of its own, and it was the first fast growing rhizobia reported to nodulate *Lablab* (Trinick, 1980). This fast-growing rhizobia which nodulate *Lablab* is called *Sinorhizobium (Ensifer) fredii* strain NGR234 (Chang *et al.*, 2011a). A study in southern China collected isolates from root nodules of *Lablab*, identified several genomic species of rhizobia that belong to *Bradyrhizobium, Rhizobium, Sinorhizobium (Ensifer)* and *Mesorhizobium* (Chang *et al*., 2011a). While, the phylogenetic position in basis of 16S rRNA gene of indigenous rhizobia isolated from *Lablab* in Algeria indicated them to belong in *Bradyrhizobium, Rhizobium* and *Mesorhizobium* genera (Benselama *et al*., 2018).

Several novel *Bradyrhizobium* spp. have been isolated from *Lablab* root nodules in different areas (Chang *et al*., 2011b; Grönemeyer *et al.*, 2017). For instance, in Namibia *Bradyrhizobium namibiense* sp. nov. strain 5-10^{T} has been identified (Grönemeyer *et al*., 2017) and in China *Bradyrhizobium lablabi* sp. nov. strain CCBUA 23086^{T}) (Chang *et al*., 2011b). These novel isolates were reported to possess several characteristics which distinguished them from other named species of genus *Bradyrhizobium* (Chang *et al.*, 2011b; Grönemeyer *et al.*, 2017). Other novel species isolated from different crops capable of forming effective nodules with *Lablab* are *Bradyrhizobium guangdongense* and *Bradyrhizobium guangxiense* from root nodules of *Arachis hypogaea* (Li *et al*., 2015). Also, an evaluation of *Lablab* inoculation with *Bradyrhizobium japonicum* also showed great performance in plant growth and yield (Benselama *et al*., 2014). Hence, these findings all suggests *Lablab* to be highly associated with genus *Bradyrhizobium* and by other genera like *Rhizobium, Sinorhizobium* and *Mesorhizobium*. In view of the above results, it is important to explore the availability of effective rhizobia of *Lablab* in our areas where the crop is grown as a way of identifying new genera and species that can be used for inoculation to promote *Lablab* production.

2.5 Characterization of indigenous rhizobia

One of the major challenge in improving nitrogen fixation is the identification and selection of elite rhizobia strains in their natural environment (Moawad & Bohlool, 1992). The isolated rhizobia display diverse characteristics, they differ with geographic location (Grönemeyer *et al*., 2014; Issa *et al*., 2018), climate difference (Saeki, 2011), and nature of seeds from which they nodulated (Puozaa *et al.*, 2017; Issa *et al.*, 2018). Identification and characterization of

indigenous rhizobia contributes to the understanding of their distribution and ecology (Grönemeyer *et al.*, 2014).

Rhizobial strains can be recognized by a combination of a large number of morphological, biochemical and physiological traits (Drew *et al*., 2011; Hamza & Alebejo, 2017). Serological characteristics are also used (Moawad & Bohlool, 1992), to determine existing taxonomic groups (Sadowsky *et al*., 1987), and relatedness of strains (Ahmad *et al*., 1984). Molecular characterization of rhizobia is a useful tool, it is used widely to determine the classification groups of rhizobia (Agrawal *et al*., 2012; Vinay & Kiran, 2013). The method has provided a successful way in identifying related strains, bringing new genera, species and regrouping of rhizobia (Howieson & Dilworth, 2016). Therefore, it is vital to characterize the indigenous rhizobia population in Tanzania, as it identifies the groups and habit of the rhizobia which suits in the given environment for proper strain selection. The knowledge of their characteristics gives a way toward identifying taxonomic relationship and diversity.

2.6 Morphological characterization

The rhizobia isolates can be distinguished by characteristic of the colony or of the cells (Naz *et al*., 2009). For example, Al-Mujahidy *et al*., (2013), observed 43 different morphologies in 260 colonies of rhizobia those were isolated from different 13 soil samples. Growth type and colony size can differentiate rhizobia, for instance, the fast-growing rhizobia will develop colonies in 2-5 days while for slow growing takes 5-10 days (Woomer *et al*., 2011). Another characteristic of the colonies can either be in shape forms like circular, oval or irregular, in elevation form for instance convex/dome, conical or flat or in and margins/edges such as entire/regular or irregular (Ngakou *et al*., 2009). With these characteristics, Ngakou *et al*. (2009) isolated rhizobia from root nodules of cowpea, soybean, groundnut and bambara groundnut and identified most rhizobia colonies having convex elevation. Rhizobia can also be distinguished by shape of cells which can either be spiral, bacteroid, rod, coccus, chain of cocci or cluster of cocci shaped (Somasegaran & Hoben, 1985). The difference in cell shape was revealed by a study of Laurette *et al*. (2015) who observed 12 isolates of bambara groundnuts, and found have rod and cocci cells.

By using opacity and colour, the colonies can be grouped as opaque or translucent with creamy, creamy-white, creamy-yellow white, or milky-white colorations. Some studies like that of Nushair *et al.* (2017), isolated cowpea rhizobia and observed the opaque colonies. While

Hamza and Alebejo (2017) had 120 isolates from rhizosphere, cowpea, elephant and *Lablab* plants the colour for most colonies was creamy-white. The surface of colonies can be rough, smooth, glistening or dull and their viscosity are gummy/mucoid, moist or firm (dry) (International Center for Tropical Agriculture [CIAT], 1988), for instance Sobti *et al*. (2015) found most of the isolates from alfalfa roots nodules had glistening surface and gummy viscosity. Observing this variation among isolates simplify understanding the diversity among isolates under study and tells the possible traits that can be used to distinguish different groups of rhizobia depending on the soil, host plant and climate.

2.7 Biochemical characterization

The biochemical characteristics are also used in characterizing rhizobia, some common characterization methods include growing them in an indicator media which are Yeast Extract Mannitol Agar (YEMA) with either Congo red (CR) or bromthymol blue (BTB) (Olsen *et al*., 1996; Woomer *et al*., 2011). These media behave differently, whereby most rhizobia incubated in darkness do not absorb Congo red (Legesse & Assefa, 2014). But, some may absorb depending on genera of the isolate, time of exposure to light at incubation or the concentration of CR (Howieson & Dilworth, 2016). YEMA plates containing BTB are green, the alkaline producing rhizobia (slow growers) turns the dye blue or not changing colour, while the acid producing (fast growers) turns the media yellow (Datta *et al*., 2015). Some studies such as that of Legesse and Assefa (2014), successfully characterized isolates from faba beans and found them to be of fast growing as they turned BTB to yellow.

Observing growth response in different modified media, such as utilization of different sugars as sole source of carbon including mannitol, sucrose, maltose, fructose, glucose, glycerol and Glucose-Peptone Agar (GPA) is also used in the characterization (Nushair *et al*., 2017). This can show difference in utilization ability even within the same species, as it was found in four derivatives of *Rhizobium japonicum*-110 (designated L1-110, L2-110, I-110 and S-110), were exhibiting different growth rates in different sugar sources such as fructose and glucose (Kuykendall & Elkan, 1976). Also, rhizobia do not grow or can grow poorly on Glucose-Peptone Agar (GPA) (CIAT, 1988; Somasegaran & Hoben, 1985). For example, Pervin *et al*. (2017) studied the ability of rhizobia isolated from *Lablab* to grow into glucose-peptone agar and observed all isolates poorly grow on that media. This growth responses can either be recorder as positive or negative depending on whether growth occurs or not (if grown in agar media) (Nahar *et al*., 2017). Alternatively, rhizobia population in these modified media can be

quantified using the optical density (OD) methods in spectrophotometer (if grown in broth media) (Laurette *et al*., 2015; Nushair *et al*., 2017). Hence, studying rhizobia in media containing different indicators becomes an important and simple method of identifying rhizobia traits. Moreover, growing them in different sources of sugar is important as it gives way of understanding the best media for culturing and multiplication of the isolates.

2.8 Physiological characterization

Different levels of temperature, pH and salt concentration can also be used as a tool for characterization of rhizobia (Fornasero *et al*., 2014; Legesse & Assefa, 2014). Whereby, cultured rhizobia are incubated at different levels of test condition and are observed whether growth occurs (+) or not (-), or quantified by spectrophotometer (Legesse & Assefa, 2014). Results can also be recorded in scores by numbers (0-5) (Fornasero *et al*., 2014) or by stars (*-***) depending on the size of growth (Kucuk *et al*., 2006). Using this approach some studies like Maatallah *et al*. (2002), found optimum growth of chick pea rhizobia at 20-30 °C, while, growth was reduced to 7% and 32% at 45 °C and 5 °C respectively. It was observed that media pH 6-7 provided sufficient growth, but, more than 50% failed to grow at pH below 5, also less than 40% isolates were able to grow at 4% salt concentration.

The combination of the cultural, morphological and biochemical characteristics is used in clustering analysis of rhizobia and producing the dendrogram to show estimated clusters at certain similarity level as was done by Wei *et al*. (2003) and Laurette *et a*l. (2015). Therefore, it is important to characterize indigenous rhizobia by morphological, physiological and biochemical characteristics for understanding rhizobia responses to different conditions and enabling the selection of high-quality strains for successful inoculation use. Moreover, it raises a chance of developing appropriate approaches for culturing, storage and application for ensuring productivity.

2.9 Molecular characterization (16S rDNA)

Molecular techniques are more reliable for evaluating genetic diversity, verification and surveying population of strains (Wilson, 1995). The 16S rDNA as a template DNA for 16S rRNA which is a constituent of 30S small subunit of ribosome has been used widely to characterize rhizobia (Poretsky *et al*., 2014). The 16S rDNA is about 1500 bp long (Clarridge, 2004), of which their sequences are genus or species specific (Jenkins *et al*., 2012). It is considered as the best for characterization studies because it has conserved (slowly-evolving)

and variable (fast-evolving) regions (Johansen *et al*., 2017), also, it is widely represented in sequence databases and has low horizontal gene transfer (Sambo *et al*., 2018). The 16S rDNA is having a long sequence which is enough for informatics purposes (Janda & Abbott, 2007; Suardana, 2014). The gene plays important roles in microbiomes phylogeny and taxonomy for identification of relationship, assessing diversity and quantification of their population (Acinas *et al*., 2004).

The 16S rDNA sequencing and phylogenetic analysis have substantial contribution to describing the novel rhizobia genera and species (Menna *et al*., 2006). For example, Gao *et al*. (2004), used sequencing and phylogenetic to study 95 rhizobia strains from *Astragalus adsurgens* and managed to identify two novel species *Mesorhizobium septentrionale* sp. nov and *Mesorhizobium temperatum* sp. nov. This methods is also used to identify species or strains those are nodulating a certain legume, for example, nine *Rhizobium* species were identified in 20 native isolates from root nodules of common beans (*Phaseolus vulgaris*) in Ecuadoran soils (Torres-Gutiérrez *et al*., 2017). The Restriction Fragments Length Polymorphism (RFLP) analysis for 16S rDNA are also used for rhizobia characterization (Rejili *et al*., 2009). For example, Rejili *et al*., 2009, analysed the RFLP of 83 isolates from root nodules of three *Lotus* spp. and found the isolated were related to *Sinorhizobium*, *Rhizobium*, and *Mesorhizobium* genera. Therefore, it is important to successfully characterize isolates from different legumes by applying molecular techniques in order to recognize their actual genetic groups and diversity, hence it will serve the purpose of inoculants production, selection and use.

2.10 Effectiveness of indigenous rhizobia

Rhizobia Effectiveness is defined as the relative ability of a given rhizobia strain to infect a legume and cause nodulation in the presence of other strains and their symbiotic ability to fix nitrogen more than others (Raposeiras *et al*., 2006). Recently, researches have based on understanding the ecological systems of plant-rhizobia interaction for maximizing nitrogen fixation (Moawad & Bohlool, 1992). There are several methods which are used to determine effectiveness of N fixation by rhizobia in legumes; these include, the determination of Total Nitrogen content (Bala & Giller, 2001), Dry matter yield (Benselama *et al*., 2013), Nodules observation (number and dry weight) (Sharma *et al*., 2013), Acetylene reduction assay, Xylem-solute technique (Hardarson & Danso, 1993), Chlorophyll content of leaves (Opabode & Akinyemiju, 2007) and ^{15}N isotopic methodologies (Peoples *et al*., 1989).

In order to improve legumes nodulation and their nitrogen fixation, the most useful way is to isolate indigenous rhizobia from legume species in question or wild legumes and use them as inoculants (Mwangi *et al*., 2011). These indigenous rhizobia can be well adapted to local environment condition such as prolonged drought, salinity and high temperature, and may have relatively high effectiveness in nitrogen fixation to improve legumes productivity (Onyango *et al*., 2015). Hundreds of native rhizobia have been tested for symbiotic efficiencies in different areas. In some areas, they have outperformed commercial stains introduced by inoculation (Chemining *et al*., 2012). For example, evaluation of symbiotic efficiency of indigenous rhizobia in common beans conducted by Kawaka *et al.* (2014) in Kenya identified indigenous rhizobia which were more efficient by 74% to 170% than commercial strain CIAT 899. Having measured the effectiveness of indigenous rhizobia, the macro or micro-symbionts as well as agronomic factors can be manipulated to maximize BNF (Hardarson & Danso, 1993). Hence, it is advisable to identify the most effective indigenous rhizobia to specific plants for proper strain selection for a particular location to ensure high effectiveness and improved crop production.

2.11 Effectiveness of rhizobia nodulating *Lablab* (*Lablab purpureus*)

Researches on the effectiveness of indigenous rhizobia have been done widely in various legume crops (Yadav *et al*., 2011). In *Lablab* the indigenous rhizobia from Savana Nigeria has justified the effectiveness in resulting high fixed N, nodules formation and dry biomass (Ojo & Dare, 2018). In another study conducted in Dhaka Bangladesh, the indigenous rhizobia isolated from *Lablab* root nodules showed optimistic effects in nitrogen fixation and plant growth, which were higher compared to non-indigenous rhizobia (Yshita, 2017). Therefore, for developing the most effective rhizobia strains to inoculate legume species, the effectiveness testing of indigenous rhizobia for different legumes is inevitable, and can bring more other strains that can be used to inoculate and improve productivity of one or more legume species.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Soil samples collection

The soil samples (4 Kg each) were collected from 15 villages in Babati district in Manyara region in Northern Tanzania (4.2078° S, 35.7461° E). Field selection was based on farms where *Lablab* was cultivated for at least three consecutive seasons. By using a spade and hand hoe, soils were taken at 0-20 cm depth from five different locations in the field to make a composite sample, that were then it was and packed into plastic bags. Plastic bags with soil samples were labeled and transferred to Nelson Mandela African Institution of Science and Technology (NM-AIST) for testing.

3.2 Soil characterization

The soil samples were submitted to Tanzania Coffee Research Institute (TaCRI) laboratory in Moshi for analysis of chemical properties. Standard methods were used to determine soil pH by 1:2.5 soil:water using pH meter, total nitrogen content of soils was determined by Kjeldahl method. Available P in soils was determined using Bray-1 method. While organic matter of the soils and exchangeable cations includes potassium ions (K^+), calcium ions (Ca^{2+}), magnesium ions (Mg^{2+}) and sodium ions (Na^+) were also analyzed as described by Anderson and Ingram (1993).

3.3 Rhizobia trapping

Each soil sample was divided into two pots that were used to grow *Lablab* in glasshouse for rhizobia trapping. *Lablab* seed variety Eldoret Black 2, acquired from Tanzania Agricultural Research Institution (TARI) - Selian, Arusha was used. At early flowering which was approximately 50 days after planting, the plants were harvested from pots and observed for active nodules (with reddish color) in their root. Active nodules were detached from roots and kept in small plastic bags that were store in deep freezer at -80 °C while waiting for isolation and characterization of rhizobia.

3.4 Media preparation

Rhizobia needs aseptic condition and suitable media to support their growth, a common and generally accepted media is Yeast Extract Mannitol Agar (YEMA) with composition as presented in Table 1 (Vincent, 1970). The pH of YEMA was adjusted to 6.8 ± 0.2 and autoclaved at 121 °C for 15 minutes and left to cool to 50 °C before it was poured in sterile petri dishes to solidify (Somasegaran & Hoben, 1985).

Table 1: Composition of YEMA in 1 L distilled water

Composition	Gram/L
Mannitol	10
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast Extract Powder	0.5
Agar Agar Bacteriological	15

3.5 Rhizobia isolation from root nodules

Rhizobia isolation was performed according to methods as described by Woomer *et al*. (2011). Firstly, the nodules from freezer were transferred in falcon tubes with sterile distilled water and kept overnight at 4 °C for imbibition. The following day nodules were surface sterilized by immersing in 95% ethanol for 10 seconds, transferred to 3.5% sodium hypochlorite for 2 minutes and serial rinsed in 6 changed sterile distilled water. In sterile petri dish with a drop of sterile distilled water, 4-6 nodules were crushed by sterile forceps. The loopful of suspension was streaked by sterile inoculation loop into petri dishes containing sterile YEMA. The cultures were incubated at room temperature for 4-7 days, with daily observation. Sub-culturing of a single, typical rhizobia colonies was done to get pure cultures.

3.6 Presumptive tests for cultures purity

Well isolated and typical rhizobia colony from each culture were further sub-cultured and streaked using a sterile inoculation loop into fresh YEMA to develop a secondary culture. The presumptive test for culture purity of the same was performed according to CIAT (1988), by streaking the secondary culture into YEMA with Congo red (CR) 25 ppm and into Glucose Peptone Agar (GPA) media under the same conditions to observe CR absorption and to growth

response in GPA. The isolates that failed to grow on GPA and failed to absorb CR were assumed to be pure rhizobia culture (Woomer *et al*., 2011).

3.7 Agar slant formation

The agar slants of YEMA were formed in 50 mL sterile falcon tubes. The slants were used to grow portion of the pure culture of which after 4-7 days of growth were stored in freezer as a reference and for future uses.

3.8 Characterization of rhizobia isolates

3.8.1 Morphological characterization

After 4-7 days of incubation pure cultures morphological characteristics of the colonies were observed. The observation made included growth type, colony form, elevation, margins, colony color, opacity, surface appearance, texture and colony size. The shape of cells and their motility was also observed under microscope as described by Howieson and Dilworth (2016).

3.8.2 Biochemical characterization

The Catalase activity test was done by spreading drops of hydrogen peroxide 30% into a petri dishes with isolates and observed for oxygen bubbles formation (Hamza & Alebejo, 2017). The other biochemical characteristics included was the response of the isolates in YEMA with CR.

3.8.3 Physiological characterization

The ability of the isolates to utilize different sugars as a sole source of carbon was tested, the sugars used were Sucrose and Maltose which substituted for mannitol in YEMA. The isolates were also streaked in selective media GPA to observe for their growth response as explained by Somasegaran and Hoben (1985). Different media pH levels 4.5, 5.5, 8.5 and 9.5 (Bernal & Graham 2001), were used to characterize the pH tolerance of isolates. The pH was adjusted by 1M NaOH and concentrated HCl. Salt tolerance was tested in YEMA with 1%, 2%, 3% and 4% NaCl (w/v) concentration (Legesse, 2016). Isolates' ability to grow under raised temperature was tested at 35 °C, 40 °C and 45 °C (Legesse, 2016). The growth responses in different physiological tests were scored as -: no growth, ⁎: weak growth, ⁎⁎: medium growth, and ⁎⁎⁎: Large growth) (Datta *et al*., 2015; Kucuk *et al*., 2006).

3.8.4 DNA extraction and 16S rDNA amplification

The equal quantity of pure colonies of the isolated rhizobia was used for DNA extraction. The full genome DNA were extracted and purified by RNAse water using the protocol developed by Mahuku (2004). The clean genomic DNA was PCR amplified for 16S rDNA by forward primer (fD1) (For-5'AGAGTTTGATCCTGGCTCAG3') and reverse primer (rD1) (Rev-5'AAGGAGGTGATCCAGCC3') as explained by Weisburg et al. (1991). These primers can amplify nearly full length of 16S rDNA of most of eubacteria and are widely used (Laguerre *et al*., 1994). The PCR reaction was carried in 25 µL reaction volume composed of 2 µL DNA (at least 50 ng/mL), 0.5 µL each primer fD1 and rD1, 9.5 µL Nuclease free water and 12.5 µL One *Taq* Quick Load Master mix. A *BIO-RAD* Thermal cycler was used for PCR reaction with temperature profile as 3 min 95 °C, 35x (1 min 94 °C, 1 min 55 °C, 2 min 72 °C and 3 min 72 °C) for initial denaturation, final denaturation, annealing, initial extension and final extension respectively (Laguerre *et al*., 1994), lid temperature 100 °C and resting at 4 °C. The PCR product 5 µL mixed with a loading dye purple and 5 µL of 1 kb DNA ladder for estimation of band size were loaded into wells of an agarose gel 1%. A gel was stained with 0.05 ppm Ethidium bromide in TBE buffer to facilitate visualization of bands pattern upon illumination with UV light. The DNA bands were separated by horizontal electrophoresis which was running at 80 Voltage for 60 min. The bands separation was visualized under UV and pictured for result presentation. The bands were observed for their possible size of base pair by comparing them to band size ruler provided by primer manufacturer.

3.9 Rhizobial isolates symbiotic effectiveness test

3.9.1 Rhizobia cells multiplication

The 60 mL Yeast Mannitol Broth (YMB) (Table 2) prepared as per Vincent (1970) was poured into 250 mL capacity conical flasks and autoclaved at 121 °C for 15 min. An equal amount of each pure rhizobia colony was transferred to YMB using sterile inoculation loop. A loopful of commercial rhizobia strains were also multiplied in broth. The broth cultures were incubated in the rotary shaker to facilitate aeration at 120 rpm, 28 °C for 5 days. Broth culture were standardized to about $1x10^8$ cells/mL (Olsen *et al*., 1996).

Table 2: Composition of YMB in 1 L distilled water

Composition	Gram/L
Mannitol	10
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast Extract Powder	0.5

3.9.2 Soil and seed preparation

Loamy soils from area with pH 7±2, no water logging history and none saline soil were collected, autoclaved and allowed to cool aseptically before use (Woomer *et al*., 2011). The cool soils were filled in surface sterilized pots of 2 L capacity. The clean and undamaged *Lablab* seeds were surface sterilized in 95% ethanol, then in 3.5% (v/v) Sodium hypochlorite and 5 times serially washed by sterile distilled water (Somasegaran & Hoben 1985). The sterile seeds were kept in a beaker with sterile distilled water and incubated in refrigerator to imbibe for 4 hours before planting (Woomer *et al*., 2011).

3.9.3 Planting and treatment application

Three (3) *Lablab* seeds were aseptically placed into each pot with sterile soil using forceps. Then, 10 mL of standardized $1x10^8$ cell/mL broth cultures of rhizobia isolates and positive control commercial strains Biofix, Legumefix beans and Legumefix soy were inoculated as described by Somasegaran and Hoben (1985). Other control treatments were pots with nitrogen and without nitrogen, they didn't receive rhizobia. Each treatment had 4 replicates, which were blocked by a complete randomized block design (CRBD). After germination the plants were thinned to 2, the remaining were left to grow for six weeks, then harvested for determination of rhizobia effectiveness.

3.9.4 Plant nutrients application

All pots were supplied with N-free nutrients solution (Table 3) at day of planting and three weeks after planting. In 10 L of sterile distilled water, 5 mL of each stock solution were added to form N-free nutrient solution (Broughton & Dilworth, 1971) which was used for supplying nutrients in potted soils. For nutrient with N, 0.05% (w/v) KNO_3 was included, that made 70 ppm N.

Table 3: The stock solutions of N-free nutrient solution

Stock solutions	Element	Formula	Gram/L	Molarity
1	Ca	$CaCl_2.2H_2O$	294.1	2
2	P	KH_2PO_4	136.1	1
3	Fe	Fe-Citrate	6.7	0.02
	Mg	$MgSO_4.7H_2O$	123.3	0.5
	K	K_2SO_4	87	0.5
	Mn	$MnSO_4.H_2O$	0.338	0.002
4	B	H_3BO_3	0.247	0.004
	Z	$ZnSO_4.7H_2O$	0.288	0.001
	Cu	$CuSO_4.5H_2O$	0.1	0.0004
	Co	$CoSO_4.7H_2O$	0.056	0.0002
	Mo	$Na_2MoO_2.2H_2O$	0.048	0.0002

3.10 Data collection

3.10.1 Plant height and stem girth

Two days before the seven weeks reached the plant height (cm) were measured using a tape measure. Also, a digital Vernier caliper was used to measure stem girth of each plant (mm).

3.10.2 Number of leave

The number of leaves were counted in each plant any leaf appeared to be formed in a plant was counted and recorded (leaves/plant).

3.10.3 Colour of leaves

Plant leaves colour was scored by looking colour of the *Lablab* leaves and gave the ranking in scale of 1 to 5 whereby 1 = chlorotic leaves, 2 = pale green, 3 = green, 4 = light green and 5 = dark green healthy leaves as done by Simon *et al*. (2014).

3.10.4 Chlorophyll extraction and determination

The chlorophyll content of leaves was determined by a method developed by Hiscox and Israelstam (1979). As small pieces of a green leaf tissue 100 mg were immersed in 15 mL capacity falcon tubes with 7 mL Dimethyl Sulphoxide (DMSO). The mixtures were kept to

extract chlorophyll at 8 °C for 3 days. The extract volume was topped to 10 mL by adding 3 mL DMSO. From each tube, the 3 mL of extract was pipetted and transferred to empty clean Eppendorf tubes. A fresh DMSO was used for calibration of a spectrophotometer. Then optical density (OD) was measured in UV spectrophotometer at 645 nm and 663 nm wavelength. The equation which was suggested by Arnon (1949), was used to calculate total chlorophyll content by using the OD values from spectrophotometer.

$$\textbf{Total chlorophyll (mg/L)} = 0.0202D_{645} + 0.00802D_{663}$$

Whereby D is the optical density measured in spectrophotometer

3.10.5 Number of active nodules

After seven weeks, the plants were harvested from potted soils. The plants were cut at section separating roots and shoots. The roots were washed in running tap water to remove the remaining soil. The active root nodules were detached and counted (nodules/plant). Nodules were carefully enclosed in small paper envelopes and properly labeled for oven dry.

3.10.6 Plant material dry weights

Shoots, roots and nodules were collected in labeled paper envelopes. Then the plant materials were oven dried at 70 ^{o}C for 3 days to attain a constant weight. The dry materials were weighed by a digital weighing balance in milligrams for nodules and in grams for shoot and roots dry weights.

3.10.7 Relative symbiotic effectiveness

Relative symbiotic effectiveness of isolates (SEi) was calculated through a shoot dry weight of inoculated plants x100 and dividing to dry weight of N fed plants as described by Somasegaran and Hoben (1985). The equations provided below was used for calculation.

$$\boldsymbol{SEi = \frac{Xi}{Xn} x\ 100}$$

For SEi, whereby *i*, and *n* =dry weight of inoculated plants and dry weight of N fed plants, respectively.

The SEi was classified as ineffective <35%; less-effective, 35 to 50%; moderately effective, 50 to 80%; and highly effective, >80% (Legesse & Assefa, 2014).

3.11 Data Analysis

The characteristics off rhizobia isolates were tabulated in excel to show differences. Data were binary coded using 1 for presence and 0 for absence of a studied traits for use in clustering of the isolates with respect to their characteristics. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used for clustering in PAST software V 3.25 (Hammer *et al.*, 2001), using Dice similarity coefficient. The symbiotic effectiveness collected plant data; plant height, stem girth, number of leaves, chlorophyll content, number of nodules, nodules dry weight and shoot dry weight were subjected to Shapiro Wilk's W to test for normal distribution. The normal distributed data were analyzed by using one-way analysis of variance [ANOVA], Fisher's least significance [LSD] was used to compare means among the treatments at $p = 0.05$ level of significance. STATISTICA version 10 software (StatSoft, Inc, 2010) was used to perform normal distribution test and ANOVA.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Soil chemical properties

Soil analysis results (Table 4) showed that most of soils have pH which was less than 7, at the same time 73% were above 6.0 which is critical level. The total nitrogen content of soils was very low (<0.1%) in all soil samples with the highest value of 0.07% in comparison with the critical level of 0.25%. The available P (ppm) was less than 20 ppm critical values, with 40% of the soils had moderate ranges, while, 60% had a low content. The exchangeable cations results (me/100 g Soil) for K^+, Mg^{2+} and Ca^{2+} of the studied soils were higher than their critical levels (0.22, 2 and 4 meq/100g respectively), while for Na^+ the values were within critical value range that is less than 1 me/100 g Soil. Furthermore, 93% of the soils were less in organic matter content, only one soil sample was having organic matter higher than 4 mg/kg a critical level.

Table 4: Chemical properties of the soil samples collected from different villages

Village	pH (H_2O) 1:2.5	Total N (%)	Av. P (ppm)	Exchangeable cation (meq/100g Soil)				OM (mg/kg)
				K^+	Ca^{2+}	Mg^{2+}	Na^+	
Sarame	4.58	0.04	11.74	0.33	1.61	2.85	0.58	0.41
Moya	5.30	0.03	14.82	0.39	1.23	1.54	0.30	0.38
Maweni	6.30	0.05	14.16	0.43	1.98	2.14	0.50	0.27
Nakwa	6.18	0.05	15.73	1.35	10.90	8.80	0.33	0.31
Manyara	6.67	0.04	16.65	1.08	9.08	3.32	0.28	2.45
Chemchemu	6.64	0.04	16.07	1.45	14.97	12.08	0.50	2.10
Mwikantsi	6.71	0.07	15.95	0.98	4.52	4.19	0.07	2.11
Riroda	6.54	0.06	14.71	0.36	6.36	3.60	0.40	0.88
Mbuyuni	7.35	0.06	19.08	0.42	30.02	2.70	0.50	0.82
Mawemairo	6.40	0.03	14.24	0.52	2.25	2.94	0.21	0.43
Mafuta	6.94	0.07	13.38	1.28	15.34	8.18	0.39	4.84
Arri	5.98	0.04	12.17	0.93	6.58	6.26	0.15	1.03
Magara	5.11	0.04	11.53	0.50	1.49	3.54	0.10	1.02
Singe	6.02	0.05	14.87	0.46	4.75	3.57	0.08	0.89
Endakiso	6.30	0.05	15.84	0.50	4.66	5.73	0.05	1.63
Critical level	**6.0**	**0.25**	**20**	**0.22**	**4**	**2**	**<1**	**4**

N=Nitrogen, Av.P=Availbale Phosphorus, K=Potassium, Ca=Calcium, Mg=Magnesium, Na=Sodium and OM=Organic Matter

4.2 Morphological characteristics

Twenty-two (22) out of thirty (30) pots with soil samples used to grow *Lablab* were found to have roots with active nodules. As shown in Table 5, there was variation in isolates growth and colonies appearance. After 4-7 days of incubation, isolates showed different colony sizes. Some isolates (BR2, BR5 BR6, BR9, BR10 and BR22) appeared to have small colonies even after 7 days and were regarded as slow growers. The rest of isolates forming large colonies after 4 days, were regarded as fast growers. The elevation of colonies was convex, flat and conical. The colonies formed regular and few irregular margins. The colonies were circular and oval in shape, with creamy, creamy-white and milky-white coloration. All colonies had smooth surfaces and dull or glistening appearance was observed. Mucus production for rhizobia isolates BR2, BR4, BR6, BR18 and BR22 was high, and was moderate to other isolates. Cells of the isolates appeared to have rod and coccus shapes, but all were motile.

4.3 Biochemical characteristics

Only one isolate (BR16) absorbed the color and formed red colonies when grown in Congo red and incubated in darkness, while the rest were white-pink. The bubbles were also formed in catalase test for all isolates, when the drops of 30% hydrogen peroxide (H_2O_2) were added to petri dishes with rhizobia culture (Fig. 1A).

4.4 Physiological and stress tolerance characteristics

The results for physiological characteristics and tolerance to pH, temperature and salt are presented in Table 6. All isolates were able to grow well in mannitol, but there was reduced growth in maltose to 41% (large), 41% (medium), 18% (weak) and in sucrose to 14% (large), 41% (medium) and 45% (weak) (Fig. 2A) when used as a sole source of carbon. There was a noted reduced growth of all isolates in acidic media than in alkaline media. At 5.5 pH there was some large growth (14%), medium growth (40%) and weak growth (46%), while at 4.5 pH no large growth was observed and recorded high weak growth (55%) and no growth (23%) (Fig. 2B). Growth was not affected in 1% NaCl, but was highly reduced at higher salt concentrations 3 and 4% NaCl (w/v) as shown in Fig. 1B and Fig. 2C. Few isolates like BR2, BR3, BR9, BR10, BR18 and BR20 were able to grow up to 4% NaCl. Temperature also had influence in rhizobia, the growth was decreased with increase in temperature. But there were some isolates that were able to grow up to 45˚C with large growth (13%), medium growth

(23%), weak growth (41%) and no growth (23%) (Fig. 2D). Isolates BR4, BR10, BR13, BR15 and BR21 were highly susceptible to high temperature.

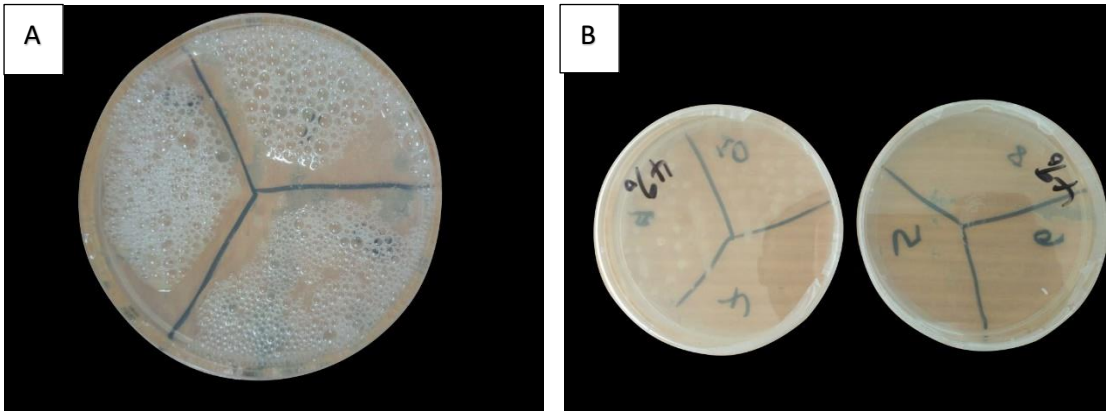
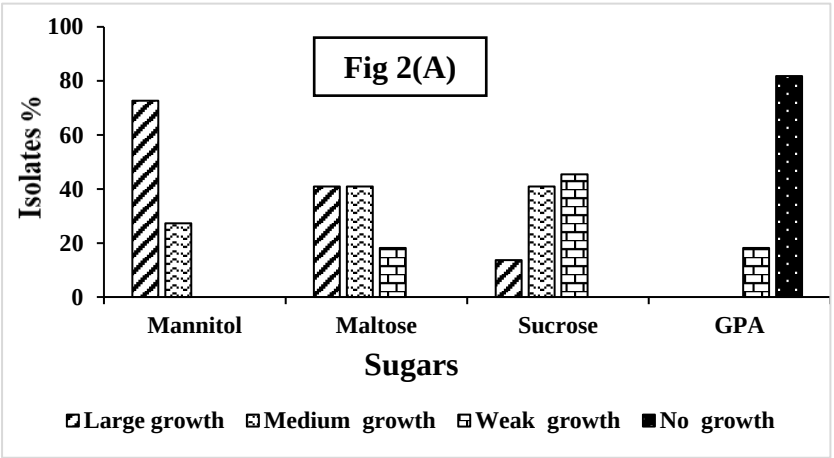
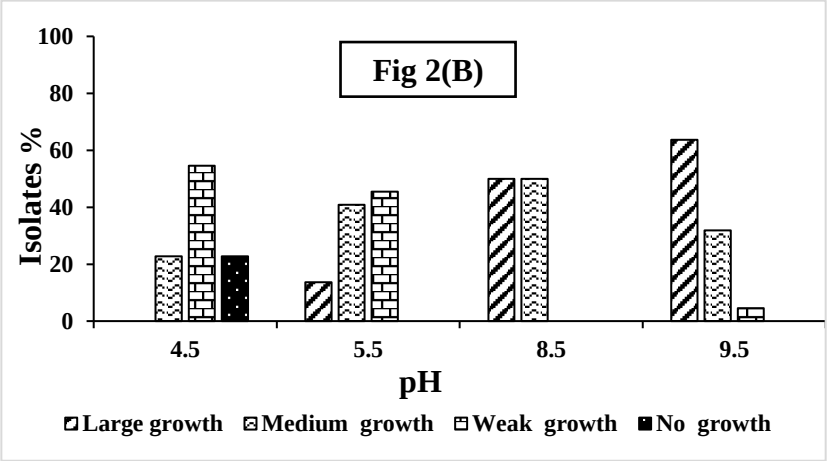
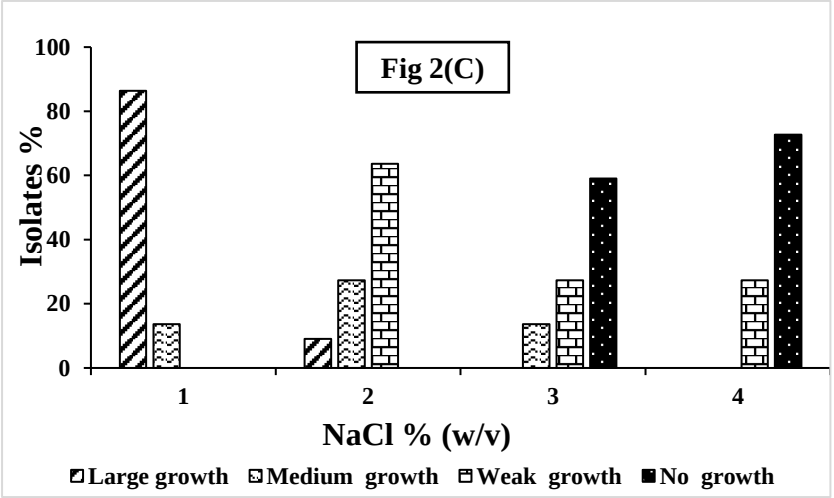


Figure 1: Response of rhizobia isolates to (A) 30% peroxide drops and (B) in YEMA with 4% NaCl







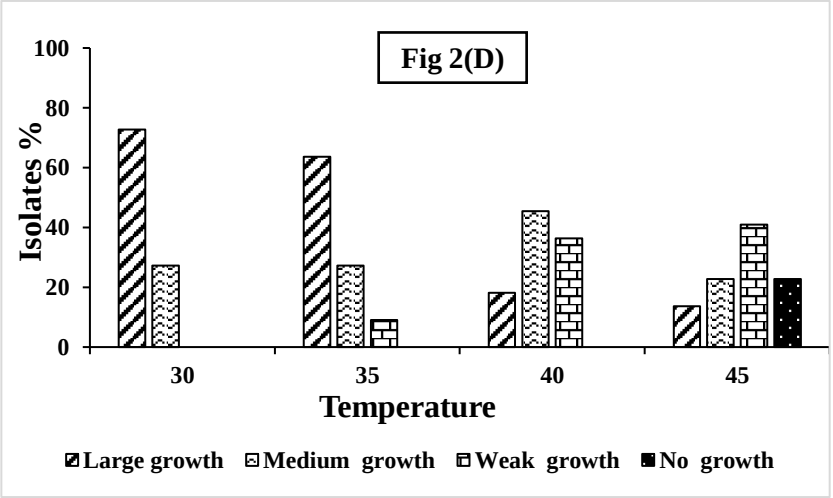


Figure 2: The percentage of rhizobia isolates as responded to media with different carbon sources 3(A), pH levels 4(B), NaCl concentration (w/v) 5(C) and Temperature 6(D)

Table 5: Morphological and Biochemical characteristics of rhizobia isolated from root nodules of *Lablab*

Isolate	Village name	Morphological Characteristics											Biochemical	
		Form	Elevation	Margin	Colour	Surface	Appearance	Opacity	Viscosity	Diam (mm)	Cell Shape	Motility	Congo Red	Catalase Activity
BR1	Maweni	Circular	Convex	Irregular	Creamy	Smooth	Glistening	Translucent	Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR2	Maweni	Circular	Flat	Regular	Creamy-white	Smooth	Glistening	Translucent	Highly mucoid	≤4	Rod	Motile	N.Abs	Positive
BR3	Chemchemu	Circular	Convex	Regular	Creamy	Smooth	Glistening	Translucent	Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR4	Chemchemu	Oval	Convex	Irregular	Creamy	Smooth	Dull	Opaque	Highly Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR5	Nakwa	Circular	Convex	Regular	Milky-white	Smooth	Dull	Translucent	Mucoid	≤4	Coccus	Motile	N.Abs	Positive
BR6	Moya	Circular	Convex	Regular	Milky-white	Smooth	Glistening	Opaque	Moist	≤4	Rod	Motile	N.Abs	Positive
BR7	Riroda	Oval	Flat	Regular	Creamy-white	Smooth	Dull	Opaque	Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR8	Mwada	Circular	Convex	Regular	Creamy-white	Smooth	Glistening	Opaque	Mucoid	≥4	Coccus	Motile	N.Abs	Positive
BR9	Endakiso	Circular	Flat	Irregular	Creamy-white	Smooth	Glistening	Translucent	Mucoid	≤4	Coccus	Motile	N.Abs	Positive
BR10	Endakiso	Circular	Flat	Regular	Creamy-white	Smooth	Glistening	Opaque	Mucoid	≤4	Rod	Motile	N.Abs	Positive
BR11	Mwikantsi	Oval	Convex	Regular	Milky-white	Smooth	Glistening	Translucent	Mucoid	≥4	Coccus	Motile	N.Abs	Positive
BR12	Mwikantsi	Circular	Convex	Irregular	Creamy-white	Smooth	Dull	Translucent	Mucoid	≥4	Coccus	Motile	N.Abs	Positive
BR13	Mbuyuni	Circular	Convex	Regular	Creamy-white	Smooth	Dull	Translucent	Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR14	Arri	Circular	Flat	Regular	Creamy-white	Smooth	Glistening	Translucent	Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR15	Arri	Circular	Conical	Irregular	Creamy-white	Smooth	Dull	Translucent	Mucoid	≥4	Coccus	Motile	N.Abs	Positive
BR16	Manyara	Circular	Flat	Regular	Milky-white	Smooth	Glistening	Opaque	Highly Mucoid	≥4	Rod	Motile	Abs	Positive
BR17	Sarame	Circular	Convex	Regular	Creamy-white	Smooth	Glistening	Translucent	Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR18	Singe	Circular	Convex	Regular	Milky-white	Smooth	Dull	Opaque	Highly Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR19	Singe	Oval	Convex	Regular	Creamy-white	Smooth	Dull	Opaque	Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR20	Matufa	Circular	Convex	Regular	Milky-white	Smooth	Glistening	Translucent	Moist	≥4	Rod	Motile	N.Abs	Positive
BR21	Mawemairo	Oval	Convex	Irregular	Creamy	Smooth	Glistening	Opaque	Mucoid	≥4	Rod	Motile	N.Abs	Positive
BR22	Mawemairo	Circular	Convex	Regular	Milky-white	Smooth	Glistening	Opaque	Highly Mucoid	≤4	Coccus	Motile	N.Abs	Positive

Note: BR=Babati Rhizobia, Diam=Colony diameter, N. Abs=Not absorbed and Abs=Absorbed

Table 6: Growth responses of rhizobia isolated from root nodules of *Lablab* to different physiological stress condition

Isolates	Village name	Growth Media				Physiological Stress Condition											
						pH				NaCl Conc. (w/v)				Temperature ˚C			
		Mann	Malt	Sucr	GPA	4.5	5.5	8.5	9.5	1%	2%	3%	4%	30	35	40	45
BR1	Maweni	***	***	**	-	*	**	***	***	***	**	*	-	***	**	*	*
BR2	Maweni	**	***	***	-	**	***	***	***	***	**	**	*	**	***	**	**
BR3	Chemchemu	***	***	***	-	*	***	***	***	***	**	*	*	***	***	***	***
BR4	Chemchemu	***	**	*	-	*	*	**	**	***	*	-	-	***	*	*	-
BR5	Nakwa	**	**	**	-	*	*	**	***	***	*	-	-	**	**	**	*
BR6	Moya	**	**	*	*	-	*	**	***	***	*	-	-	**	***	*	**
BR7	Riroda	***	*	*	-	*	**	**	***	**	*	-	-	***	***	**	*
BR8	Mwada	***	**	**	-	*	*	**	***	**	*	-	-	***	***	**	*
BR9	Endakiso	**	*	**	-	*	**	**	***	***	***	*	*	**	***	***	**
BR10	Endakiso	**	***	**	-	*	*	***	***	***	**	**	*	**	**	*	-
BR11	Mwikantsi	***	**	*	-	**	**	***	***	**	*	-	-	***	**	*	*
BR12	Mwikantsi	***	**	*	-	-	*	***	***	***	*	-	-	***	***	**	**
BR13	Mbuyuni	***	**	*	-	**	*	***	**	***	*	-	-	***	**	*	-
BR14	Arri	***	***	*	-	*	*	**	***	***	*	-	-	***	***	**	*
BR15	Arri	***	*	*	-	-	*	***	**	***	*	-	-	***	***	*	-
BR16	Manyara	***	***	**	-	*	**	***	**	***	**	*	-	***	**	***	**
BR17	Sarame	***	*	**	*	*	**	**	**	***	*	-	-	***	***	**	*
BR18	Singe	***	**	**	-	**	**	**	**	***	**	*	*	***	***	**	***
BR19	Singe	***	***	*	*	-	**	**	*	***	*	-	-	***	***	**	*
BR20	Matufa	***	***	***	-	**	***	***	***	***	***	**	*	***	***	***	***
BR21	Mawemairo	***	***	**	-	-	*	**	**	***	*	*	-	***	*	*	-
BR22	Mawemairo	**	**	*	*	*	**	***	***	***	*	-	-	**	***	**	*

Note: BR (Babati Rhizobia), Mann (Mannitol), Malt (Maltose), Sucr (Sucrose), GPA (Glucose Peptone Agar), - (No growth), * (Weak growth), **(Medium growth) and ***(Large growth)"

4.5 Dendrogram of clustering

The combination of morphological, biochemical and physiological characteristics presented in Table 1 and Table 2, were used to generate dendrogram to show relationship between the isolates. The dendrogram formed 2 groups (A and B) at 55% similarity and there was a total of 7 clusters at 70% similarity level (Fig. 3). All groups composed slow and fast-growing isolates. Group A with total of 8 isolates formed Cluster I and II, also there were 4 unpaired (BR9, BR16, BR1 and BR18) isolates. Group B is composed of 14 isolates, with 5 clusters (III-VII) and one isolate (BR11) was independent. The cluster with higher number of isolates formed in dendrogram was cluster IV with 4 isolates (BR7, BR19, BR14 and BR 17), followed by cluster VII with 3 isolates, all other remaining clusters were having 2 isolates. For group A, the member isolates were all the acidic, salt and high temperature tolerant. All of them were capable to grow in 3% NaCl and 6 out of 8 isolates grew in 4% NaCl. Only one isolate BR21 of group B was able to grow at 3% NaCl.

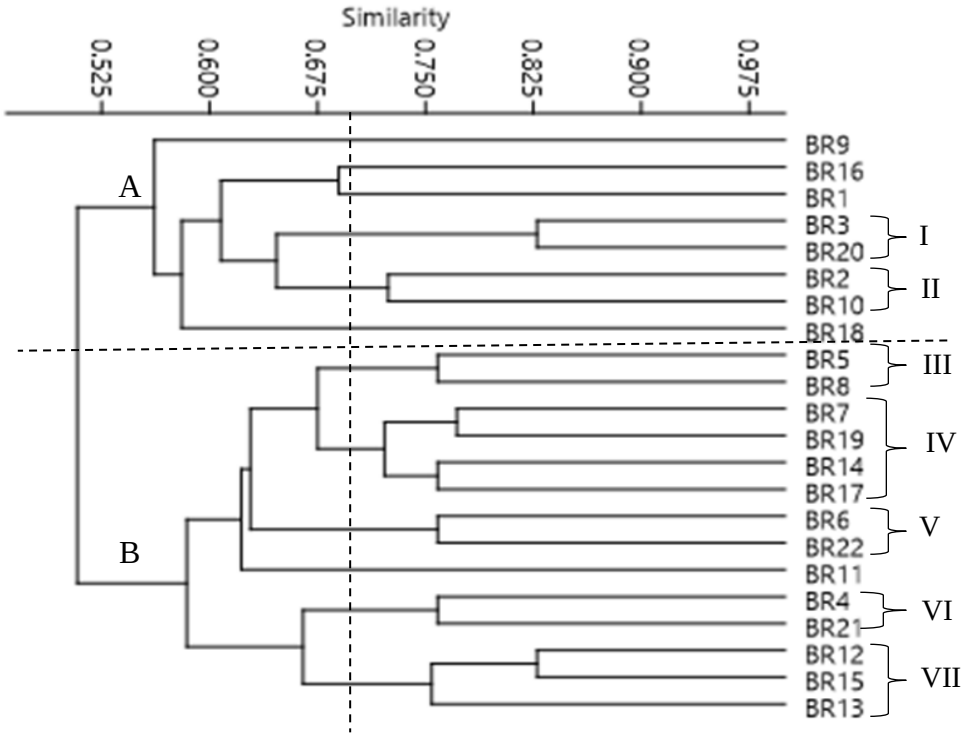


Figure 7: A dendrogram showing similarities between the indigenous rhizobia isolates

4.6 PCR amplification of 16S rDNA

The 16S rDNA of 18 isolates out of 22 were amplified (Fig. 4 showing a single band of nearly expected full length gene (1500 bp). The remaining 4 isolates namely, BR3, BR7, BR11 and BR14 were not amplified.

Figure 8: Amplification of 16S rDNA from sampled isolates

The numbers (1 to 22) represent isolates number and a villages of origin BR1=Maweni, BR 2=Maweni, BR3=Chemchemu, BR4=Chemchemu, BR5=Nakwa, BR6=Moya, BR 7=Riroda, BR8=Mwada, BR9=Endakiso, BR10=Endakiso, BR11=Mwikantsi, BR12=Mwikantsi, BR13=Mbuyuni, BR14=Arri, BR15=Arri, BR16=Manyara, BR17=Sarame, BR18=Singe, BR19=Singe, BR20=Matufa, BR21=Mawemairo and BR22=Mawemairo

4.7 Plant nodulation

All *Lablab* plants inoculated with rhizobia isolates formed nodules. There was significant difference in nodulation capacity between the isolates at $p < 0.001$ (Table 8). The isolate BR15 showed a large nodulation capacity with 40 nodules per plant, followed by BR8 (34 nodules/plant), BR1 (29 nodules/plant), BR14 (28 nodules/plant) and BR11 (27 nodules/plant), while BR2 was the least in nodules number forming only 3 nodules/plant. Among all commercial inoculants used, Biofix (for beans) formed 14 nodules per plant. This was high than other 10 indigenous isolates. There were no nodules in uninoculated and N-fed plants.

4.8 Nodules dry weight

The dry weight of nodules was statistically significant at $p < 0.01$ among the isolates (Table 8), it was highest at 51.83 mg/plant in isolate BR16 and the lowest was 3.88 mg/plant in BR2. The other isolates those performed better were isolates BR1 (43.85 mg/plant), BR8 (37.73 mg/plant), BR11 (34.75 mg/plant) and BR12 (34.73 mg/plant). In different commercial inoculants which were used, biofix beans had nodules with dry weight 21.2 mg/plant, which was higher compared with nodules dry weight of other 10 isolates which were used.

4.9 Shoot dry weight

Shoot dry weights for plants as affected by rhizobia inoculated were differing significantly between treatments at $p < 0.01$. As presented in Table 8, the shoot dry weight ranged from the highest 3.28 g/plant for BR4, followed by 3.1 g/plant (BR3) 3.1 g/plant (BR19), finally 2.88 g/plant (BR8) and 2.85 g/plant (BR16) respectively. The lowest shoot dry weight was observed in BR 22 with 1.7 g/plant. Among the commercial inoculants used, Biofix beans had shoot dry weight of 2.65 g/plant which was higher than other 17 indigenous isolates. The N-fed plants had higher shoot dry weight of about 1.7 g/plant than some other isolates like BR10, BR21, BR6 and BR22. Shoot dry weight under negative control was very low than in any other treatment with 0.83 g/plant.

4.10 Root dry weight

The *Lablab* roots were significantly different at $p < 0.001$ between the treatments (Table 8), as it was much higher in BR17 with 649.15 mg/plant followed by BR20, BR16, BR15 and BR4 with 595.78 mg/plant, 544.6 mg/plant, 466.88 mg/plant and 466.88 mg/plant respectively. The *Lablab* inoculated with commercial inoculants Biofix beans had higher roots dry weight than other strains, and it was higher than in some 13 indigenous isolates. Rhizobia BR21 and BR22 even have lower root dry weight than N-fed plant, while the lowest root dry weight was for negative control with 144.95 mg/plant.

4.11 Plant height

The isolate BR14 was found to have higher plant height of 118.43 cm/plant, followed by isolates BR7 (117.93 cm/plant), BR10 (117.0 cm/plant), BR16 (116.93 cm/plant) and BR8 (112.6 cm/plant) respectively. The plant height of 86.2 cm/plant in isolate BR12 was the lowest among the isolates. *Lablab* inoculated by Legumefix beans commercial inoculants formed 97.38 cm/plant, which was exceeding other commercial inoculants and other indigenous isolates like BR18, BR9, BR1, BR2, BR15 and BR12. The negative control was the least in plant height with 44.68 cm/plant, N fed plant with 86.83 cm/plant which was less than most of the isolates and higher that BR12 only. The results were significant different at $p < 0.001$.

4.12 Chlorophyll content

The chlorophyll content of the leaves was statistically different between the treatments at $p < 0.001$. The highest chlorophyll content was identified in a plant inoculated with commercial inoculant Biofix beans 0.0232 mgChl/L which was also followed by the indigenous rhizobia BR1 (0.0231 mgChl/L), BR3 (0.0215 mgChl/L) and BR4 (0.0208 mgChl/L). N fed plant was found to have high chlorophyll (0.0163 mgChl/L) than 10 different isolates. An isolate that caused a low chlorophyll content was BR20 (0.0131 mgChl/L), which was even less than of the N fed, and less than all commercial inoculants. The negative control was having the lowest chlorophyll content with 0.01102 mgChl/L.

4.13 Colour of plant leaves, stem girth and number of leaves

The colour of *Lablab* leaves was statistically different at $p < 0.001$ (Table 7), whereas leaves were greener in BR4, BR9 and BR19 all with 5 units. The plants treated with Biofix beans, Legumefix beans and N were having 4.25 units of green color that was more than some of the isolates like BR13, BR14, BR16, BR2, BR17, BR18 and BR6. The negative control was almost going to yellowish as it scored 1.5. Stem girth was not significantly different at $p < 0.05$, all plants were found to be nearly equal in their girth between the treatments. The number of leaves per plant were significant different at $p < 0.001$ between the treatment, with biofix beans forming more plant leaves (26 leaves/plant) than other rhizobia at early flowering. The isolate BR6 was having few leaves (14 leaves/plant) and close to negative control (11 leaves/plant) (Fig. 5).

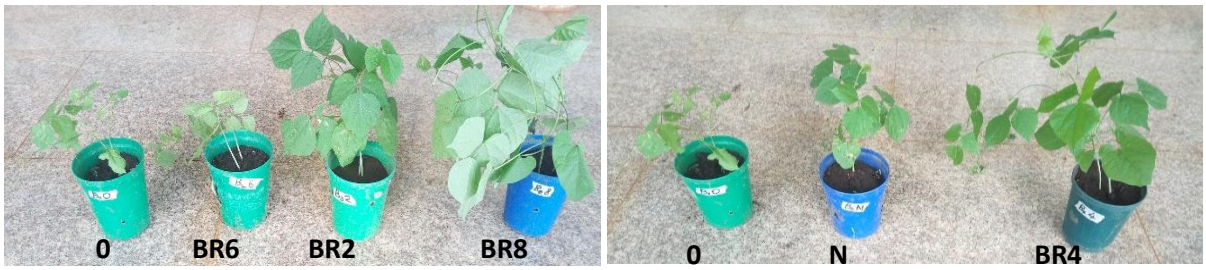


Figure 9: Influence of rhizobia inoculation in plant growth (0=No nitrogen or rhizobia inoculated, N=Nitrogen fed, BR=Babati Rhizobia)

Table 7: Influence of rhizobia inoculation on plant height, number of leaves, stem girth, colour of leaves and chlorophyll content

Isolates	Village name	Parameters/(Mean±SE)				
		Plant height (cm)	No. of leaves/plant	Stem girth (mm)	Colour of leaves	Chlorophyll (mgChlg/L)
Neg control		44.68 ± 2.73f	11.50 ± 1.32h	1.83 ± 0.13d	1.50 ± 0.29g	0.0110 ± 0.0000j
N fed		86.83 ± 4.13e	16.50 ± 0.87efgh	2.67 ± 0.09abc	4.25 ± 0.48abcd	0.0163 ± 0.0007defghi
Legfix Soy		96.35 ± 6.01abcde	18.00 ± 0.71defg	2.47 ± 0.11bc	3.75 ± 0.25cde	0.0132 ± 0.0008ij
Legfix Beans		97.38 ± 11.07abcde	19.50 ± 1.66cdef	2.50 ± 0.11bc	4.25 ± 0.48abcd	0.0205 ± 0.0006abcde
Biofix Beans		86.38 ± 7.32e	26.25 ± 1.25a	2.64 ± 0.11abc	4.25 ± 0.48abcd	0.0232 ± 0.0018a
BR1	Maweni	94.13 ± 7.66bcde	20.25 ± 1.25cdef	2.88 ± 0.28ab	4.25 ± 0.48abcd	0.0232 ± 0.0014ab
BR2	Maweni	89.98 ± 11.76cde	20.75 ± 2.56bcdef	2.57 ± 0.24abc	3.75 ± 0.48cde	0.0181 ± 0.0002cdefgh
BR3	Chemchemu	108.35 ± 6.96abcde	23.00 ± 2.45abcd	2.83 ± 0.07ab	4.75 ± 0.25ab	0.0215 ± 0.0026abc
BR4	Chemchemu	112.13 ± 10.68abc	24.50 ± 2.6abc	2.84 ± 0.14ab	5.00 ± 0.00a	0.0208 ± 0.0028abcd
BR5	Nakwa	102.6 ± 11.82abcde	21.5 ± 2.87abcde	2.62 ± 0.17abc	4.25 ± 0.48abcd	0.0159 ± 0.0013efghi
BR6	Moya	100.48 ± 8.9abcde	14.00 ± 1.22gh	2.58 ± 0.38abc	2.75 ± 0.48f	0.0163 ± 0.0031defghi
BR7	Riroda	117.93 ± 11.28a	25.75 ± 3.25ab	2.76 ± 0.19ab	4.25 ± 0.48abcd	0.0141 ± 0.0002ghij
BR8	Mwada	112.6 ± 6.57abc	22.75 ± 1.25abcd	2.82 ± 0.11ab	4.75 ± 0.25ab	0.0165 ± 0.0009defghi
BR9	Endakiso	94.4 ± 8.42bcde	21.50 ± 0.87abcde	2.81 ± 0.16ab	5.00 ± 0.00a	0.0168 ± 0.0003defghi
BR10	Endakiso	117 ± 3.89ab	23.25 ± 2.56abcd	2.49 ± 0.15bc	4.50 ± 0.29abc	0.0174 ± 0.0018cdefghi
BR11	Mwikantsi	110.33 ± 4.79abcd	20.00 ± 2.74cdef	2.63 ± 0.17abc	4.25 ± 0.25abcd	0.0165 ± 0.0013defghi
BR12	Mwikantsi	86.2 ± 6.62e	22.00 ± 1.41abcd	2.66 ± 0.25abc	4.50 ± 0.29abc	0.0159 ± 0.0011efghi
BR13	Mbuyuni	111.75 ± 3.66abc	22.25 ± 0.75abcd	2.44 ± 0.26bc	4.00 ± 0.41bcde	0.0159 ± 0.0011efghi
BR14	Arri	118.43 ± 9.51a	19.75 ± 1.44cdef	2.50 ± 1.10bc	4.00 ± 0.00bcde	0.0163 ± 0.0017defghi
BR15	Arri	88.03 ± 9.23de	23.00 ± 1.22abcd	2.14 ± 0.14cd	4.50 ± 0.29abc	0.0189 ± 0.0035abcdef
BR16	Manyara	116.93 ± 11.19ab	22.50 ± 2.18abcd	2.61 ± 0.26abc	4.00 ± 0.00bcde	0.0188 ± 0.0037abcdefg
BR17	Sarame	111.28 ± 3.73abc	18.75 ± 1.89defg	3.09 ± 0.23a	3.50 ± 0.50def	0.0144 ± 0.0009fghij
BR18	Singe	95.83 ± 6.18abcde	16.00 ± 0.58fgh	2.72 ± 0.18ab	3.25 ± 0.25ef	0.0151 ± 0.0005fghij
BR19	Singe	103.15 ± 1.67abcde	19.75 ± 2.1cdef	2.68 ± 0.18ab	5.00 ± 0.00a	0.0135 ± 0.0004hij
BR20	Matufa	98.3 ± 6.7abcde	18.50 ± 1.55defg	2.67 ± 0.12abc	4.50 ± 0.29abc	0.0131 ± 0.0003ij
BR21	Mawemairo	98.5 ± 14.88abcde	20.50 ± 2.78bcdef	2.51 ± 0.31bc	4.50 ± 0.29abc	0.0178 ± 0.0009cdefghi
BR22	Mawemairo	116 ± 7.56ab	24.25 ± 0.95abc	2.55 ± 0.02bc	4.50 ± 0.29abc	0.0185 ± 0.0019bcdefg
	F Statistics	**3.52*** **	**3.299*** **	**1.55**	**4.718** **	**3.242*** **

Values with dissimilar letter(s) in a column are significantly different by Fisher LSD at P=0.05

Table 8: Influence of rhizobia inoculation on number of nodules, root dry weight, shoot dry weight and nodule dry weight

Isolates	Village name	Parameters/(Mean±SE)			
		Nodules/Plant	Root dry weight (mg)	Shoot dry weight (g)	Nodule dry weight (mg/plant)
Neg control		0.00 ± 0.00i	144.95 ± 13.90g	0.83 ± 0.11g	0.00 ± 0.00f
N fed		0.00 ± 0.00i	214.05 ± 54.09efg	1.70 ± 0.32defg	0.00 ± 0.00f
Legfix Soy		10.00 ± 4.76efghi	227.85 ± 56.36efg	1.78 ± 0.47cdefg	11.23 ± 5.64def
Legfix Beans		2.75 ± 0.75hi	215.93 ± 49.77efg	1.50 ± 0.23efg	1.15 ± 1.02f
Biofix Beans		14.50 ± 3.33cdefghi	352.98 ± 102.99cdefg	2.65 ± 0.38abcd	21.20. ± 5.02bcdef
BR1	Maweni	29.75 ± 7.33abc	412.50 ± 88.36bcde	2.43 ± 0.08abcdef	43.85 ± 20.75ab
BR2	Maweni	3.50 ± 0.65ghi	303.30 ± 90.27defg	1.88 ± 0.50cdefg	3.88 ± 1.86ef
BR3	Chemchemu	13.75 ± 4.85cdefghi	381.65 ± 42.43cdef	3.10 ± 0.39ab	18.98 ± 5.13cdef
BR4	Chemchemu	16.50 ± 8.05cdefghi	466.63 ± 89.49abcd	3.28 ± 0.05a	21.88 ± 7.71bcdef
BR5	Nakwa	12.25 ± 5.51defghi	252.45 ± 52.18efg	2.18 ± 0.41abcdef	17.75 ± 4.35cdef
BR6	Moya	4.75 ± 1.31ghi	252.78 ± 86.78efg	1.43 ± 0.19efg	11.48 ± 3.25def
BR7	Riroda	14.50 ± 6.03cdefghi	264.78 ± 54.67defg	2.55 ± 0.88abcde	18.73 ± 4.80cdef
BR8	Mwada	34.50 ± 16.52ab	325.58 ± 59.95defg	2.88 ± 0.34abc	37.73 ± 16.27abc
BR9	Endakiso	9.00 ± 2.94efghi	418.98 ± 107.59bcde	2.18 ± 0.14abcdef	17.723 ± 6.92cdef
BR10	Endakiso	10.00 ± 3.54efghi	243.63 ± 48.27efg	1.65 ± 0.49defg	16.85 ± 4.95cdef
BR11	Mwikantsi	27.50 ± 10.78abcd	261.63 ± 65.76defg	2.45 ± 0.67abcdef	34.75 ± 11.37abcd
BR12	Mwikantsi	22.25 ± 3.82bcdef	279.43 ± 74.59defg	2.38 ± 0.64abcdef	34.73 ± 13.16abcd
BR13	Mbuyuni	19.25 ± 4.61bcdefgh	269.85 ± 48.48defg	2.03 ± 0.35bcdef	28.45 ± 9.26abcd
BR14	Arri	28.50 ± 10.47abcd	287.25 ± 27.90defg	2.03 ± 0.34bcdef	31.33 ± 9.89abcd
BR15	Arri	40.25 ± 9.52a	466.88 ± 56.92abcd	2.13 ± 0.28bcdef	33.83 ± 14.38abcd
BR16	Manyara	26 ± 4.74abcde	544.60 ± 132.44abc	2.85 ± 0.42abc	51.83 ± 10.79a
BR17	Sarame	20 ± 3.63bcdefg	649.15 ± 169.27a	2.23 ± 0.23abcdef	32.78 ± 6.00abcd
BR18	Singe	17.50 ± 5.19bcdefgh	306.13 ± 88.75defg	2.05 ± 0.16bcdef	26.45 ± 8.16bcde
BR19	Singe	12.25 ± 4.05defghi	415.90 ± 66.01bcde	3.10 ± 0.50ab	14.23 ± 5.75cdef
BR20	Matufa	23.00 ± 5.35bcde	595.78 ± 41.84ab	1.90 ± 0.37cdefg	26.70 ± 7.08bcde
BR21	Mawemairo	12.25 ± 3.25defghi	165.70 ± 32.22g	1.58 ± 0.31defg	18.68 ± 4.80cdef
BR22	Mawemairo	5.75 ± 0.48fghi	170.68 ± 32.93fg	1.33 ± 0.48fg	11.25 ± 1.21def
	F Statistics	**2.95*** **	**2.975*** **	**2.155** **	**2.399** **

Values with dissimilar letter(s) in a column are significant by Fisher LSD at P=0.05

4.14 Relative symbiotic effectiveness

Relative symbiotic effectiveness of the rhizobia which were inoculated in *Lablab* was calculated by using the shoot dry weight of the harvested plants. There was observed differences in their relative effectiveness (Fig. 6), one isolate BR4 recorded the highest relative effectiveness 193%, followed by BR3 and BR19 (182%), BR8 (169%) and BR16 (168%) while the lowest was found in isolate BR22 (78). The total of 17 indigenous isolates were found to be highly effective as they made a relative symbiotic effectiveness of above 100%, and the 5 remaining isolates were effective by the 70-100% relative symbiotic effectiveness. Among the commercial inoculants, biofix beans was the best by 153% relative effective than some other isolates.

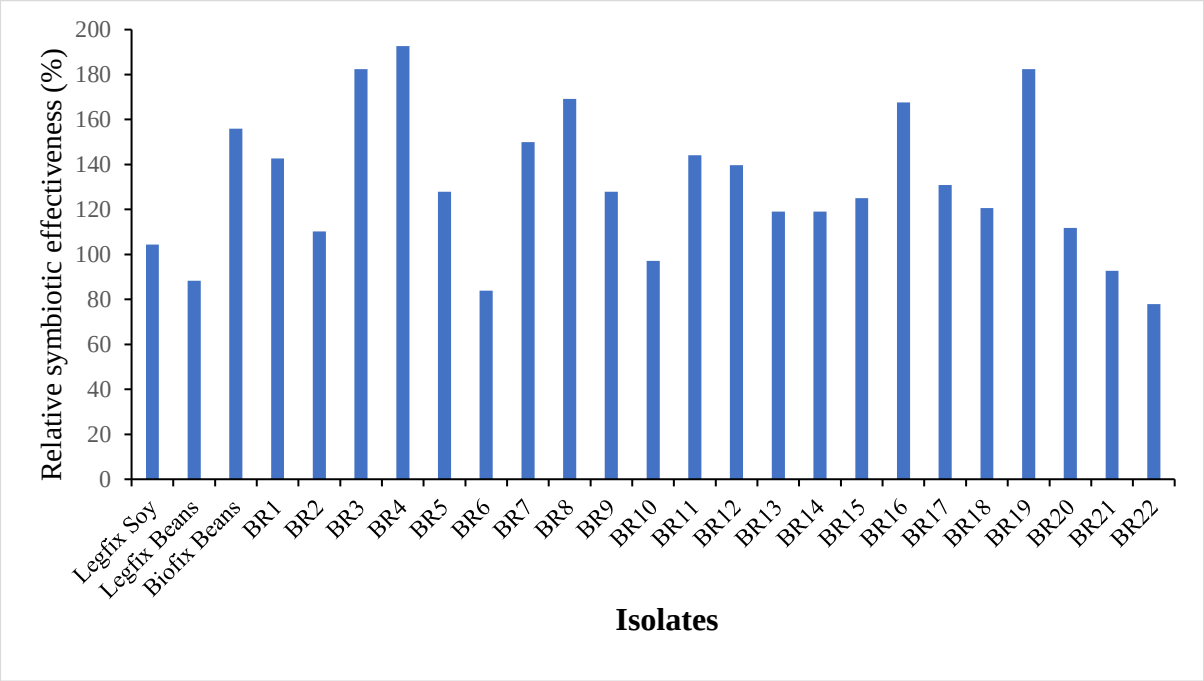


Figure 10: Relative symbiotic effectiveness commercial and indigenous rhizobia used for inoculation (BR stands for Babati Rhizobia)

4.15 Discussion

This study reports on characteristics and symbiotic effectiveness of 22 rhizobia isolates of *Lablab purpureus* from villages of Babati district, Tanzania. The analysis for chemical properties of the tested soils which were used to test for nodulation of *Lablab puperleus*, showed to have low N and P, while, their pH was slightly acidic. In most of the soil their pH values ranged between 5.5 to 7.0, this range is considered ideal for agriculture purposes, but

also can also support growth of rhizobia and successful nodulation in legumes (Argaw, 2012; Hadad, 1984; Onyango *et al*., 2015). The process such as leaching of N and P adsorption due to the low pH could influence low level of these nutrients (Mowo *et al*., 2006). Despite the deficiencies in total N and available P, the soils had other good properties which can support plants growth. The finding of this work concurs to those of (Makoi, 2016; Meya *et al*., 2020; Mowo *et al*., 2006; Ndakidemi & Semoka, 2006), that most of the soils in northern Tanzania have low N and P to maintain adequate soil fertility and support plant growth although the exchangeable cations K^+, Ca^{2+}, Mg^{2+} and Na^+ were adequate within the established critical levels. Critical level of the nutrients is needed for improving plant performance, but will also improve rhizobia survival and effectiveness (Gentili *et al.,* 2006; Míguez-Montero *et al.,* 2019; Robson *et al.*, 1981; Valverde *et al*., 2002), as sufficient P stimulates nodulation through its essential roles in plants and as source of energy (Gentili and Huss-Danell, 2002; Ssali and Keya, 1983), while excessive N will depress ability of rhizobia forming effective nodules and N fixation (Gentili & Huss-Danell, 2003; Tewari, 1965). Other factors such as shifting and over cultivation with little or no input supply also contributes in nutrients depression (Nyoki & Ndakidemi, 2016; Seresinhe & Pathirana, 2002). Efforts are needed to improve nutrients and other soil properties for ensuring sustainable use of available soils for agricultural purposes. To meet this demand, practice for improving soil nutrients are needed which may include application of manure and recommended synthetic fertilizers. Additionally, culturing and identifying the elite strains of indigenous rhizobia which can be used for legume seed inoculation, will be a necessary step to improve N fixation so that to add N in the soils.

The studied isolates showed the characteristics of fast (73%) and slow (27%) growing rhizobia. The result informs that *Lablab* can be nodulated by either of them. This concurs with finding by Benselama *et al.* (2013). The dominance of fast-growing over slow growing rhizobia was contrary to Jaiswal and Dakora (2019) who reported that slow growing rhizobia (*Bradyrhizobium*) were dominant in some African soils. The different on dominance in rhizobia can be caused by soil properties from where the rhizobia are native (Grönemeyer *et al*., 2014; Issa *et al*., 2018), soil management practices and seeds they nodulate (Puozaa *et al*., 2017; Issa *et al.*, 2018). As it has been reported that *Lablab* can be nodulated by different rhizobia species including *Rhizobium* spp. which are fast growing rhizobia (Benselama *et al*., 2018). The variation in viscosity of colonies as high and moderate mucoid among the rhizobia isolated from the same plant species were like those observed by Teixeira *et al.* (2010). This feature is important in rhizobia as it helps in adapting to stress conditions and it is vital for nodulation capacity (Ondieki *et al.*, 2017).

It is widely documented that rhizobia do not absorb congo red, although, some few rhizobia can still absorb the dye, hence, it can be used as one of distinctive feature (Somasegaran & Hoben, 1994). In our study, only one isolate (BR16) absorbed congo red, the observations align to that of Kneen and Larue (1983) on *R. trifolii* and *R. melilot* to absorb congo red. So, BR16 could be related to them hence, further investigation is recommended on this isolate to identify its group. All 22 isolates formed bubbles when drops of 30% hydrogen peroxide (H_2O_2) were added on plates. This showed the positive catalase activity as the enzyme hydrolyzes hydrogen peroxide (H_2O_2) to water and oxygen gas (O_2) (Wadhwa *et al.*, 2017; Elzanaty *et al*., 2015). This is an important feature as it enables rhizobia to prevent from this harmful forms of oxygen (Elzanaty *et al*., 2015). Mannitol was the best source among other sugars used, and it has been used widely as standard sugar for rhizobia culturing. This was also recommended by Vincent (1970) as a standard media and source of carbon for rhizobia cultures. The insufficient growth of rhizobia in the maltose and sucrose (disaccharides) can be due to the inability of disaccharides uptake by some rhizobia (Dekak *et al*., 2018). Similar findings were reported by Naz *et al.* (2009) and Patel and Dubey (2014).

Growth of the isolates was hampered in acidic than in alkaline media. Acidic conditions have been reported to affect growth of rhizobia, and the optimum growth is found at pH 6.5-7 (Laurette *et al.*, 2015). Our findings aligns those reported by Dias *et al*. (2019) who found acidity negatively affected growth while alkalinity was not a problem. More studies are needed on isolates which were able to grow in pH below 5, as they can have high contribution to improving nitrogen fixation for legumes in low pH soils.

All rhizobia isolates had large growth at 1% NaCl, while at 2% reduced growth started to occur. The large growth in low salt concentration was caused by less salt effects in media and physiology of rhizobia. More reduced growth was observed in 3% and 4% NaCl, as 59% and 73% respectively of the isolates did not grew (Figure 3). Increased salt concentration in growth media could have affected rhizobia growth through the mechanisms related to toxicity and osmotic stress (Zahran, 1999). Despite the toxicity in higher salt concentration, some few slow growing (BR2, BR9 and BR10) and fast-growing (BR3, BR18 and BR20) isolates tolerated and grew at 4% NaCl and these were considered as salt tolerant isolates. Similar findings for *Rhizobium* and *Bradyrhizobium* growing at 4% NaCl was reported by Datta *et al*. (2015), Kucuk *et al.* (2006) and Laurette *et al.* (2015). They described tolerance to high NaCl concentration as an important feature for rhizobia competitiveness in high salt rhizosphere as

it helps in the survival and nodulation ability in the host plant. Further studies are recommended on isolates which are tolerant to higher salt concentration in order to develop inoculants with salt tolerance that can be used in high saline soils.

Temperature has effect on survival, growth and on physiological activities of rhizobia (Simon *et al*., 2014). In a current study, optimal temperature for the isolates' growth was observed to be 30 °C. The same optimum temperature was also reported by Benselama *et al.* (2013) in studies involving *Lablab* rhizobia. Isolates BR3, BR18 and BR20 were more temperature tolerant than others, growing vigorously up to 45 °C. These results are encouraging because such isolates once used can support successive nodulation and nitrogen fixation, hence improving the final plant growth in high soils regions.

In a dendrogram all groups composed combination of slow and fast-growing isolates. The largest cluster formed was cluster IV with 4 isolates, followed by cluster VII with 3 isolates, and all other remaining clusters were having 2 isolates. Being at different groups and clusters for the fast and slow growing isolates, it implies that they could show some similarities in morphological, biochemical and physiological traits. All isolates of group A were able to grow in acidic (pH 4.5) media, and in high NaCl with 3% salt concentration. Also, isolates in group A showed tolerance against high temperature and acidic media by forming large colonies. Additionally, the isolates in group A were found to have ability to grow vigorously in maltose and sucrose sugars. This was different in group B isolates which showed susceptibility to higher salt concentration by 3% NaCl. Under this concentration only one isolate which is BR21 was able to grow in good condition. Moreover, their tolerance to acidity was highly reduced at pH 5.5, while, some failed to grow at pH 4.5. Their ability to utilize maltose and sucrose was less compared to group A, they were dominated by medium to small growths. Physiological characteristics showed to have greatly contributed to grouping and clustering of the isolates into respective groups and clusters. The grouping and the differences between the two groups was related to the study by Maatallah *et al*. (2002) who found physiological characteristics relating to adaptation such as acidity and salinity can be used to group rhizobia.

Isolates from same origin were placed in different clusters, while isolates from different origins were clustered together. Several studies such as Rai *et al.* (2012) found similar diversity in rhizobia from same origin not clustered together, which reflects a wide diversity among isolates from same area. Also, rhizobia in a geographic area presents high diversity as it has been reported that different rhizobia can be found in the same root nodule (Denison & Kiers, 2011).

Hence, the diversity of rhizobia that has been identified is an indicator that rhizobia are very diverse and more exploration is needed to identify the best candidate isolates which can suit our farming system and improve sustainability.

The PCR amplification of 16S rDNA from different isolates by primer tells the presence of rhizobia spp. those can nodulate *Lablab* in Tanzania. In this study, PCR products in agarose gel formed a single band at similar band size about 1500 bp as expected for 16S rDNA of most rhizobia spp., although some isolates did not show amplification. Primers fD1 and rD1 are designed for most eubacteria (Weisburg *et al*., 1991) as they amplify nearly a full length rDNA for most bacteria genera (Rejili *et al*., 2009). Therefore, failure in amplification to some isolates can be due to presence of the 16S rDNA which cannot be amplified by present oligonucleotides (Damanka *et al.*, 2019). The differences in 16S rDNA can be caused by gene multiple copies in some bacterial genomes that make it prone to genetic recombination and horizontal gene transfer (Aserse *et al*., 2012). From this study, it is recognized that rhizobia which can effectively nodulate *Lablab* are present in Babati district, Tanzania, since most of them were able to be amplified by primers. Sequencing of 16S rDNA from the isolates of the same area is needed for further characterizations and identification of rhizobia groups which are present and can be used as potential biofertilizers in improving *Lablab* production.

The isolates of indigenous rhizobia have shown high contribution to different plant parameters which were measured to determine symbiotic effectiveness. All parameters showed significant difference between treatments except for stem girth, which indicate that girth was not directly affected by inoculation treatments or N supply. The number of effective nodules/plant and nodules dry weight (mg) varied between isolates, this showed the influence of different rhizobia to nodulate legumes including *Lablab*, it was corresponding to study of Hardarson and Danso (1993) that different rhizobia has varied ability to nodulate legumes. The variation in other effectiveness parameters such as number of leaves/plants, chlorophyll content (mgChl/g), plant height (cm), color of plant leaves and root dry weight (mg) between different treatments, suggests that these attributes were influenced by rhizobia nitrogen fixation. Accumulation of the fixed nitrogen in plant tissue stimulates plant healthy, growth and chlorophyll content in the leaves (Rajput & Patil, 2017), hence makes plants with higher nitrogen content to have more green leaves and expected to have good photosynthesis capacity than those with less nitrogen as it was observed in leaves of uninoculated plants (Rajput & Patil, 2017). The observations in this study have sufficiently convinced on the ability of the isolated indigenous

rhizobia to improve nitrogen fixation which in turn contributes to total crop production and soil improvement through N incorporation in soil and sufficient organic matter from plant residues.

When compared with N fed plants and commercial inoculants which were used (biofix beans, legumefix beans and legumefix soy) the indigenous rhizobia outperformed both in number of nodules/plant, root dry weight, shoot dry weight and nodules dry weight which are important symbiotic effectiveness parameters, implying that the indigenous isolates had higher N fixing efficiency than the commercial inoculants. The findings concur with studies by Ojo and Dare, (2018), Simon *et al*. (2015) and Yadav *et al.* (2011). Shoot dry weight is used widely as a measure to reflect the symbiotic effectiveness of rhizobia (Somasegaran & Hoben, 1985). This is due to the role of nitrogen in influencing plant growth, dry matter and final yield (Rajput & Patil, 2017; Santos *et al.*, 2018). The differences observed in shoot dry weight represents a different ability of rhizobia to influence nitrogen fixation through symbiotic relationship with their host plants (Thrall *et al.*, 2011; Wielbo *et al.*, 2012). In this study, shoot dry weight was found to be highe in plants inoculated with isolates BR4, BR3, BR19, BR8 and BR16. These isolates outperformed both N fed plant and the commercial inoculants, while, a shoot dry weight for negative control was the lowest. Similarly to this study, Karaca and Uyanöz (2012) reported the same growth differences for *Lablab* inoculated with indigenous rhizobia, commercial inoculants and negative control. The isolates performance has shown an undoubted performance and provide possibility of having strains which can be used for inoculation in crops to improve yield while maintaining soil fertility for ensuring sustainability.

The possible potential of indigenous rhizobia in nitrogen fixation in relation with of N fed plants was observed in relative symbiotic effectiveness assessment. According to Legesse (2016) ratings for relative symbiotic performance, 19 isolates out of 22 were highly effective. They contribute more dry matters in plants up to 193% compared with N fed plants, while, only few isolates ranged in moderate effectiveness. The effective and compatible rhizobia population is required for the symbiotic effectiveness in a host plant to be achieved (Bala & Giller, 2001). With the existence of depleted soils with low N in Tanzania and other parts of sub-Saharan Africa, inoculation with elite indigenous rhizobia can have marvelous output on the legumes production. This will promote sustainable farming practices rather than depending on N fertilizers for plant nutrition.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In this study, I found a high diversity among the rhizobia isolates nodulating *Lablab* through their morphological, biochemical and physiological characteristics. They were morphologically distinct, and all tested positive to catalase activity, while, one isolate absorbed red color of media in YEMA with congo red. Their utilization ability for alternative sources of carbon was different as mannitol was the best source followed by maltose, while, sucrose was less preferred. Different levels of tolerance were observed against stress condition such as high/low pH, salt concentration and temperature above optimum levels. Only 6 isolates were not able to grow in acidic media of pH 4.5, while all isolates were able to grow in alkaline conditions of pH 9.5. All isolates grew at salt concentration of 1% and 2% NaCl (w/v), but for the later there was a reduced growth, while only 6 isolates were tolerant at 4% salt concentration. In a 45 °C temperature, only 13% of the isolates formed large growth. The dendrogram was developed based on the observed characteristics, in which 2 groups and 7 clusters were formed at 55% and 70% similarity level respectively, while 5 isolates did not pair in any cluster. Being indigenous they present important characteristics of rhizobia in Tanzania, there is possibility of having numerous species in Tanzanian soils. They also provide useful information which can be used in selection of isolates that need further characterization to identify their potential and relation to other rhizobia. The PCR products of 16S rDNA showed bands for the 18 isolates, the remaining 4 failed to amplify. The failure to amplify for some isolates could be caused by presences of bacteria having genome with multiple copies which makes them susceptible to genetic recombination and horizontal gene transfer.

All isolates were symbiotically effective, formed active root nodules and their effects on nitrogen fixation were observed by differences in growth parameters which reflected effectiveness of rhizobia isolated. Plants inoculated by indigenous rhizobia showed satisfying performance than those supplied by N, while 5 isolates (BR4, BR3, BR19, BR8 and BR16) were more effective in influencing shoot dry weight than commercial inoculants and any other in this study. The relative symbiotic performance was determined and 96% of the isolates were highly effective with more than 80% of the symbiotic effectiveness when compared with the N fed plants. Up to 193% relative symbiotic effectiveness was recorded in isolate BR4. This

study has demonstrated the presence of indigenous rhizobia in Tanzania which can nodulate *Lablab* more effectively than the tested commercial inoculants. Further testing of the isolates under different field condition is recommended.

5.2 Recommendations

The nitrogen fixation by legume plants has been a very important biological process in agriculture systems as it reduces the need for inorganic fertilizers. The process occurs when the macro symbiont (host plant) is infected by the compatible micro symbionts (rhizobia) and results in active nodules which can covert atmospheric nitrogen into a form which can be readily available for plants uptake. For indigenous rhizobia to be more efficient the threshold population is needed, also, there must be favorable environment. The indigenous rhizobia can be processed into commercial inoculants, but their characteristics vary with location and nature of seeds they nodulate.

Therefore, characterization of indigenous rhizobia associated with *Lablab* from different areas in Tanzania is recommended in order to acquire superlative candidates which can be used as biofertilizers for improving *Lablab* yield. There is a need to further test and validate isolates which can tolerate harsh environmental conditions such as elevated temperatures and higher NaCl concentrations and low pH.

Moreover, symbiotic efficiency is important for selection of the elite rhizobia isolates. More studies are recommended to be performed in different field condition to determine symbiotic ability in such environment changes. For the isolates which showed effectiveness, field trials must be conducted in order to test their competitiveness in the soil to fix nitrogen in the presence of other rhizobia. This will give the evidence on amount of nitrogen that can be fixed by isolates of indigenous rhizobia.

Finally, farmers and agricultural officers in Babati district this can be a useful information in step forward towards improving soil productivity. They need to implement sustainable farming practices which will not affect microbials survival and maintain them to improve their effectiveness.

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RESEARCH OUTPUTS

Output one: Research paper

Malugu, E. D., Venkataramana, P. B. & Ndakidemi P. A. (2020). Morphological, biochemical and physiological characterization of indigenous rhizobia nodulating *Lablab purpureus. International Journal of Biosciences, 16*(3), 662-671.

Output two: Poster presentation.