CHARACTERISATION OF *Bradyrhizobium liaoningense* OF WILD SOYA BEANS (*Glycine soja*) AND DETERMINATION OF ITS INOCULATION EFFECT ON GROWTH, CHLOROPHYLL CONCENTRATION, NODULATION AND YIELD OF *Glycine max*

BY

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DECLARATION

I hereby declare that this thesis is my original work and has not been presented for the award of a degree in any other university or institution. All sources of information have been duly acknowledged in the reference.

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CERTIFICATION

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DEDICATION

This thesis is dedicated to my wife, Violah Sigei, whose love and counsel has been my strength, to our two children: Shirleen Kemunto Jelagat and Malcolm Okora Kipkorir, may they grow to be instruments of societal change. To my beloved parents Mr. Ombati William Gataga and Mrs. Kemunto Mary Gataga, who through their efforts, guidance and care nurtured me into a reliable focused and hard working person, they taught me that even the largest task can be accomplished through one's determination.

ABSTRACT

Soya beans are important staple food crops in western Kenya. Legumes play a key role in agriculture by fixing nitrogen in the root nodules in symbiosis with rhizobia. Currently Kenya is experiencing low yield of soya beans, approximately 0.8 t/ha, due to low soil fertility and high cost of inorganic fertilizers and it is a threat to agricultural productivity. Symbiotic rhizobia can increase yields through biological nitrogen fixation. However, the potential of *Bradyrhizobium liaoningense* in improving yield and productivity of soya beans is not known. Although Rhizobia seem to be widely distributed in the soil, only specific strains of Rhizobia are compatible to specific legumes. There is need to characterize rhizobia species from the wild soya bean to identify effective and compatible rhizobia isolates to host plant to boost nitrogen fixation in the soil hence promote growth and yield of soya beans. Rhizobia bacteria are known to improve growth and yield of several other crops but the effect of inoculating soya beans with *B. liaoningense* on growth, chlorophyll concentration, nodulation and yields has not been determined. This study aimed at characterization of *B. liaoningense* bacteria from wild soya beans and determining its inoculation effect on growth, chlorophyll content, nodulation and yields of soya beans (*Glycine soja*). Sterile root nodules of *B. liaoningense* obtained from *G. soja* plants were crushed with pestle and mortar separately by adding small aliquots of sterile water. The purified isolates were inoculated in petri plates containing sterile yeast mannitol agar medium with congo red. Inoculated plates were incubated at 29± 2 °C for 7 days. Pure cultures of the isolates were obtained through re-streaking then the rhizobium characterized morphologically and biochemically. Nine isolates were obtained by preparing pure cultures. Growth tests were carried out in the greenhouse using 5 litres plastic pots of height 25 cm and 23 cm diameter. Pots were each filled with 7kg of top soil. The seeds were treated as: un-inoculated (control), $1.07x10^7$ cfu/ml, $1.19x10^7$ cfu/ml, $1.31x10^7$ cfu/ml and $2.67x10^7$ cfu/ml inoculation of *B. liaoningense*. The rhizobial colony forming units were established through serial dilution. The treatments were replicated three times and the experiment laid out in the green house in a completely randomised design. Ten seeds of soya beans were sown in each pot. After two weeks of germination, the seedlings were thinned to three plants per pot. Watering was done daily with 200 ml of water per pot up to the end of their physiological maturity. Data on plant height, number of leaves, leaf area, shoot and root fresh weight and dry weight, chlorophyll concentration, number of nodules and number of pods were determined. Data was subjected to analysis of variance. Treatments means were separated and compared using Least Significant Difference at ($P = 0.05$). Isolates from the wild soya bean nodules had entire margin. The bacterial colony was rod shaped and white in colour. The colonies had raised elevation under microscopic examination. The nine bacterial isolates tested negative by gram staining. Indole test, catalase test, carbohydrate fermentation test, potassium hydroxide test and methyl red test were positive while citrate test and starch hydrolysis test were negative confirming that the nine isolates belonged to the same *Bradyrhizobium spp.* Soya bean growth and yields after inoculation treatments were found to be significantly different among treatments ($P \leq 0.05$). Plant height, number of leaves and leaf area were highest in 2.67×10^7 cfu/ml of *B. liaoningense* inoculation followed by $1.31x\ 10^7$ cfu/ml inoculation, $1.19x10^7$ cfu/ml, $1.07x10^7$ cfu/ml and lowest in un-inoculated (control). Inoculation at 2.67×10^7 cfu/ml significantly increased soya bean root and shoot fresh weight and dry weights. Chlorophyll concentration was found to be highest in 2.67×10^7 cfu/ml inoculation and lowest in un-inoculated plants. Inoculation at 2.67×10^7 cfu/ml increased number of nodules of soya beans with increasing rhizobia inoculation. Number of pod per plant were highest at 2.67×10^7 cfu/ml and lowest in un-inoculated (control). These findings show that *B. liaoningense* inoculation is effective in improving growth, chlorophyll concentration, number of nodules and number of pods in soya beans. From the study it is strongly recommended that *B. liaoningense* from wild soya bean may be used to improve productivity of soya beans by smallholder farmers.

TABLE OF CONTENTS

LIST OF ABBREVIATION AND ACRONYMS

ANOVA:	Analysis of Variance
ATP:	Adenosine triphosphate
BNF:	Biological Nitrogen Fixation
Cfu:	Colony forming unit
IITA:	International Institute of Tropical Agriculture
Ppm:	Part per million
T/ha:	Tonnes per hectare
YEMA:	Yeast extract Mannitol
NA:	Nutrient agar
LSDT:	Least significant differences test.
MT:	Metric ton
N:	Nitrogen
Spp:	Species
LA:	Leaf area
UV:	Ultra violet

LIST OF TABLES

LIST OF APPENDICES

CHAPTER ONE

INTRODUCTION

1.1 Background information

Soya bean (*Glycine max*) is one of the world's most important legumes in terms of production and trade and has been a dominant oil seed since the 1960s (Getnet, 2019). Historic data on Kenya's soya bean production is poor and scanty, especially in the years before 1990 (Nyaguthii, 2017). After 1990, data suggests that production, area and yield have remained almost stagnant, with little annual change (Nyaguthii, 2017). Currently Kenya is experiencing low yield of soya beans, approximately 0.8 t/ha, due to low soil fertility and high cost of inorganic fertilizers and it is a serious threat to agricultural productivity (Chianu *et al.*, 2018). The production is low compared to food crops like corn (3t/ha) and wheat (2.5t/ha) (Nyaguthii, 2017). Symbiotic rhizobia can increase yields and contribute to the improvement of the soil nitrogen balance through biological nitrogen fixation. Although Rhizobia seem to be widely distributed in the soil, soils in different places contain different strains of rhizobia but only those that are compatible and effective will fix nitrogen in the soil. For that reason, characterization then inoculation of the seed is required to ensure effective nitrogen fixation. Previous studies by Küçük, and Kivanc (2008) indicate that *Bradyrhizobium spp* from chickpea nodules obtained from same plant species and soil were isolated and characterized on the basis of morphological, cultural and biochemical characteristics and found to hve similar characteristics. Morphologically, they were found to be rod shaped, appeared white with raised elevation and gram negative while biochemically they were found to be indole positive, catalase positive and citrate negative. However, it is not known if similar findings will be found if *Bradyrhizobium liaoningense* is characterized morphologically and biochemically. There is need to characterize *Bradyrhizobium liaoningense* from the wild soya

bean in order to identify effective and compatible rhizobia strain with host plant to boost nitrogen fixation in the soil hence promote growth and yield of soya beans.

Western Kenya stands out as the leading soya bean growing region in Kenya, accounting for nearly 50 % of total national smallholder planted area (Nyongesa *et al*., 2017). The main soya bean growing counties are Kakamega, Busia, Bungoma, Trans Nzoia, Kakamega, and Vihiga (Nyongesa *et al*., 2017). Soya beans are important and staple food crop in western Kenya (Nyongesa *et al*., 2017). Soya bean plants require a large amount of nitrogen as the seeds contain high concentrations of protein and the total amount of nitrogen accumulating in the shoot is proportional to the seed yield (Alam *et al*., 2015). Soya bean has a relative specificity for rhizobia among other leguminous crops (Andrews and Andrews 2017). Soya beans can suffer from nitrogen deficiency under field conditions, particularly at flowering when the nodules start to senescence or when seeds are either planted without inoculation of soil with proper symbiotic bacteria, particularly in areas where soya bean has not been grown before (Ntambo *et al*., 2017). Inoculation of soya bean with *Bradyrhizobium japonicum* has been found to increase growth rate of soya beans significantly since inoculation introduced more number viable and efficient rhizobium bacteria in the soil rhizosphere which promoted nitrogen fixation, uptake of minerals as well as cell division and elongation. Despite such positive results, it is yet to be established whether inoculation of soya beans with *Bradyrhizobium li*aoningense will give similar results.

Higher plants such as soya beans contain chlorophyll is a crucial pigment that gives plants their characteristic green colour and occupies a unique role in the physiology, productivity and economy of green plants (Nursu'aidah, 2014). Amount of chlorophyll in leaf tissue is influenced by nutrient availability and environmental stresses such as drought, salinity, cold and heat (Fortunato *et al*., 2018). Chlorophyll content in leaf is a good indication of the nitrogen fixation (Kataria and Baghel, 2016). Previous findings by Hussain *et al.* (2018) have

shown that *Bradyrhizobium japonicum* inoculation may influence growth conditions of leguminous plants such as soya beans by increasing leaf photosynthesis and chlorophyll contents in the leaves. Bambara and Ndakidemi (2010) found that scowpeas inoculated with *Bradyrhizobial japonicum* had great positive response in leaf chlorophyll content as compared with the control. For example, Bambara and Ndakidemi (2010) found that the leaf Chlorophyll concentration for the greenhouse experiment increased significantly with rhizobial inoculation by 123% and 178% for the field experiment relative to the un-inoculated control. Increase in chlorophyll concentration in inoculated soya bean plants could be due to nitrogen uptake, which is crucial in synthesis of photosynthetic pigments. However, it is not known if similar results could be found when soya beans are inoculated with *Bradyrhizobium liaoningense*.

Soybean production in western Kenya is further constrained by poor nodulation due to absence of appropriate *Bradyrhizobium spp*. Inoculation of legumes is especially critical when compatible rhizobia are present, when population densities are low, or when native rhizobia are not effective (Regus *et al.,* 2017). The goal of inoculation is to provide high numbers of viable effective rhizobia in the rhizosphere to allow rapid colonization, nodulation and nitrogen fixation by the selected inoculant strain in order to maximize legume yield potential (Asamoah, 2015). Although bradyrhizobial inoculation technology is simple and extremely cost effective for soybean production legume inoculation is not practised by most farmers. Information on the inoculation of soya bean and their response to growth and yield is lacking. A practical alternative to inoculation has been the development o f soybean varieties capable of forming effective symbiosis with indigenous cowpea Bradyrhizobia. Rurangwa *et al*. (2018) and Kühling *et al*. (2018) reported significant responses to inoculation and higher nodule count in *Bradyrhizobium japonicum* inoculated soya beans compared with the control. The high response of nodulation with increasing inoculation may be due to less nitrogen level

and few indigenous rhizobia in the soil before inoculation which is in agreement with Ngeno (2018) who reported that great response to inoculation could be achieved if the number of indigenous rhizobia population is less than 10 cells/gram of soil. However, it has not been ascertained if similar results can be obtained when soya beans seeds are inoculated with *Bradyrhizobium liaoningense*.

The most important constraint limiting crop yield in developing nations and especially among resource poor farmers is soil fertility decline (Itelima *et al*., 2018). Unless the fertility is restored in the soil, farmers will gain little benefit from the use of improved varieties and even more productive agricultural technologies (Vanlauwe *et al*., 2015). The application of inorganic fertilizers may provide an option to overcome soil infertility, but long-term use of inorganic fertilizers causes decline in soya bean productivity (Gwenzi *et al*., 2015). In recent years, biofertilizer have emerged as a promising component of integrating nutrient supply system in agriculture (Itelima *et al*., 2018). Biofertilzers offer environmentally friendly and sustainable agricultural practices (Itelima *et al*., 2018). Sustainable agriculture based on the use of microbial products is an effective option for overcoming problems of soil fertility (Masso *et al*., 2015). Previous findings by Zimmer *et al*., 2016 and Htwe *et al*., 2015 indicates that inoculation of soya beans with rhizobia species such as *Bradyrhizobium japonicum* improves soya bean yield. Optimal soils conditions especially soil nitrogen levels are however important for the success of any inoculant application to boost soya bean yield. However, there is limited research and documentation on how inoculation of soya beans with *Bradyrhizobium li*aoningense influences the yield of soya beans.

1.2 Statement of the problem

Soya bean growth and production in Kenya has remained low, partly due to soil nutrient depletion and degradation, which is a serious threat to agricultural productivity (Yebasa *et al*., 2019). Symbiotic rhizobia is known to increase yields and contribute to the improvement of the soil nitrogen balance through biological nitrogen fixation. Soils in different environments contain different strains of rhizobia hence those that are compatible and effective will fix nitrogen in the soil. There is lack of information on *Bradyrhizobiun liaoningense* characterization which is vital in identifying strains that can be useful for sustainable cultivation of soya beans in western Kenya. This prompts characterization and inoculation of soya bean seeds with rhizobia species such as *Bradyrhizobiun japonicum* to ensure effective nitrogen fixation, such information will provide an opportunity to manage production and increase yields. Nitrogen enhances nutrients uptake of P, K, Ca, Mg, S, Mn, Fe, Cu, Zn, B, and Mo by plants through synergistic effects (Rampim *et al*., 2015). Studies have shown that productivity of soils in western Kenya is limited by a deficiency of nutrients like nitrogen. Recent studies by Meegalla and Yapa, (2020) have shown that inoculation of soya beans with Bradyrhizobium spp. can increase growth and yield of soya beans. Therefore, there is a need for adoption of sustainable agriculture through seed inoculation with effective and compatible rhizobia spp. which has emerged as a promising component of integrating nutrient supply system in agriculture to boost soya bean growth and yield. There is lack of information on soya beans growth response to *Bradyrhizobia liaoningense* inoculation which could lead to low production. Soya beans grown in nutrient depleted soils faces a problem of low chlorophyll concentration in the leaves, a pigment that offers unique role in the physiology, productivity and economy of green plants. Recent study by Hussain *et al.* (2018) has shown that inoculation of soya beans with *Bradyrhizobia japonicum* positively impacted on chlorophyll concentration in soya beans. However, there is lack of information on the effect

of *Bradyrhizobia liaoningense* inoculation on soya beans chlorophyll concentration which may affect its productivity. Nutrient depleted soils tend to restrict nodule formation in soya bean plants which leads to reduced nitrogen fixation levels in soya beans plants leading to reduced growth and yield of soya beans plants. Promiscuous soya bean varieties are known to nodulate with a wide range of rhizobial strains such as *Bradyrhizobia elkanii*, *Bradyrhizobia japonicum* and *Sinorhizobium fredii*. However, little is known on the effect of *Bradyrhizobia liaoningense* inoculation on the nodulation of soya beans in western. Intensification of land use by small-scale farmers with minimal nutrient inputs has led to declining soya beans yield and productivity. There is high demand of soya bean production in western Kenya due to the growing population prompting farmers to practice continues cropping that depletes soil nutrients which contribute to low yield of soya beans. Soya bean yield in most Kenya is faced with different constraints such poor nodulation with native rhizobia in the soil, inherent low levels of essential nutrients, low availability and lack of awareness on use of inoculants (Nyaguthii, 2017). There is little information on the effect of *Bradyrhizobium liaoningense* inoculation on soya bean which may translate to increase in yield and production. As such, there is need to understand the inoculation effect of *Bradyrhizobium liaoningense* on soya beans yield under green-house conditions.

1.3 Objectives of the study

1.3.1 Broad objective

The main objective of the study was to characterise the *Bradyrhizobium liaoningense* of wild Soya beans and to determine its inoculation effect on growth, chlorophyll concentration, nodulation and yield of soya beans.

1.3.2 Specific objectives

i. To morphologically and biochemically characterise *Bradyrhizobium liaoningense* strains of wild soya beans.

ii. To determine the effect of inoculating soya beans with *Bradyrhizobium liaoningense* on growth of soya beans.

iii. To determine the effect of inoculating soya beans with *Bradyrhizobium liaoningense* on chlorophyll concentration of soya beans leaves.

iv. To determine the effect of inoculating soya beans with *Bradyrhizobium liaoningense* on number of nodules.

v. To determine the effect of inoculating soya beans with *Bradyrhizobium liaoningense* on yield of soya beans.

1.4. Hypotheses

i. There are no morphological and biochemical difference in strains of *Bradyrhizobium liaoningense* of wild soya beans.

ii. Inoculating soya beans with *Bradyrhizobium liaoningense* has no effect on growth of soya beans.

iii. Inoculating soya beans with *Bradyrhizobium liaoningense* has no effect on chlorophyll concentration of soya beans.

iv. Inoculating soya beans with *Bradyrhizobium liaoningense* has no effect on number of nodules of soya beans.

v. Inoculating soya beans with *Bradyrhizobium liaoningense* has no effect on yield of soya beans.

1.5. Justification

In Kenya about 80% of soya bean is consumed by the livestock industry with human consumption accounting for about 20 to 30% (Jackson, 2016). By the year 2016, demand for soya bean consumption rose to about 150, 000 tons per year (Jackson, 2016). This trend,

therefore, calls for a need to increase soya bean production to supply the deficit, which is normally met through imports (Bender *et al*., 2015). There is reported decline in soya bean production due to low soil fertility and reduced area under farming. Increasing the area of land under soya bean to boost production is not favourable due to increasing population growth leading to decline in arable land (Nyongesa *et al*., 2015). Deficiencies in nitrogen has affected many soils in Kenya (Koskey *et al*., 2017). This is attributed to naturally low inherent levels in the soil, continuous cropping, lack of crop rotation, removal of crop residue from the fields, non-application of sufficient organic and inorganic fertilizers, reduction of fallow period and soil erosion (Koskey *et al*., 2017). Inoculation with efficient and effective strains of *Bradyrhizobia spp* have shown improvement in soya bean production (Zimmer *et al*., 2016). Optimal soils conditions especially soil nitrogen levels are important for the success of the inoculant application (Souza *et al*., 2015). Determining optimal soil nitrogen levels for soya bean to respond to rhizobia inoculation and use of soil amendment at low fertile soils will therefore aid in guiding the best nitrogen management strategy to boost soya bean production. The research is important because inoculation technique will unravel the soil fertility issue and contribute to food security among farmers since there will be increased productivity (Dakora *et al.*, 2015).

1.6. Significance of the study

Symbiotic rhizobia can increase yields and contribute to the improvement of the soil nitrogen balance through biological nitrogen fixation. Although Rhizobia seem to be widely distributed in the soil, soils in different places contain different strains of rhizobia but only those that are compatible and effective will fix nitrogen in the soil. These findings show that *B. liaoningense* inoculation is effective in improving growth, chlorophyll concentration, number of nodules and number of pods in soya beans.

1.7. Scope and limitations of the study

The study involved inoculation of soya beans with *Bradyrhizobium liaoningense* to investigate its effect on growth, chlorophyll concentration, nodulation and yield.

However, the following limitations were encountered.

- The research did not ascertain the indigenous rhizobia population in the soil before inoculation which might have given more direction on inoculation application levels for more effective results. The soil used in this study was collected from one place therefore no variation expected since control treatment was used.
- The result only focused on the effect of inoculation on soya bean yields but the mechanism of nitrogen fixation was not determined, so it was difficult to ascertain the mechanism through which nitrogen was fixed in the soil that gave high yield.
- The study did not carry out molecular characterization of *Bradyrhizobium liaoningense* of the wild soya beans, but only focused on morphological and biochemical characters to identify *Bradyrhizobium spp* hence it was difficult to ascertain bacterial strain.

CHAPTER TWO

LITERATURE REVIEW

2.1. General overview on Legume nitrogen fixation

Soya bean *Glycine max (*L.) Merrill is a cultivated species belonging to order Fabales and family Fabaceae. The legumes contain seeds rich in proteins and oils (Kumar *et al*., 2018). Soya bean is a low temperature growing species that can give best productivity at 20-28°C (Hough, 2016). Nodule formation and nitrogen fixation in soya beans are very sensitive to external nitrogen sources including fertilizers and available soil nitrogen (Bhat *et al*., 2015). Previous study by Gresshoff *et al*. (2015) showed that available nitrogen in high concentration is known to suppress nitrogen fixation while on the other hand low nitrogen levels also affect plant development and nitrogen fixation. According to Craine *et al*. (2015), when the supply of nitrogen available from the soil and fertilizer increases, the amount of nitrogen fixed by the plant decreases. Previous studies by Leghari *et al*. (2016) showed that low levels of available nitrogen may have little impact on nodulation and fixation; however, when the combined levels of available soil and fertilizer nitrogen reach approximately 40 kg/ha, any additional nitrogen reduce nodulation and fixation. Previous research by Carter and Tegeder (2016) showed that the application of combined nitrogen especially nitrate to soya bean has shown to strongly inhibit nodule formation, growth and nitrogen fixation. Furthermore, Carter and Tegeder (2016) reported that combined levels of soil and fertilizer nitrogen greater than 55 kg/ha can dramatically delay nodulation and reduce or eliminate nitrogen fixation. Rahima *et al*. (2016) reported that soya bean yield responses to starter nitrogen fertilization are extremely variable, depending on the efficiency of *Bradyrhizobium spp*, soya bean cultivars, soil NO_3-N content and N rates. Furthermore, most effective rhizobia host plant symbiosis fixes little or no nitrogen at all if the soil nitrogen is sufficient to meet the nitrogen demand of the crop. Previous findings by Tairo and Ndakidemi (2013) indicate that

soya bean treatments supplied with *Bradyrhizobium japonicum* inoculation had great positive response in leaf chlorophyll content and growth parameters measured such as plant height, number of leaves per plant, stem girth, number of days to 50% flowering and number of days to 50% pod formation as compared with the control. *Bradyrhizobium liaoningense* being a new species may have certain effects on growth, chlorophyll content, nodulation and yield of legumes, which may support or counteract previous findings if inoculated.

2.2. Effect of *Bradyrhizobium spp* inoculation on growth of legume plants

Studies by Shrivastava and Kumar (2015) showed that *Bradyrhizobium japonicum* increases shoot length, leaf area, root and shoot dry weight of a legume since it enhances rapid cell division and elongation as well as increases growth stimulating hormones such as Auxins and Gibberellins. For one to realize increased growth of plant during inoculation an effective strain of rhizobium has to be used to inoculate the legume. Previous findings by Tairo and Ndakidemi, (2013) indicated that Inoculating soya bean with *Bradyrhizobium japonicum* significantly increases leaf area by 31 and 157 % in the glasshouse and field experiment respectively relative to the control. Other findings by Mfilinge *et al*. (2014) indicated that inoculation of cowpea with *Bradyrhizobium elkanni* and *Bradyrhizobium japonicum* significantly improved the plant height measured at four, six and eight weeks after planting (WAP) in both screen house and field experiments relative to the control treatment. *Bradyrhizobium liaoningense* being a new species may have certain effects on growth of legumes, which may support or counteract previous findings if inoculated. There is therefore need for a study to help shed light on the effect of *Brandyrhizobium liaoningense* on growth parameters of soya beans such as shoot length, leaf area and leaf number.

2.3. Effect of *Bradyrhizobium spp* on plant leaf chlorophyll concentration

According to Mmayi *et al.* (2015), chlorophyll is a very important pigment in soya bean productivity since it helps in absorption of light which is used in photolysis of water molecule that is an important raw material for photosynthesis.

According to Ganeshamurthy *et al*. (2015), chlorophyll deficiency is associated with symptoms of yellowing, dropping of leaves, poor growth, delayed flowering and fruiting. Research by Mmbaga *et al*. (2015) indicated that inoculation with appropriate strain(s) of *Bradyrhizobium spp* may be an effective way of increasing leaf chlorophyll content in legumes. Insufficient levels of nitrogen may hinder chlorophyll accumulation, which limits photosynthesis (Voitsekhovskaja and Tyutereva, 2015). Furthermore, studies involving different types of crops by Yamori *et al.* (2016) revealed that when nitrogen is limited, the most prominent effects is chlorosis hence loss of chlorophyll pigments in a leaf. According to Shrivastava and Kumar (2015), inoculation with effective rhizobium strains such as *Pseudomonas syringae* and *Bradyrhizobium japonicum* can be a solution to increased productivity and yield of any legume plant through accumulation of more chlorophyll that promote photosynthesis. Tairo *et al.* (2017) studied the effects of *Bradyrhizobium japonicum* inoculation on Cowpea (*Vigna unguiculata* (L.) and found that the total leaf chlorophyll content of cowpea measured after planting increased.

Previous studies by Nyoki and Ndakidemi, (2014) indicated that *Bradyrhizobium japonicum* significantly increased leaf chlorophyll content of cowpea by 26% in screen-house and 37.9% on the field. Beneficial rhizobia bacteria may thus influence the physiological growth conditions of leguminous plants by increasing chlorophyll contents in leaves and finally ending up with improved plant growth. The encouraging results obtained from previous studies demonstrates that rhizobia inoculation may substitute the expensive inorganic N fertilizers in improving plant growth and chlorophyll synthesis. *Bradyrhizobium liaoningense*

being a new species may have certain effects on chlorophyll concentration of legumes, which may support or counteract previous findings if inoculated.

2.4. Effect of *Bradyrhizobium spp* inoculation on number of nodules in legumes

Previous study by Hungria *et al*. (2015) indicate that root infection is a precursor to nodulation which is a multi-step process that involves specific plant and bacterial gene expression. Before nodulation begins, the two partners; -Rhizobium, the micro symbiont, and a host plant, the macro symbiont each exist as individual organism (Abd-Alla *et al.*, 2014). The process starts with the multiplication of the bacteria in the rhizosphere (Yergeau *et al.*, 2014). Before infection can proceed, the plant and compatible rhizobia must recognise each other, and the rhizobia must colonise the root surface and attach themselves to the root hairs (Saha *et al.*, 2017).

Infection and nodule organogenesis occur simultaneously during root nodule formation. During the course of this interaction, the invading rhizobia attached to the emerging root hairs release nod factors that induce a pronounced curling of the root hair cells (Miao *et al.*, 2018). The cells most susceptible to infection are those located just above the region of root elongation (Ibáñez *et al.*, 2017). The rhizobia become enclosed in a small compartment formed by the curling of the root hair and stimulate non-dividing root cortical cells to divide and form a distinct area in the cortex, called a nodule primordium from which the nodule develops. The infection and differentiation of the cortical cells causes the induction of a series of plant genes to provide functions for the developing nodules (Marks *et al*., 2013).

The infection thread with proliferating rhizobia elongates through the root hair towards specialised cells in the developing nodule where it fuses with the plasma membrane, divides and begins to branch. Branching of the infection thread enables the bacteria to infect many cells (Marks *et al*., 2013). The bacteria then stop dividing and begin to enlarge and

differentiate into N fixing endosymbiotic organelles called bacteroids. The infection process requires and is regulated by a range of molecular signals between the bacterium and the host plant (Marks *et al*., 2013).

The nodule as a whole develops such features as a vascular system (which facilitates the exchange of fixed N produced by the bacteroids for nutrients contributed by the plant) and a layer of cells to exclude oxygen from the root nodule interior (Liu, 2014). The nodule meristem contributes to the shape of the nodule, with some temperate legumes like peas having nodules which are cylindrical and elongated while the nodules of tropical legumes such as soybean, common bean and peanut (*Arachis hypogaea* L.) lack a persistent meristem and are spherical (Liu, 2014). Rhizobia generally enter the plant through root hairs except in genera like Arachis and Stylosanthes where they enter through the sites of lateral root emergence (Mateos *et al.*, 2011).

Thilakarathna and Raizada (2017) found that soya bean inoculated with rhizobium showed increase in nodule numbers as compared to uninoculated soya bean seeds. Recent studies by Musyoka *et al*. (2020) indicated that inoculation of green grams' seeds with rhizobia increased both the size and number of nodules. *Bradyrhizobium liaoningense* being a new species may have certain effects on nodulation of soya beans, which may support or counteract previous findings if inoculated.

2.5. Effect of *Bradyrhizobium spp* inoculation on yield of legumes

Recent studies by Thilakarathna and Raizada, (2017) showed that inoculation of seeds by *Rhizobium species* prior to planting is a key factor in enhancing high grain yield. A study conducted by Stephanie *et al*. (2015) showed that bean seeds inoculated with Rhizobium strain had higher number of seeds per pod, number of pods per plant and grain yield as compared to those of un-inoculated crops.

Bambara and Ndakidemi (2010) reported high dry bean seed yield of 1679 kg /ha with inoculated crop compared to 758 kg /ha from the control obtained comparable results. Bambara and Ndakidemi (2010) further indicated that higher yields obtained with inoculation was critical in supplying nitrogen to legumeswhich is a better option for resource-poor farmers who cannot afford to purchase expensive inputs. O'Callaghan (2016) argues that negative results were associated with inoculation failure due to loss of viability of Rhizobia in the inoculant caused by exposure to heat or prolonged storage, environmental and management factors or use of unsuitable rhizobium strain for that legume. Previous findings by Mfilinge *et al*. (2014) working on soya bean found that *Bradyrhizobium elkanii* and *Bradyrhizobium japonicum* culture positively influenced the growth and yield of soya beans like number of pod bearing branches per plant, number of pods per plant, number of seeds per pod and 1000-seedweight. Other findings by Nyoki and Ndakidemi (2018) indicated that *Bradyrhizobium japonicum* inoculation significantly improved soybean yield attributes such as the number of pods, seed weight, biological yield and grain yield of soybean. *Bradyrhizobium liaoningense* being a new species may have certain effects on yield of soya beans, which may support or counteract previous findings if inoculated.

2.6. Glycine soja as source of *Bradyrhizobium liaoningense*

Wild soya beans (*Bradyrhizobium liaoningense*) are annual plants in the legume family (Gu *et al*., 2017). It is the closest living relative of soya beans (*Glycine max)* (Valliyodan *et al*., 2017). Wild soy bean is known to have *Bradyrhizobium liaoningense* in their root nodules (Naamala *et al.* 2016) and high ability to form nodules as they obtain the bacteria from soil and fix it in the nodules (Cardoso *et al*., 2018). According to Zhang *et al*. (2017), a good source of the *Bradyrhizobium liaoningense* is the *Glycine soja*, a bacterium that is compatible and effective to be used in inoculating soya beans and give the best inoculants compared to other inoculants such as *Rhizobium leguminosarum*. Previous studies by Ali *et al.* (2016)

points out that *Bradyrhizobium liaoningense* is a species of legume-root nodulating, micro symbiotic nitrogen-fixing bacterium which was first isolated from *Glycine soja* root nodules in China.

2.7. Soya bean and rhizobia inoculation

Soya bean is an important nitrogen-fixing leguminous crop, due to its high-quality protein and input of combined N_2 into the soil (Getachew *et al*., 2017). Recent studies by Zou *et al.* (2019) show that the symbiotic relationship of rhizobia and soya bean roots and the subsequent nitrogen fixation is among the vital physiological processes, which occur in the growth and development of soya bean. According to Thilakarathna and Raizada (2017), soya bean is a crop that was recently introduced in the tropics and it is important to inoculate the seed with appropriate *rhizobium spp* if no soya bean crop has previously been grown in the field. Studies by Regus *et al*. (2017) showed that inoculation of legumes is especially critical when compatible rhizobia are absent, when population densities are low, or when native rhizobia are not effective. Legume inoculation is an established agricultural practice that has been used for more than a century to introduce rhizobia into the soil (Hungria *et al*., 2015). Inoculants are produced commercially in many countries. Their quality depends on both the number of rhizobia they contain and their effectiveness in fixing N with the target host (Herrmann and Lesueur 2013). Symbiotic effectiveness is one of the most important factors when selecting an inoculant strain (Htwe *et al.*, 2015). Stajkovic *et al.* (2011) found that inoculation of soya bean guaranteed increased nodulation and nodule occupancy by the inoculated strains and increased N_2 fixation and crop yield.

The goal of inoculation is to introduce a large number of viable host-specific Rhizobia in order to increase infection rates, which ultimately leads to higher yields (Thilakarathna and Raizada, 2017). Inoculants are produced in powdered, granular or liquid forms (Yadav and Chandra 2014). They can be applied directly onto seed, which is the traditional and most

commonly used method of inoculation, on mineral granules or into the seed bed (Bashan *et al.,* 2014). The quality of an inoculant is evaluated by the number of viable rhizobia it contains (Penna *et al.,* 2011). However, high quality inoculants are produced and are available in powdered or liquid form in North America, Europe, Australasia and some other countries (Yadav and Chandra 2014). Rhizobial inoculants supports and maintains optimum viable count ($1.04x10^7$ cfu/g) for up to 6 months (Yadav and Chandra, 2014). Legume seeds may be inoculated by farmers immediately prior to sowing or custom inoculated by local seed merchants with coating facilities to be sown within a week (Deaker *et al.,* 2004). Inoculation techniques used in legumes are highly variable. Seed inoculation immediately prior to sowing is by far the most popular method used (Hungria *et al.*, 2020). However, the addition of adhesives humus to the inoculant results in the retention of more bacteria on the seed coat and prevents the sloughing-off of the coating material and reduces damage to the cotyledons (Bennett and Lloyd 2015).

Inoculation of annual crop legumes has produced variable results when different inoculation methods were used. Granular seed inoculation gave increased seed yield compared with peat-based seed inoculants in dry peas (*Pisum sativum* L.) (Jambhulkar *et al*., 2016). In common bean, the most common method of inoculation is to apply the culture of *Rhizobium spp*. to seed prior to sowing (Mulas *et al*., 2015). Soil application of inoculum has been successful in the annual legumes; soy bean and field peas (*Pisum sativum* L.) (Alam *et al.,* 2015).

Studies by Sinclair and Nogueira (2018) have shown that Biological Nitrogen Fixation which is enhanced by inoculation of rhizobia to compatible host legume leave residual nitrogen in the soil which improves soil organic matter for the following cropping seasons of cereals and other legumes. According to Zimmer *et al*. (2016), the maximization of biological nitrogen fixation has been obtained by inoculating legumes seeds with efficient *Bradyrhizobium spp* inoculants in low nitrogen soils without nitrogen fertilizer application. Inoculation of soya

beans with efficient strains of *Bradyrhizobium japonicum* have shown to increase plant dry matter, nitrogen concentration, nitrogen accumulation and grain yields (Egamberdieva *et al*., 2016).

According to Zimmer *et al*. (2016), *Bradyrhizobium* inoculation increased soya bean seed yield by 85 % over control. Similarly, Egamberdieva *et al.* (2016) reported that nodule number, nodule dry weight, and soya bean shoot yield were increased when seeds were inoculated with *Bradyrhizobium*. According to Zimmer *et al*. (2016), inoculation may not be required in fields where soya beans have been previously grown and inoculated for many years. Inoculation of legumes with *Bradyrhizobium spp* introduces nitrogen in the soil which has a significant effect on the soil chemistry and enhances nutrients uptake of P, K, Ca, Mg, S, Mn, Fe, Cu, Zn, B, and Mo by plants through synergistic effects (Rampim *et al*., 2015). According to Mazid and Khan (2015), seed inoculation with rhizobia bacteria such as *Bradyrhizobium liaoningense* could be an alternative for use of expensive commercial nitrogen fertilizers and realization of optimal productivity in legumes.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Collection of plant materials and transportation

Nine nodulated plants of wild soya bean (*Glycine soja)* were collected from uncultivated farm in Maseno University, latitude 0°1'N-0°12'S and longitude 34°25'E-34°47'E. Nodules were sampled from same plant species on the same site during the late flowering and early pod setting stages. Random Sampling method was used to select the wild soya bean plant where nodules were obtained. Uprooting was carefully done using a hoe and the wild soya bean plants were transported to Maseno University botany laboratory in zipped polythene bags for rhizobia bacteria isolation. Healthy nodule possessed a yellow colour according to Yang *et al.* (2018). After carefully washing wild soya beans plant roots, fresh and yellow nodules were carefully removed from the roots, wrapped in sterilized absorbent paper, and placed in nine different sterilized Petri dishes labelled as RH-1, RH-2, RH-3, RH-4, RH-5, RH-6, RH-7, RH-8 and RH-9.

3.2. Isolation and purification of bacteria isolates from *Glycine soja* nodules

Isolation and purification of soya beans bacteria isolates followed the procedure of Yang *et al*. (2018). Five healthy nodules of wild soya beans were collected from each plant for rhizobia isolation. Nodules were first washed thoroughly with tap water to get rid of soil debris, followed by rinsing with sterile distilled water. Selected nodules per plant were sterilized by immersing in 95% ethanol for 5-10 seconds and in 4 % sodium hypochlorite for 3 minutes and then finally rinsed five times with sterile distilled water. The nodules were then transferred separately into sterile test tubes each containing 5 ml of sterilized distilled water. Using a sterile glass rod, nodules from nine different test tubes were separately crushed in different test tubes, after which a loopful of the crushed nodule materials were sieved and streaked separately on to nine different plates containing Yeast Extract Mannitol (YEM) agar.

YEM agar was prepared from 10g mannitol, 0.5 g di-potassium hydrogen phosphate (K2HPO4),0.2 g Magnesium sulphate(MgSO4.7H2O), 0.1 g Sodium chloride (NaCl), 1 g yeast extract and 15g agar suspended in 1 litre distilled water as described by Khaitov *et al*. (2016). The plates were then incubated for 48-72 hours in the dark in an incubator at 29± 2°C. Single colonies were identified and re-streaked on the fresh YEMA agar medium; this process was repeated until pure single bacterial colonies were obtained. The single colonies were subjected to different morphological and biochemical tests for bacterial identification.

3.3. Characterization of pure bacteria isolates from *Glycine soja* root nodules

3.3.1. Morphological Characterization

Morphological characterization of the rhizobia was done to determine their growth rate (slow or rapid), mucous production (quantity of mucous and elasticity), and colony characteristics. The formation of colonies on YEMA plates was monitored daily for 14 days, and the pH change of the growth medium was scored on YEMA plates containing 0.25 mg/l Congo red dye. The cultures were incubated for 14 days at 28°C and observed for colour change on a daily basis. All the isolates cultured on YEMA medium containing Congo red dye produced colonies that were whitish to pale pink indicating that the isolates did not absorb the dye when incubated in the dark. After incubation at 28°C for 7 days, distinct colonies were characterized based on their size, colour, shape, transparency, and elevation (Datta *et al.*, 2015).

3.3.1.1. Gram staining technique

Gram staining and microscopy were carried out to determine if the cultures were Gram negative or positive. Staining was done following the method described by Jangra, (2017). A colony of bacterial culture was picked with a sterile inoculating wire loop, and a thin smear was prepared in a drop of water on a clean glass slide. The smear was air-dried, heat fixed,

stained with crystal violet for one minute, and then washed with distilled water. The smear was flooded with iodine solution for one minute followed by one-minute decolorization with ethanol (95% v/v), then washed with distilled water to stop the action of alcohol, and counterstained with safranin for 20 minutes. The slide was washed with distilled water, dried, and observed under light microscope at 1000x magnification using oil immersion. The ability of the isolates to absorb Congo red was tested by adding 1% Congo red solution on prepared and autoclaved YEMA media before pouring into sterile Petri plates.

3.3.1. 2. Congo Red Test

The purity of the rhizobial isolates was detected by adding Congo red in YEMA media. An aliquot of 2.5 ml of 1% solution of the dye in H_2O was added to a litre of YEMA. Most rhizobia absorb the dye only weakly whereas contaminants including Agrobacteria, will absorb strongly. Isolated bacterial strain were streaked on YEMA plated and incubated for 48-72 hours at 29 +/- 2˚C as described by Hamza and Alebejo. (2017).

3.3.2. Biochemical characterization

The isolates were investigated using different biochemical characteristics which included: Indole Test, Citrate Test, Methyl Red -Voges Proskauer test, Carbohydrate fermentation test, Catalase test, Starch hydrolysis and Potassium hydroxidase test, according to procedure by Rohomania *et al.* (2015).

3.3.2.1. Indole Test

Indole production test was done to determine the ability of microorganisms to degrade the amino acid tryptophan by the enzyme tryptophanase. For indole test each indole broth containing 6ml of peptone, sodium chloride was taken. Using sterile technique, small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of loop inoculation method with an inoculating loop. The tubes were then incubated for 24 hours at

37ºC. In order to detect the indole production, 10 drops of Kovac reagent was added to all the tubes. If red reagent layer develops then it indicates indole positive and absence of red color indicates that the substrate tryptophan was not hydrolyzed and it indicates indole negative reaction (Chowdhury, 2018).

3.3.2.2. Citrate Test

Citrate utilization test was done to differentiate among enteric organisms based on their ability to ferment citrate as a sole source of carbon by the enzyme citrase. For citrate utilization test, each test tube containing 2.5 ml of Simmons citrate agar was taken. Using sterile technique, small amount of the experimental bacteria from 24-hours fresh culture was inoculated into the test tubes by means of a streak inoculation method with an inoculating loop. The test tubes were then incubated at 37ºC for 24-48 hours. After 48 hours incubation, if the Prussian blue colour developed then it indicates the citrate positive result, which means the organism, was capable of fermenting citrate as a sole source of carbon. If the colour remained green then it indicates citrate negative result (Chowdhury, 2018).

3.3.2.3. Voges Proskauer test

The Voges-Proskauer (VP) test was done to determine if an organism produces acetyl methyl carbinol from glucose fermentation. For Voges-Proskauer test each VP broth containing dipeptone, dextrose and potassium phosphate was taken. Using sterile technique, each test tube was inoculated by fresh culture of experimental bacteria by means of loop inoculation method. The tubes were then incubated for 48 hours at37ºC. After 48 hours, 10 drops of Barritt's reagent A was added to each tube and the tubes were shaken. Then immediately 10 drops of Barritt's reagent B was added and the tubes were shaken. The colour was observed after 15-30 minutes of the reagent addition. If red colour developed, then it indicates that the organism was capable of fermenting glucose with ultimate production of acetyl methyl

carbinol and it indicates positive result. If no colour developed then it indicates voges-proskauer negative result (Chowdhury, 2018).

3.3.2.4. Carbohydrate fermentation test

Carbohydrate fermentation test was performed to test the ability of the bacteria to utilize carbohydrates, according to the procedure described by Musyimi *et al*. (2017). Each test tube was aseptically inoculated with the test microorganism using an inoculating loop. The test tubes were incubated 37°C for 24 hours. After incubation the liquid in the test tube turns yellow for positive fermentation test while retention of red colour indicates a negative test.

3.3.2.5. Catalase test

The differentiation of bacteria that produce the enzyme catalase from non-catalase producers is achieved using this test. Catalase acts as a catalyst in the breaking down of hydrogen peroxide to Oxygen and water, two to three ml of 3% hydrogen peroxide solution was poured into a test tube. A 24-hour culture of the test organism from the nutrient agar was emulsified in the hydrogen peroxide solution. The release of bubbles immediately indicated a positive test while it was negative when no bubble was formed (Chowdhury, 2018).

3.3.2.6. Starch hydrolysis

Starch hydrolysis test was done to observe if the microbes can use starch, a complex carbohydrate made from glucose, as a source of carbon and energy for growth. Use of starch is accomplished by an enzyme called alpha-amylase. Soluble starch media was dissolved in a small amount of water and was heated slowly with constant stirring. Then all the ingredients were added to it and was transferred into a conical flask and sterilized by autoclaving at 121.5°C. The sterilized agar medium was poured into the sterilized Petri plates and was allowed to solidify. Each plate was inoculated at the center with the bacterial inoculum. Plates were incubated at 37°C for 24–48 hrs. To test the hydrolysis of starch, each plate was

flooded with iodine. An appearance of clear zone around the growth is considered as positive result (Chowdhury, 2018).

3.3.2.7. Potassium hydroxidase test

Bacteria was aseptically removed from petri plates with an inoculating wire loop, placed on glass slide in drop of 3% KOH solution, stirred for 10 seconds and observed for the formation of slime threads according to the procedure of Kapembwa *et al.* (2016). The presence of slimy thread after 24 hours incubation indicated a positive potassium hydroxidase test while absence of slimy thread indicated a negative test.

3.4. Preparation of *Bradyrhizobium liaoningense* inoculants

Preparation of different concentration of *Bradyrhizobium liaoningense* followed the procedure of Miao *et al*. (2018). One ml of the incubated Tryptone-Yeast broth/bacterial suspension mixture was placed in a sterile 10 ml tube with ten ml of sterile water. The original broth culture was designated 10^0 and the first dilution 10^{-1}. One ml of well mixed broth was taken from 10^{-1} dilution and added to nine ml of sterile distilled water and thoroughly mixed to give 10^{-2}. One ml of well mixed broth was taken from 10^{-2} dilution and added to 10 ml of sterile distilled water and thoroughly mixed to give 10^{-3}.

3.5. Bacterial enumeration

Bacterial enumeration was done using the procedure of Thomas *et al*. (2015), an aliquot of 0.5 ml of each dilution was pipetted onto different Tryptone-Yeast agar plates and lightly spread as evenly as possible over the whole plate with a sterile spreader. Three replicates were prepared of each dilution. Plates were sealed with plastic cling film and incubated in the dark at 28 ^{o} C, for 24 hours until the number of colony forming units (cfu) were determined.

Bacterial numbers expressed as colony forming units ml^{-1} broth of different dilution (10^0, 10^{-1}, 10^{-2} and 10^{-3}) were obtained as 2.67 x 10^7 cfu/ml, 1.31x 10^7 cfu/ml, 1.19x10^7 cfu/ml, 1.07x10^7 cfu/ml respectively.

Calculations

1 ml of original bacterial suspension were added to 9 ml of diluent

Dilution factor = Final volume/sample volume

= 10ml/1ml

= 10

Total dilution factor for the 4 dillution = 10x10x10x10 = 10^4

Cfu / ml= (Number of colonies x dilution factor)/ volume of the culture plate

= 267 x 10^4/0.1 ml

= 2.67 x10^7 cfu / m

Number of colonies	**Serial dilution**	**Cfu/ml**
267	10^0	2.67 x10^7 cfu/ml
131	10^{-1}	1.31 x10^7 cfu/ml
119	10^{-2}	1.19 x10^7 cfu/ml
107	10^{-3}	1.07 x10^7 cfu/ml

3.6. Seed inoculation procedure in the laboratory

Different bacterial concentrations of the pure isolates were mixed with humus (bacteria carrier), sugar solution (sticker substance) and one hundred and fifty sterilized soya bean seeds (Pastor-Bueis *et al*., 2019). The mixture was thoroughly stirred using a sterilized wooden spoon for 30 seconds. The inoculant mixture was sprinkled on to the seeds and gently turned to ensure entire coating of soya bean seeds. The sugar solution which was prepared by dissolving 50 g of sugar in 1 litre of distilled water ensured the bacteria inoculant stick on the seeds during inoculation as described by Hassen *et al*. (2014).

3.7. Seed germination experiment in the laboratory

A preliminary germination test was conducted on the nine wild soya beans isolates with five different treatments replicated three times to determine the most effective isolate for seed inoculation in the green house experiment (O'Callaghan, 2016). A total of one hundred and thirty five soya bean seeds, five per treatment per replicate were placed in each sterilized petri dishes lined with layers of moistened Whatsman no.1 filter papers. The seeds were subjected to different serially diluted *Bradyrhizobium liaoningense* inoculants, 2.67 $x10^7$ cfu/ml, 1.31 $x10^7$ cfu/ml, 1.19 $x10^7$ cfu/ml, 1.07 $x10^7$ cfu/ml and sterilized distilled water (control) on the nine bacterial isolates which were replicated three times. Data on seeds germination and radical length were determined as described by Finch-Savage and Bassel (2016). The isolate that gave the highest germination percentages and radical length (RH-3) was selected for green house experiment (Appendix 1 and 2).

3.8. Experimental design and treatment in the green house

Fifteen -5 litre plastic pots with dimensions 25 cm in height and 23 cm in diameter were each filled with 7 kg of top soil collected from Maseno University botanic garden to make up 3 inches soil depth for better root anchorage, Ambede *et al*. (2012). The soils are classified as acrisol, deep reddish brown friable clay with pH ranging from 4.5 to 5.5, soil organic carbon and phosphorus contents are 1.8% and 4.5 mg kg-1, respectively. The soils are well drained and deep, with high extractable Ca and K ions as described by Ambede *et al*. (2012). The soil was sterilized through solarisation method where the soil was spread on polythene bags and left in the greenhouse open and undisturbed for 2 weeks (Katan, 2015). Three pots with top soil and uninoculated seeds were used as control experiment and the remaining twelve pots with top soil contained soya bean seeds mixed with different serially diluted bacteria inoculants containing different numbers of colony forming unit per ml (2.67 $x10^7$ cfu/ml, 1.31 $x10^7$ cfu/ml, 1.19 $x10^7$ cfu/ml, 1.07 $x10^7$ cfu/ml) of *Bradyrhizobium liaoningense* as described by Miao *et al*. (2018). The experiment was laid out in a completely randomized design (CRD), since it yields maximum degree of freedom for experimental error. Ten seeds of Soya beans coated with *Bradyrhizobium liaoningense* inoculants were sown in each pot containing soil. After two weeks of growth, the seedlings in each pot were thinned to three plants per pot. Watering was done daily with 200 ml tap water per pot up to the end of their physiological maturity as described by Grassini *et al.* (2015). The water was sufficient to avoid washing away of rhizobia inoculants from the soil. Data on growth parameters, Chlorophyll concentration, nodule number and yields were taken. The greenhouse daily mean temperature was maintained at 29^0C with diurnal amplitude of ± 5^0C. The relative humidity of air inside the greenhouse ranged between 50 and 95% during the experiment.

3.9. Measurement of growth parameters of soya bean plant

3.9.1. Plant height

Plant height was measured from soil level to the upper point of the terminal bud of the seedling using a meter rule every fourteen days up to the end of the experiment. Two randomly selected plants in each pot were tagged and measured as described by Musyimi *et al*. (2017).

3.9.2. Number of leaves

Number of mature leaves of two randomly selected plants in each pot were counted and recorded after every fourteen days up to the end of experiment, according to Musyimi *et al*. (2017).

3.9.3. Leaf area

Leaf area of two randomly selected plants in each pot was determined after every fourteen days. Leaf area was calculated using the formula: LA=0.5(L X W) Where L=length of leaf W=maximum width (Musyimi *et al*., 2017).

3.9.4. Root and shoot fresh weight

Ninety days after sowing, two randomly selected plants in each pot were carefully uprooted from the soil, cleared off debris, separated into shoot and root and weighed separately using electronic weighing balance according to Musyimi *et al*. (2017).

3.9.5. Root and shoot dry weights

Ninety days after sowing, two randomly selected fresh plants in each pot were uprooted packed separately in envelopes and dried in an oven at constant temperature 60^0C for three days. The roots and shoot were allowed to cool in a dry environment then weighed on an electronic weighing balance according to Musyimi *et al*. (2017).

3.10. Leaf chlorophyll concentration

Determination of chlorophyll followed the method of Ali *et al.* (2012). The third fully expanded leaf from shoot apex was collected from all the treatments after every 14 days. Three grams of leaves were grounded in 10 ml of 80% (V/V) acetone using mortar and pestle. They were left overnight for 24 hours to allow maximum extraction of chlorophyll. The resulting extracts were read at 645 nm and 664 nm using UV-visible spectrophotometer. Chlorophyll a, b and total concentration were calculated as follows:

Chlorophyll a =13.19 A664-2.57 A645 (mgg^{-1} fresh weight)

Chlorophyll b =22.1 A664-5.26 A664 (mgg^{-1} fresh weight)

Total Chlorophyll =7.93 A664+19.53 A645 (mgg^{-1} fresh weight)

Where A664 is the absorbance at 664nm and A645 is the absorbance at 645 nm.

3.11. Number of nodules per plant

Mature nodules from two randomly selected plants in each pot were counted and recorded 90 days after sowing. This was achieved by uprooting the plants and removing all the soil from the roots of selected plants by washing before counting (Hao *et al*. 2014).

3.12. Number of pods per plant

At the end of the experiment, 90 days after planting mature pods of soya bean plant of two randomly selected plants in each pot were counted for each plant and recorded according to Yang *et al*. (2018).

3.13. Statistical analysis

Data obtained from the study on soya growth, chlorophyll concentration and yield parameters was subjected to Statistical analysis of variance using SAS version 9.1. A one-way factorial analysis of variance (ANOVA) was used to determine whether there was any significant effect among the different soya bean treatments. Means that were considered significantly different were separated using Fisher's LSD at $P \leq 0.05$.

CHAPTER FOUR

RESULTS

4.1. Morphological characterization of *Bradyrhizobium liaoningense* of *Glycine soja*

4.1.1. Isolation and characterization of bacteria isolates

Nine isolates were obtained from the root nodules of wild soya bean on YMA plates. They were designated as RH-1, RH-2, RH-3, RH-4, RH-5, RH-6, RH-7, RH-8 and RH-9. The colony characteristics of all the nine-rhizobial isolates had the same characteristics (Table 4.1). All the isolates had a mucoid texture and the individual bacteria under light microscope appear rod-shaped. All the isolates appeared white with entire margin on Yeast Mannitol Agar (YMA) media within 4-5 days of incubation. All the isolates had colonies size ranged between 0.2 to 1mm (Table 4.1). Gram staining revealed that all the bacteria in the isolate were gram negative. Congo red Dye test revealed that the all bacteria isolates of absorbed the dye weakly. In addition, the result indicated that all the isolates had raised elevation. All the isolates appeared as transparent bodies in a grey background studied with engrossing. These distinct characteristics indicated that *Bradyrhizobium liaoningense* was present in the isolate from wild soya beans.

Table 4.1. Morphological characterization of bacteria isolates from *Glycine soja*

I No.	TXR	Mgn	SP	Color	GS	Ele'n	BS	CS (mm)	C R absorption
RH-1	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly
RH-2	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly
RH-3	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly
RH-4	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly
RH-5	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly
RH-6	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly
RH-7	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly
RH-8	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly
RH-9	Mucoid	Entire	Rod	White	-ve	Raised	Transparent	0.2-1	weakly

KEY

(-ve) Negative, **(+ve)** Positive

I No. – Isolate number, **TXR-** Texture, **Mgn**- Margin, **SP**-Shape, **Ele'n**-Elevation **GS-** Gram staining, **BS**-Background staining **CS-**Colony size, **CR**-Congo red, e

4.2. Biochemical characterization of bacteria isolates from wild soya bean (*Glycine soja*)

Biochemical tests were done in the laboratory to study the biochemical characteristics of pure bacteria isolates. Results in Table 4.2 indicate that all the nine isolates were *Bradyrhizobium liaoningense*.

4.2.1. Indole test

Indole test of all the bacteria isolates showed a development of a red ring at top layer of the medium (Table 4.2). This indicated that the bacteria were Indole test positive.

4.2.2. Citrate test

Citrate test of bacteria isolates showed that all the nine isolate were unable to utilize citrate as sole carbon source as the media, they retained the green colour after incubation period indicating the bacteria were citrate negative bacteria (Table 4.2).

4.2.3. Voges Praskauer test

The VP test showed that, medium incubated with all isolates changed to red. This indicated a positive result for methyl red test (Table 4.2)

4.2.4. Carbohydrate fermentation test

Carbohydrate fermentation test of the bacteria isolates from nodules of wild Soya showed that all the isolates were able to utilize sucrose, maltose, lactose or dextrose. This was confirmed by the colour change from red to yellow. This indicated a positive result for Carbohydrate fermentation test (Table 4.2).

4.2.5. Catalase test

Catalase test was done to determine aerobic and anaerobic bacteria. All the nine isolate produced bubbles during the tests. This indicated a positive result for Catalase test (Table 4.2).

4.2.6. Starch hydrolysis test

All bacteria isolate changed colour to dark purple after four minutes. This was an indication of negative test. The bacteria did not hydrolyse starch. This indicated a negative result for starch hydrolysis test (Table 4.2)

3.2.7. Potassium hydroxidase test

All the nine isolate formed slime threads on their surfaces on addition of 3% KOH solution, this confirmed the identity of the bacterial isolate. This indicated a positive result for Potassium hydroxidase test (Table 4.2)

Table 4.2. Biochemical characterization of bacteria isolates from Wild soya bean (*Glycine soja*)

Isolate No.	**I**	**Ci**	**MR**	**CF**	**C**	**SH**	**PH**	**Microbe present**
RH-1	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*
RH-2	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*
RH-3	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*
RH-3	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*
RH-5	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*
RH-6	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*
RH-7	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*
RH-8	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*
RH-9	+ve	-ve	+ve	+ve	+ve	-ve	+ve	*B. liaoningense*

Key

(-ve) =Negative; (+ve) = Positive, **I. No** –isolates, **I** – Indole test, **Ci** –Citrate test, **MR** - Methyl red Voges Proskauer test, **CF** –carbohydrate fermentation test, **C-** Catalase, **SH** – starch hydrolysis, **PH-** potassium Hydroxidase test.

4.3. Determination of Soya bean growth parameters at inoculation treatments

4.3.1. Effect of inoculating soya beans with *Bradyrhizobium liaoningense* on height of soya beans

Soya bean plant height varied significantly in different treatment of rhizobia inoculation. At day 14 after emergence the soya bean plant height was highest at $2.67x10^7$ cfu/ml rhizobia inoculation which had a significant different ($P \leq 0.05$) with $1.31x10^7$ cfu/ml, $1.19x10^7$ cfu/ml $1.07x10^7$ cfu/ml, and un-inoculated (control). At $1.31x10^7$ cfu/ml rhizobia concentration soya bean plant height was higher than $1.19x10^7$ cfu/ml rhizobia inoculation which was also higher than the $1.07x10^7$ cfu/ml rhizobia inoculation. Un-inoculated (control) recorded the lowest soya bean plant height that was significantly different with $1.07x10^7$ cfu/ml rhizobia inoculation but significantly different with $2.67x10^7$ cfu/ml, $1.31x10^7$ cfu/ml and $1.19x10^7$ cfu/ml. The soya beans plant height at day 28, 42, 56 and 70 showed significant differences across all the treatments with highest height at $2.67x10^7$ cfu/ml rhizobia inoculation and lowest in un-inoculated (control) (Table 4.3)

Table 4.3. Effect of inoculating soya beans with *Bradyrhizobium liaoningense* on plant height of soya beans

	Plant height (cm)					
Inoculation treatments	**14**	**28**	**42**	**56**	**70**	Overall mean
Un-inoculated (control)	11.87d	21.13e	28.37e	36.40e	45.20e	28.59e
1.07x10^7 cfu/ml	14.80d	23.70d	31.67d	41.17d	52.10d	32.69d
1.19x10^7 cfu/ml	16.80c	25.60c	34.90c	45.97c	58.47c	36.35c
1.31x10^7 cfu/ml	20.03b	29.73b	39.73b	50.23b	62.83b	40.51b
2.67x10^7 cfu/ml	23.00a	34.60a	44.10a	56.10a	67.70a	45.10a
LSD	**0.8937**	**0.6285**	**0.6758**	**0.9133**	**0.9987**	**1.8193**
P.value	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0001**

Means with the same letter down the column are not significantly different at P≤0.05.

4.3.2. Effect of inoculating soya beans with *Bradyrhizobium liaoningense* on leaf number of soya beans

The leaf number of soya beans increased steadily from day 14 to day 70 of measurement with increase in concentration of rhizobia inoculant, 2.67x10^7 cfu/ml rhizobia inoculation had highest and significantly different soya bean plant leaf number across treatments while 1.31x10^7 cfu/ml, 1.19x10^7 cfu/ml, 1.07x10^7 cfu/ml and un-inoculated (control) had no significant differences in leaf number at day 14. At day 28, 2.67x10^7 cfu/ml rhizobial concentration registered the highest and significantly different (P≤0.05) soya bean plant leaf number while 1.31x10^7 cfu/ml, 1.19x10^7 cfu/ml and 1.09x10^7 cfu/ml rhizobial inoculation had no significant differences (P≥0.05). At day 42, 2.67x10^7 cfu/ml and 1.31x10^7 cfu/ml inoculation was not significantly different (P≥0.05) but significantly different from other treatments, 1.31x10^7 cfu/ml and 1.19x10^7 cfu/ml were not significant (P≥0.05) while un-

inoculated (control) had the lowest soya bean leaf number means, which was significantly different (P≤0.05) from other treatments. At day 56 and 70, the soya bean leaf number was highest at 2.67x10^7 cfu/ml rhizobia inoculation which was significantly different (P≤0.05) across treatments and lowest in un-inoculated (control) except for day 56 where 1.07x10^7 cfu/ml and un-inoculated (control) had no significant differences (P≥0.05) (Table 4.4)

Table 4.4. Effect of inoculating soya beans with *Bradyrhizobium liaoningense* on leaf number of soya beans

Leaf number						
Inoculation treatments	**14**	**28**	**42**	**56**	**70**	Overall mean
Un-inoculated (control)	3.00b	3.33c	5.00c	6.33d	8.00e	5.13c
1.07x10^7 cfu/ml	3.00b	4.33b	5.67bc	7.33cd	9.00d	5.87bc
1.19x10^7 cfu/ml	3.00b	4.33b	6.333b	7.667c	10.00c	6.27abc
1.31x10^7 cfu/ml	3.00b	5.00b	7.67a	8.67b	10.67b	7.00ab
2.67x10^7 cfu/ml	4.00a	6.00a	8.00a	10.33a	11.66a	7.99a
LSD	**0.9401**	**0.8136**	**1.1506**	**1.0504**	**0.6643**	1.8193

Means with the same letter down the column are not significantly different at P≤0.05.

4.3.3. Effect of inoculating soya beans with *Bradyrhizobium liaoningense* on leaf area of soya beans

Inoculation of soya beans at 2.67x10^7 cfu/ml rhizobia concentration had the highest significantly different (P≤0.05) soya bean plant leaf area compared to the rest of the treatments (1.31x10^7 cfu/ml, 1.19x10^7 cfu/ml, 1.07x10^7 cfu/ml and control) of rhizobia inoculation as the days increased. At different days of treatments, soya bean plant leaf area showed a significant difference (P≤0.05) as the concentration of inoculant increased. Un-inoculated (control) treatment had the lowest leaf area in all the days of treatments (Table 4.5).

Table 4.5. Effect of inoculating soya beans with *Bradyrhizobium liaoningense* on leaf area of soya beans

	Leaf area (cm^2)					
Inoculation treatments	**14**	**28**	**42**	**56**	**70**	**Overall mean**
Un-inoculated (control)	26.27e	34.27d	35.23e	39.17e	43.27e	35.642d
$1.07x10^7$ cfu/ml	33.40d	37.50c	40.67d	45.43d	48.83d	43.108c
$1.19x10^7$ cfu/ml	35.53c	40.00c	43.87c	48.50c	53.13c	44.210c
$1.31x10^7$ cfu/ml	38.53b	43.20b	48.27b	52.93b	57.53b	48.090b
$2.67x10^7$ cfu/ml	41.97a	46.00a	50.40a	56.17a	62.10a	51.330a
LSD	**0.7589**	**2.7086**	**0.5167**	**0.6372**	**0.6267**	2.3012
P.value	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0.0001**

Means with the same letter down the column are not significantly different at P≤0.05.

4.3.4. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* on shoot fresh weight

The shoot fresh weight increased steadily as the inoculation increased. There was significant difference (P≤0.05) of root fresh weight at different treatment of rhizobia inoculant (control, $1.07x10^7$ cfu/ml, $1.19x10^7$ cfu/ml, $1.31x10^7$ cfu/ml and $2.67x10^7$ cfu/ml). $2.67x10^7$ cfu/ml inoculation had the highest shoot fresh and significantly different from the other treatments, $1.31x10^7$ cfu/ml had a higher shoot fresh weight than $1.19x10^7$ cfu/ml rhizobial inoculant concentration, followed by $1.09x10^7$ cfu/ml inoculatio, un-inoculated (control) rhizobia had the lowest shoot fresh weight which was significantly different with the other treatments (Table 4.9).

Table 4.6. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* **on shoot fresh weight**

Inoculation treatments	**Shoot fresh weight (g)**
Un-inoculated (control)	41.33e
1.07x10^7 cfu/ml	55.73d
1.19x10^7 cfu/ml	58.10c
1.31x107 cfu/ml	61.43b
2.67x10^7 cfu/ml	64.43a
LSD	**1.3127**
P.Value	**<.0001**

Means with the same letter down the column are not significantly different at P≤0.05.

4.3.5. Effect of Inoculating Soya beans with *Bradyrhizobium liaoningense* on Shoot Dry weight

The shoot dry weight of soya bean increased as the concentration of rhizobial inoculant increased. There was significant difference (P≤0.05) with different treatment of rhizobia inoculant (control, 1.07x10^7 cfu/ml, 1.19x10^7 cfu/ml, 1.31x10^7 cfu/ml and 2.67x10^7 cfu/ml). 2.67x10^7 cfu/ml rhizobia concentration had the highest shoot dry weight which was significantly different from the other treatments. 1.31x10^7 cfu/ml rhizobia inoculant concentration recorded a higher shoot dry weight than 1.19x10^7 cfu/ml rhizobia inoculant concentration. Also, 1.07x10^7 cfu/ml rhizobia inoculant concentration recorded a higher shoot dry weight as compared to un-inoculated (control) soil. Therefore, un-inoculated (control) treatment had the lowest shoot dry weight which was significantly different from other treatments. (Table 4.10)

Table 4.7. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* **on shoot dry weight**

Inoculation treatments	**Shoot dry weight (g)**
Un-inoculated (control)	18.00e
1.07x10^7 cfu/ml	24.80d
1.19x10^7 cfu/ml	26.53c
1.31x10^7 cfu/ml	28.03b
2.67x10^7 cfu/ml	29.46a
LSD	**0.7675**
P.Value	**<.0.0001**

Means with the same letter down the column are not significantly different at p≤0.05.

4.3.6. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* on root fresh weight

The soya bean responses on root fresh weight indicated that as the concentration of rhizobia inoculant increased, the shoot fresh weight also increased. There was significant difference (P≤0.05) with different treatment of rhizobia inoculant (un-inoculated, 1.07x10^7 cfu/ml, 1.19x10^7 cfu/ml, 1.31x10^7 cfu/ml and 2.67x10^7 cfu/ml). 2.67x10^7 cfu/ml rhizobia concentration had the highest root fresh weight which was significantly different from the other treatments, 1.31x10^7 cfu/ml rhizobia concentration had a higher root fresh weight than 1.19x10^7 cfu/ml rhizobia concentration which was also higher than 1.07x10^7 cfu/ml rhizobia concentration, un-inoculated (control) treatment had the lowest root fresh weight which was significantly different with the other treatments (Table 4.11).

Table 4.8. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* on root fresh weight

Inoculation treatments	Root fresh weight (g)
Un-inoculated (control)	12.80e
$1.07x10^7$ cfu/ml	15.56d
$1.19x10^7$ cfu/ml	16.73c
$1.31x10^7$ cfu/ml	18.96b
$2.67x10^7$ cfu/ml	20.33a
LSD	**0.5997**
P.Value	**<.0.0001**

Means with the same letter down the column are not significantly different at P≤0.05.

4.3.6. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* on root dry weight

The soya bean responses on root dry weight showed that, increased rhizobia inoculant concentration increased the shoot fresh weight steadily. There was significant difference (P≤0.05) of root dry weight in different treatment of rhizobia inoculant (control, $1.07x10^7$ cfu/ml, $1.19x10^7$ cfu/ml, $1.31x10^7$ cfu/ml and $2.67x10^7$ cfu/ml). $2.67x10^7$ cfu/ml rhizobia concentration had the highest root dry weight, which was significantly different from the other treatments. Un-inoculated (control) treatment had the lowest root dry weight which was also significantly different with the other treatments (Table 4.12).

Table 4.9. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* on root dry weight

Inoculation treatments	Root dry weight (g)
Un-inoculated (control)	3.86e
$1.07x10^7$ cfu/ml	5.40d
$1.19x10^7$ cfu/ml	5.90c
$1.31x10^7$ cfu/ml	6.50b
$2.67x10^7$ cfu/ml	7.86a
LSD	**0.3787**
P.Value	**<.0.0001**

Means with the same letter down the column are not significantly different at P≤0.05.

4.4. Effect of inoculating soya beans with *Bradyrhizobium liaoningense* on chlorophyll concentration of soya bean plants

Inoculation of soya beans at $2.67x10^7$ cfu/ml had the highest and significantly different ($P \leq 0.05$) chlorophyll concentration, while un-inoculated (control) had the lowest chlorophyll concentration in all the days of treatments. In days 14, there was a significant difference ($P \leq 0.05$) in chlorophyll concentration across all the treatments ($2.67x10^7$ cfu/ml, $1.31x10^7$ cfu/ml, $1.19x10^7$ cfu/ml, $1.09x10^7$ cfu/ml and control). The same trend was observed on day 28, 42, 56 and 70 of the experiment.

Table 4.10. Effect of inoculating soya beans with *Bradyrhizobium liaoningense* on chlorophyll concentration of soya bean plants

Inoculation treatments	Chlorophyll concentration (mgg^{-1} fresh weight)					
	14	**28**	**42**	**56**	**70**	**Overall mean**
Un-inoculated (control)	35.07e	36.00e	37.47e	39.27e	37.53e	37.068c
$1.07x10^7$ cfu/ml	36.70d	37.30d	38.33d	39.53d	38.57d	38.086bc
$1.19x10^7$ cfu/ml	38.40c	39.50c	40.30c	41.27c	40.57c	40.008abc
$1.31x10^7$ cfu/ml	39.57b	40.70b	41.47b	42.30b	41.60b	41.128ab
$2.67x10^7$ cfu/ml	40.90a	42.07a	42.63a	43.33a	42.00a	42.186a
LSD	**0.1558**	**0.1694**	**0.1243**	**0.1243**	**0.1819**	**3.0442**
P. Value	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0.0001**	**<.0.0001**

Means with the same letter down the column are not significantly different at $P \leq 0.05$.

4.5. Effect of Inoculating Soya beans with *Bradyrhizobium liaoningense* on number of nodules

The nodule formation responses of soya bean resulting from inoculation with *Bradyrhizobium liaoningense* are presented in Table 4.8. Inoculation of the soya seeds with different concentrations of the inoculants (un-inoculated soil, $1.07x10^7$ cfu/ml, $1.19x10^7$ cfu/ml, $1.31x10^7$ cfu/ml and $2.67x10^7$ cfu/ml) indicated significant differences ($P \leq 0.05$) in their effects on number of soya bean nodules. Un-inoculated (control) had the lowest number of nodules, while $2.67x10^7$ cfu/ml inoculation had the highest nodule number and significantly different across all treatments.

Table 4.11. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* on number of nodules of soya bean plant

Inoculation	**Number of nodules**
Un-inoculated (control)	10.33e
$1.07x10^7$ cfu/ml	12.67d
$1.19x10^7$ cfu/ml	15.67c
$1.31x10^7$ cfu/ml	18.33b
$2.67x10^7$ cfu/ml	21.00a
LSD	**1.2428**
P.Value	**<.0.0001**

Means with the same letter down the column are not significantly different at $P \leq 0.05$

4.6. Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* on number of pods in soya bean plants

The number of pods of soya bean plants recorded a significant difference ($P \leq 0.05$) with different inoculations (un-inoculated soil, $1.07x10^7$ cfu/ml, $1.19x10^7$ cfu/ml, $1.31x10^7$ cfu/ml and $2.67x10^7$ cfu/ml). $2.67x10^7$ cfu/ml inoculation had the highest number of soya bean pods

number followed by $1.31x10^7$ cfu/ml, $1.19x10^7$ cfu/ml, $1.09x10^7$ cfu/ml and un- inoculated (control) which had the lowest soya bean plant pods number (Table 4.7)

Table 4.12 Effect of inoculating Soya beans with *Bradyrhizobium liaoningense* on number of pods in soya beans

Inoculation treatments	Number of pods
Un-inoculated (control)	14.33e
$1.07x10^7$ cfu/ml	18.33d
$1.19x10^7$ cfu/ml	21.67c
$1.31x10^7$ cfu/ml	23.33b
$2.67x10^7$ cfu/ml	26.00a
LSD	**1.2428**
P.Value	**<.0.0001**

Means with the same letter down the column are not significantly different at $P \leq 0.05$.

CHAPTER FIVE

DISCUSSION

5.1. Morphological characterization of *Bradyrhizobium liaoningense* isolated from nodules of *Glycine soja*

This study reports on the morphological characteristics of Bradyrhizobia species. Nine isolates of rhizobia were recovered from root nodules of wild soya beans (Table. 4.1). These isolates were designated as rhizobia on the basis of their colony characteristics, cell morphology, and inability to absorb Congo red dye (Ondieki *et al*., 2017). All the isolates were Gram-negative and had rod-shaped cells. In addition, all the isolates cultured on YEMA medium containing Congo red dye produced colonies that were whitish to pale pink indicating that the isolates did not absorb the dye when incubated in the dark. The inability of the isolates to absorb Congo red dye is a distinctive character of rhizobia. Species of rhizobia do not absorb Congo red dye or may absorb little amount to give a pale pink appearance (Njeru *et al*., 2020). Njeru *et al*. (2020) also observed whitish or pale pink colonies of faba bean rhizobia isolates on YEMA media containing Congo red dye.

Based on the growth rate on YEMA medium, the Rhizobiaceae family of bacteria can be divided into two major groups, namely, fast- and slow-growing rhizobia. In the present study, the rhizobia species were classified as slow growers. Slow-growing isolates formed colonies after 7–14 days of incubation that were small to medium sized, white or milky, and translucent and were raised with smooth margins. These are characteristics of *Bradyrhizobium* spp. as described by Ondieki *et al*. (2017).

Slow-growing isolates showed mucus production that ranged from high to intermediate with some isolates being dense and elastic and others diffuse and nonelastic. Mucus production by most rhizobia isolates is a fundamental characteristic that is associated with nodulation (Chen

et al., 2020). This suggests that rhizobia isolate with high mucus production ability have high competitive advantage in the initial infection, colonization, and root nodules formation. The identity of the bacteria was further ascertained by similar characteristics by Xu *et al*. (1995) as extra slow-growing and same source of isolation as *Glycine soja*. Xu *et al*. (1995) further identified the species on the basis of genomic DNA G+C content analysis, DNA-DNA hybridization experiments, a partial 16S rRNA sequence analysis, a serological analysis, an N and C content analysis, and an N/C ratio analysis of members of the three groups of soya bean rhizobia, where the name *Bradyrhizobium liaoningense* was proposed.

5.2. Biochemical characterization of *Bradyrhizobium liaoningense*

Bradyrhizobium liaoningense was found to be indole positive (Table 4.2) which may be attributed to the presence of tryptophanase enzyme which degrades tryptophan reagent (amino acid) to pyruvic acid, indole and ammonia. Indole reacted with Kovac's to form a red ring. Similar results were reported by Roychowdhury *et al.* (2015) where Indole reacted with Kovac's to form a red ring in the presence of tryptophanase enzyme.

Bradyrhizobium liaoningense was identified as citrate negative (Table 4.2). The use of citrate as a carbon source showed no colour change, exhibiting negative citrate test. Datta *et al.* 2015 found that citrate utilization as a carbon source was positive in *Rhizobium phaseoli* and *Rhizobium trifolii* (the fast growing *Rhizobia*). However, slow growing *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* showed no colour change, exhibiting negative citrate test. *Bradyrhizobium liaoningense* being among the slow growing rhizobia could explain the bacteria as negative for citrate test.

Bradyrhizobium liaoningense was found to be Voges- Proskauer positive (Table 4.2) possibly due to possession of different metabolic systems by bacteria isolate which enable them to metabolize pyruvic acid, producing acetoin, a neutral reacting end product enabling

smaller quantities of mixed acids to form in the process. In the presence of atmospheric oxygen and 40% potassium hydroxide was converted to diacetyl, under the catalytic action of alpha-naphthol and creatine, was converted forming a red complex. The development of the red colour was a result of acetyl-methyl carbinol (acetoin) production. Results of the current study support those of Karthik *et al*. (2017) who reported that Voges- Proskauer in rhizobia was positive producing a red colour.

Bradyrhizobium liaoningense was positive in carbohydrate fermentation test (Table 4.2), changing colour from red to yellow. This is an indication of presence of enzymes in the bacteria isolates, which enabled them to oxidise environmental nutrients sources such as dextrose, sucrose and maltose and lactose. The kind of enzyme produced in the bacteria enabled them to utilise diverse carbohydrates in their environment helping them in the identification. This is in agreement with Fossou *et al*. (2020) who indicated that carbohydrate fermentation test was positive in rhizobia changing colour from red to yellow.

Bradyrhizobium liaoningense was Catalase positive (Table 4.2) this was an indication of presence of Catalase enzyme in the bacteria isolates, the Catalase enzyme utilise the bactericidal effect of hydrogen peroxide and protects them against the toxic effect of hydrogen peroxide. Bubbling of gas due to production of oxygen gas. catalase test was found to be positive due to bubble formation around bacterial colonies. Datta *et al.* (2015 also observed bubble formation around bacterial colonies of four rhizobial strains.

Bradyrhizobium liaoningense was negative in starch hydrolysis test (Table 4.2) as it could hydrolyze starch (amylose and amylopectin) using the enzymes a-amylase and oligo-1,6-glucosidase. In order to use these starches as a carbon source, bacteria must secrete a-amylase and oligo-1,6-glucosidase into the extracellular space. These enzymes break the starch molecules into smaller glucose subunits which can then enter directly into the glycolytic

pathway. In order to interpret the results of the starch hydrolysis test (Table 4.2), iodine was added to the agar. *Bradyrhizobium liaoningense* did not hydrolyse starch in the presence of iodine confirming starch hydrolysis negative. There was no clear zone around isolates was observed under starch hydrolysis assay, which was performed to determine the production of reducing sugar from starch in bacteria. Similarly, Datta *et al.* (2015) found no clear zones around colony growth zone in *Rhizobium leguminosarum*.

Bradyrhizobium liaoningense was positive in potassium hydroxide test (Table 4.2). KOH dissolved the thin layer of peptidoglycan of the cell walls of gram-negative bacteria. Disintegration of *Bradyrhizobium liaoningense* cell walls lysed the cell and released its contents, including the DNA making the solution very viscous thus sticking on to the inoculating loop when touched. The results are in agreement with previous results by Dash and Payyappilli (2016) who observed presence of slimy thread in Gram negative bacterial cell *Pseudomonas aeruginosa*.

5.3 Effect of Inoculating Soya beans with *Bradyrhizobium liaoningense* on growth parameters

Soya bean plant height increased under *Bradyrhizobium liaoningense* inoculation (Table 4.3) resulting from adequate supply of nitrogen in the soil. The findings are in agreement with Mondal and Bose (2019) who indicated that plant height is an indicator of vegetative growth and that reduced plant height might be due to the result of unavailability of nitrogen and other nutrients required by the plants for their normal growth and development. The significant increase in plant height because of inoculation was probably due to nitrogen fixation induced by inoculated isolates of *Bradyrhizobia liaoningense*, which play a vital role in the vegetative growth of soybean. The significant effect of inoculation on soybean height could probably be

due to the reduced competitiveness of the indigenous rhizobia in the soil, which was outcompeted by the inoculated rhizobium isolate, even though the native rhizobia population in the experimental soil in the pots was not assessed.

Mondal and Bose (2019) further observed that an increase in nitrogen levels through inoculation positively affected the plant height character, which might be due to the role of nitrogen in cell division and cell enlargement. The findings are also in agreement with findings of Fituma (2015) who concluded that *Bradyrhizobium japonicum* inoculation increased soybean plant height up to 112% compared to control. Inoculation of seeds before planting has been found to be the best method in promoting soybean growth and development (Nimnoi *et al*., 2014). Kuryata *et al*. (2019) stated that inoculation with *Bradyrhizobium japonicum* in soybean resulted in an increased height of crop especially at seedling, production of flowers through to fruiting stages. Abera *et al*. (2019) observed better performance of inoculated soybean in terms of height than the uninoculated control. The improved soya bean plant in the inoculated treatments over the control is an indication that *Bradyrhizobium liaoningense* were efficient in fixing nitrogen which is a building block of plant proteins many of which determine the size and structure of plant tissues and consequently plant growth.

Soya bean inoculation had a significant increase in number of leaves (Table 4.4). The increase in leaf number could be attributed to the availability of nitrogen source resulting from *Bradyrhizobium liaoningense* inoculums which enhanced the formation of nodules enhancing nitrogen fixation while un-inoculated soya beans lacked adequate supply of nitrogen required for multiplication of leaves number. The results are in agreement with Kumawat *et al*. (2017) who indicated that application of *Bradyrhizobium liaoningense* inoculums resulted in optimum growth of soya bean leaves. Several researchers have reported

on the importance of inoculation on number of leaves and branches. Turuko and Mohammed (2014) reported that inoculation of seeds before planting increased the soya beans number of leaves, leaf area and branches. Similar reports have been made in other studies including Tairo *et al*. (2017), Abera *et al*. (2019) and Nimnoi *et al*. (2014). Soya beans grown in nitrogen-depleted soils through leaching have numerous deficiency symptoms such as stunted growth which lead to reduced number of leaves (Yu *et al*., 2019). Considering the detrimental environmental effects of fertilizers, *Bradyrhizobium liaoningense* inoculation can provide an alternative option to fertilizers in increasing the leaf number in soya bean.

Inoculated soya bean plants had larger leaf area than un-inoculated soya beans (Table 4.5). *Bradyrhizobium liaoningense* inoculation enhanced nitrogen fixation rates and thus led to development of leaves. Correlation between N uptake and leaf growth in soya bean was supported by the findings of Jaga and Sharma (2015) who reported that increased nitrogen uptake by soya bean plants resulted in higher leaf area per plant. Leaf area is directly related to shoot growth and the two are directly influenced by root growth. Large root system and continued production of root hairs in plants are required for maximum response to nutrient supply that positively correlates with improved shoot growth and consequently increase in leaf area. Such results were observed in inoculated soya bean plants that produced larger leaves than the control experiment. Shoot growth retardation occurs when roots are exposed to stressful conditions such as low nutrient levels which possibly reduces optimum nutrient export to the shoots for luxuriant growth. The control experiment where soya bean plants had reduced leaf area may have exposed the plant to low nutrient levels leading to reduced leaf area. The findings are in agreement with Gai *et al*. (2017) who reported that increased nitrogen uptake by soya bean plants led to increased photosynthesis and partitioning of more dry matter to leaves and thus, resulted in higher leaf area per plant.

Inoculated soya beans recorded a higher root (Table 4.8) and shoot fresh weigh (Table 4.6) as compared to un-inoculated soya beans (Table 4.6 and 4.7). Active shoots ensure a sufficient supply of carbohydrate to roots and maintain root function which can in turn, improve shoot characteristics by supplying sufficient amount of nutrients, water, and phytohormones to shoots which improved shoot and root fresh weight of inoculated soya beans in comparison to un-inoculated soya bean plants. The findings are in agreement with Yang *et al*. (2018) who showed that effective rhizobia bacteria isolates were able to fix nitrogen, increase soya bean root and shoot fresh weight and nitrogen content of the roots and shoots.

Inoculated soya beans recorded a higher root (Table 4.9) and shoot dry weight (Table 4.7) as compared to un-inoculated soya beans. The increase in root and shoot dry weight of the inoculated treatment over the control concurs with a study conducted by Samago *et al*. (2018) who revealed that root and shoot dry matter of the inoculated treatments was significantly greater than that of the control because of an increase in nodulation. Based on the levels of nitrogen in plant shoots, it was evident that the symbiotic effectiveness was high when high concentration of rhizobial inoculant was applied on soya bean. The root and shoot dry weight obtained from soya beans confirmed an increase in biomass through inoculation. The results are in agreement with those of Koskey *et al*. (2017) who indicated that nitrogen accumulation in legumes affect positively the root and shoot dry weight of soya beans plants. The increased dry matter yield after inoculation can be attributed to enhanced effect on stem growth in length and width and the ability of the inoculation to significantly influence nodulation. These findings are further supported by Koskey *et al*. (2017) who reported root and shoot biomass increase in legumes as a result of nitrogen accumulation by rhizobia-inoculated plants. Other studies by Ntambo *et al*. (2017) reported higher plant shoot and root dry matter of soybean after being subjected to application of *Bradyrhizobium japonicum*.

5.4. Effect of Inoculating Soya beans with *Bradyrhizobium liaoningense* on chlorophyll concentration

Inoculating soya beans with *Bradyrhizobium liaoningense* increased chlorophyll concentration in the soya bean leaves as compared to un-inoculated soya bean plants leaves (Table 4.10). The higher chlorophyll content in the inoculated soya beans could be attributed to applied inoculation which availed important nutrients such as nitrogen, which is important in the synthesis of chlorophyll in the chloroplast. Nitrogen is a component of the enzymes associated with chlorophyll synthesis and chlorophyll concentrations reflects relative N status in the soil, which was greatly enhanced through soya bean inoculation. The findings are in agreement with those of Kalaji *et al*. (2018) who indicated that optimum availability nutrients such as N, plays a vital role in the formation of active photosynthetic pigments, including chlorophyll. The findings are also in agreement with Nyoki and Ndakidemi (2018) who indicated significant improvement on the total leaf chlorophyll content of cowpea as a result of *Bradyrhizobium japonicum* inoculation in both screen house and field experiment relative to un-inoculated treatments. The findings are in agreement with Moriwaki *et al*. (2019) who indicated that addition of N promotes the formation of active photosynthetic pigments by increasing the amounts of stromal and thylakoid proteins in tomato leaves, as well as increasing the formation of chloroplasts during leaf growth.

5.5. Effect of Inoculating Soya beans with *Bradyrhizobium liaoningense* on nodulation

Inoculating soya bean with *Bradyrhizobium liaoningense* increased nodulation significantly compared to the un-inoculated soya bean plants (Table 4.11), which is in agreement with previous research by Yang *et al*. (2018) who indicated that improvement in nodulation resulting from inoculation of the soya bean may be due to the benefits of the soya bean-

Bradyrhizobia japonicum interaction from growth promoting substances such as auxins, flavonoid - like compounds and siderophores, which enhance root proliferation and provide more infection sites for the *rhizobia* and in synchronism enhancing the survival and activity of micro-symbiont in the soya bean rhizosphere. Increase in nodule number was also found by Nimnoi *et al*. (2014) who indicated that rhizobial inoculation significantly increased nodule number and dry weight over control in soya bean plants. Similarly, Argaw (2016) indicated that response of soybean to inoculation is greatly affected by the number of effective rhizobia in the soil. The high response of nodulation with increasing inoculation concentration in this study may be attributed to lesser indigenous rhizobial count in the soil before inoculation and the observations are in agreement with Ngeno (2018) who reported that great response to inoculation could be achieved if the number of indigenous *rhizobia* population is less than 10 cells/gram of soil.

Ntambo *et al*. (2017) indicated that *Rhizobium spp* inoculated soya beans plots had higher growth characteristics especially high number of nodules while un-inoculated check plots recorded the lowest number of nodules. Similar study was observed in $2.67x10^7$ cfu/ml inoculation treatment in this study, which reduced significantly with reduction in the concentration levels while control experiment had the lowest nodule number count.

5.6. Effect of Inoculating Soya beans with *Bradyrhizobium liaoningense* on number of pods

The number of pods per plant were more in inoculated soya bean plant as compared to un-inoculated one (Table 4.12). The increase in pod number in this study could be attributed to the complementary effects elucidated by the organisms to enhance the nitrogen fixation performance, as well as nutrient availability and uptake from soil and a healthy rhizosphere,

which may have resulted in the production of substances like hormones, siderophores, phosphate solubilisation and improvement of nutrients and water uptake. The increase in pod number in inoculated soya bean plants over un-inoculated may also be attributed to rhizobuim inoculant, which increases the level of nitrogen absorption by the soya bean plant, that has a direct effect in the biomass accumulation in the plant parts including the pods.

Deshwal *et al*. (2013) reported that rhizobium inoculation resulted in 11% increase in pod number in soya bean over control. Similar findings were reported by Owusu-Mensah (2017) who indicated that inoculation of soya bean cultivars with different bacteria strains had a significant difference in pod number per plant. Ahlijah (2017) on contrary found that inoculation with *Bradyrhizobium japonicum* did not significantly affect pod number and pod weight of soybean, a fact that was attributed to non-promotive effect of the inoculants applied on growth and dry matter accumulation and lower rates of nitrogen applied.

CHAPTER SIX

CONCLUSION, RECOMMENDATION AND SUGGESTIONS FOR FURTHER RESEARCH

6.1. Conclusion

1. Morphological characteristics of the isolates based on gram staining confirmed the standard morphological characteristics of *Bradyrhizobium liaoningense* as being rod-shaped bacteria, which were about 0.2-1mm with no spore formation and gram negative. The colonies were circular, entire, transparent, raised elevation, mucoid texture on Yeast Mannitol Agar with a weak absorption of the Congo red dye characteristic. Biochemically, *Bradyrhizobium liaoningense* was found to be Indole positive, Vorges-Proskauer positive, Methyl red positive, Carbohydrate fermentation positive, Catalase positive, Starch hydrolysis negative and Potassium hydroxide positive that further affirmed the identity of the bacteria under study as *Bradyrhizobium liaoningense.*
2. Inoculation of soya beans with *Bradyrhizobium liaoningense* increased plant height, number of leaves, leaf area, shoot and root fresh weights, shoot and root dry weights
3. Chlorophyll concentration of soya bean leaves increased with increasing *Bradyrhizobium liaoningense* inoculation.
4. There was a significant increase in number of nodules and total number of pods as the number of colony forming units increased.
5. From the study, it could be concluded that growth and yield advantages were gained by inoculation of soya beans with *Bradyrhizobium liaoningense.*

6.2. Recommendations

From the findings of the study, it can be recommended that: -

1. *Bradyrhizobium liaoningense* from *Glycine soja* nodules, were found to be effective and efficient in promoting growth in soya beans hence *Bradyrhizobium liaoningense* from wild soya beans could be used as a source of bioferlizer for growing soya beans instead of expensive inorganic fertilizers to improve growth of soya beans in nutrient depleted soils.
2. It is recommended to use *Bradyrhizobium liaoningense* from *Glycine soja* to improve soya beans Chlorophyll synthesis so as to achieve better productivity of soya beans.
3. It is recommended to use *Bradyrhizobium liaoningense* from *Glycine soja* to improve soya beans nodulation which will enhance nitrogen fixation and achieve higher productivity of soya beans.
4. It is recommended to use *Bradyrhizobium liaoningense* from *Glycine soja* to increase number of pods in soya beans.

6.3. Suggestions for Future Research

1. The research did not ascertain the indigenous rhizobia population in the soil before inoculation. Future research should ensure that initial rhizobia population in the soil is determined before inoculation.
2. Future studies should look into the mechanism of *Bradyrhizobium liaoningense* nitrogen fixation in soya beans.
3. Future studies should also focus on Molecular characterization of *Bradyrhizobium liaoningense* to establish the specific strains of *Bradyrhizobium liaoningense* from the wild soya beans.

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APPENDICES

Appendix 1: Germination pre-lab test for screening rhizobium activity

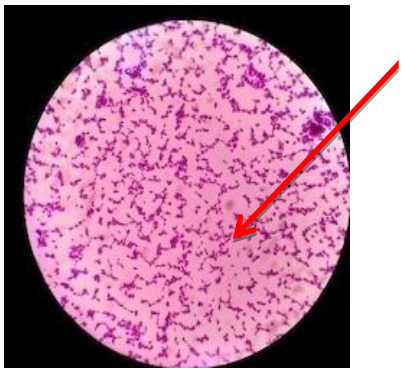
Inoculation Treatments	RH-1	RH-2	RH-3	RH-4	RH-5	RH-6	RH-7	RH-8	RH-9	Overall mean
Un-inoculated (control)	53.33	53.33	73.33	53.33	53.33	50	56.67	60	53.33	**56.29**
$1.07x10^7$ cfu/ml	56.67	63.33	80	56.67	60	56.67	60	66.67	53.33	**61.48**
$1.19x10^7$ cfu/ml	60	63.33	86.67	60	66.67	60	66.67	70	56.67	**65.56**
$1.31x10^7$ cfu/ml	66.67	66.67	93.33	63.33	73.33	63.33	70	73.33	60	**70.00**
$2.67x10^7$ cfu/ml	73.33	73.33	96.33	66.67	76.67	70	73.33	76.67	63.33	**74.41**
Overall mean	**62.00**	**64.00**	**85.93**	**60.00**	**66.00**	**60.00**	**65.33**	**69.33**	**57.33**	

Appendix 2: Radical length pre-lab test for screening rhizobium activity

Inoculation treaments	RH-1	RH-2	RH-3	RH-4	RH-5	RH-6	RH-7	RH-8	RH-9	Overall mean
Un-inoculated (control)	7.47	7.6	10.5	7.5	7.4	7.1	7.4	7.27	6.33	**7.62**
$1.07x10^7$ cfu/ml	8.73	8.3	11.8	7.9	8.07	7.87	8.03	7.93	7.4	**8.45**
$1.19x10^7$ cfu/ml	9.83	8.8	12.7	8.53	8.8	8.47	8.6	8.47	8	**9.13**
$1.31x10^7$ cfu/ml	10.7	9.8	13.5	9.47	9.9	9.6	9.33	9.27	8.57	**10.01**
$2.67x10^7$ cfu/ml	11.5	10.5	14.5	9.9	10.77	10.23	10.4	10.5	9.03	**10.82**
Overall mean	**9.65**	**9.01**	**12.60**	**8.66**	**8.99**	**8.65**	**8.75**	**8.68**	**7.87**	

Appendix 3: Pure bacterial isolate RH-3 on YEM medium and the cells mg. ×1000 showing morphological characteristics

Pure rhizobia isolate RH-2 of *Brandyrhizobia spp*



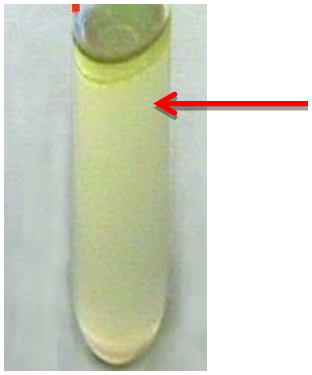
Pink coloured stain of gram-negative *Brandyrhizobia spp*

Appendix 4: Pure isolate of bacteria, RH-3 on Congo Red test

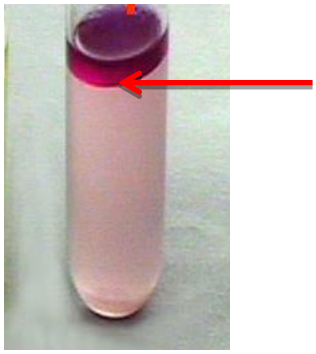
Uninoculated (control)

Congo red positive bacteria

Appendix 5: Pure isolate of bacteria, RH-3 on Indole Test

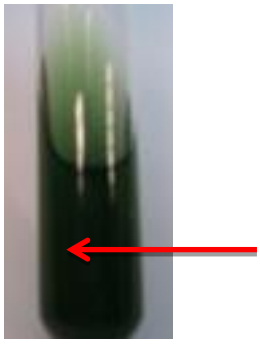


Pure rhizobia isolate (control)



Red ring confirming indole positive bacteria

Appendix 6: Pure isolate of bacteria, RH-3 on Citrate Test



Control (Pure isolate)

Green colour indicating citrate negative bacteria

Pure bacteria isolate (control)

Citrate negative bacteria

Appendix 7: Pure isolate of bacteria, RH-3 on Voges- praskauer Test

Whitish colour (control)

Red colour indicating VP positive bacteria.

Whitish colour of pure rhizobia isolates (control)

Red colour indicating mv-vp positive bacteria.

Appendix 8: Pure isolate of bacteria, RH-3 on Carbohydrate Fermentation Test

Control (-ve)

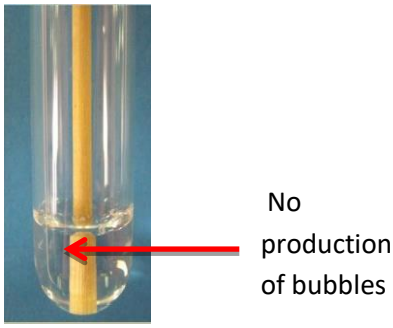
Red colured showing unfermented rhizobia bacteria isolate

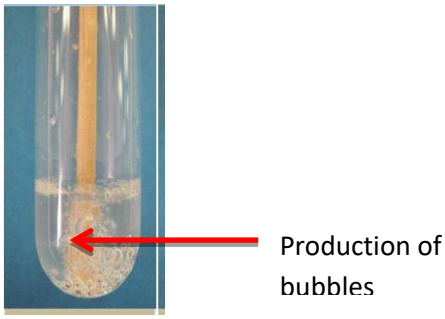
Gas produced

Yellow colour (+ve)

Yellow colour indicating fermentation occurred

Appendix 9: Pure isolate of bacteria, RH-3 on Catalase Test



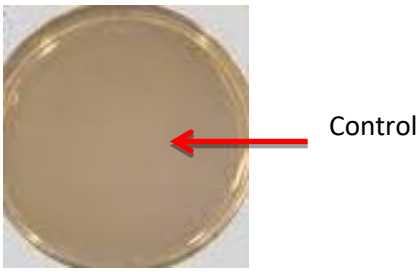


No Bubbles indicating negative catalase bacteria isolate.

Production of bubbles indicating positive catalase test

Appendix 10: Pure isolate of bacteria, RH-3 on Starch Hydrolysis Test

.



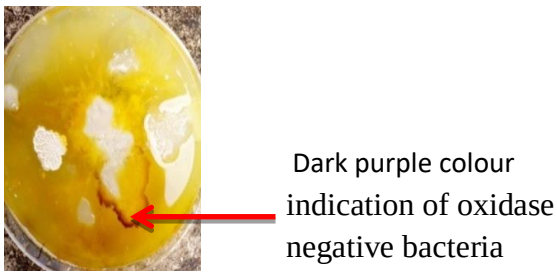
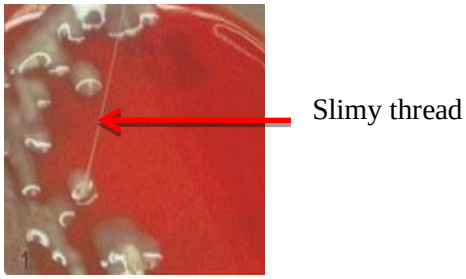


Plate 16 Pure isolate of bacteria (control)

No starch hydrolysis

Appendix 11: Pure isolate of bacteria, RH-3 Potassium Hydroxide Test



Uninoculated (control)

Slimy threads(+Ve)

Appendix 12: Determination of Chlorophyll concentration using light Spectrophotometer

Appendix 13: A Picture of wild soya bean in uncultivated far, Maseno university

Appendix 14: A Picture of wild Soya Bean with mature

Appendix 15: Analysis of variance showing Soya bean height

Parameter	Day	Source	DF	SS	MS	F Value	Pr>F
Soya bean height (cm)	14	Model	4	227.95	56.99	236.13	<.0001
		Error	10	2.41	0.24		
		Corrected total	14	230.36			
		Inoculation	4	227.95	56.99	236.13	<.0001
	28	Model	4	337.46	84.37	706.98	<.0001
		Error	10	1.93	0.11		
		Corrected total	14	338.66			
		Inoculation	4	337.46	84.37	706.98	<.0001
	42	Model	4	472.5	118.12	885.97	<.0001
		Error	10	1.38	0.13		
		Corrected total	14	473.88			
		Inoculation	4	472.5	118.12	885.97	<.0001
	56	Model	4	706.35	176.59	700.74	<.0001
		Error	10	2.52	0.25		
		Corrected total	14	708.87			
		Inoculation	4	706.35	176.59	700.74	<.0001
	70	Model	4	940.74	235.19	780.48	<.0001
		Error	10	3.01	0.301		
		Corrected total	14	943.76			
		Inoculation	4	940.74	235.19	780.48	<.0001

Appendix 16: Analysis of variance showing Soya bean leaf number

Parameter	Day	Source	DF	SS	MS	F Value	Pr>F
Soya Bean Leaf number	**14**	Model	4	2.4	0.6	3.03	<.0001
		Error	10	0	0		
		Corrected total	14	2.4			
		Inoculation	4	2.4	0.6	3.03	<.0001
	28	Model	4	11.6	2.9	14.5	0.0004
		Error	10	2	0.2		
		Corrected total	14	13.6			
		Inoculation	4	11.6	2.9	14.5	0.0004
	42	Model	4	19.73	4.93	12.33	0.0007
		Error	10	4	0.4		
		Corrected total	14	23.733			
		Inoculation	4	19.73	4,93	12.33	0.0007
	56	Model	4	27.6	6.9	20.7	<.0001
		Error	10	3.33	0.33	20.7	
		Corrected total	14	30.93			
		Inoculation	4	27.6	6.9	20.7	<.0001
	70	Model	4	24.4	6.13	45.75	<.0001
		Error	10	1.33	0.13		
		Corrected total	14	25.73			
		Inoculation	4	24.4	6.13	45.75	<.0001

Appendix 17: Analysis of variance showing Soya bean leaf area

Parameter	**Day**	**Source**	**DF**	**SS**	**MS**	**F Value**	**Pr>F**
Soya bean Leaf area cm^2	**14**	Model	4	423.66	105.91	608.72	<.0001
		Error	10	1.74	0.174		
		Corrected total	14	425.4			
		Inoculation	4	423.66	105.91	608.72	<.0001
	28	Model	4	255.52	63.88	28.82	<.0001
		Error	10	22.16	2.21		
		Corrected total	14	277.68			
		Inoculation	4	255.52	63.88	28.82	<.0001
	42	Model	4	439.97	109.99	1363.55	<.0001
		Error	10	0.8	0.08		
		Corrected total	14	440.77			
		Inoculation	4	439.97	109.99	1363.55	<.0001
	56	Model	4	524.78	131.19	1069.54	<.0001
		Error	10	1.22	0.12		
		Corrected total	14	526.01			
		Inoculation	4	524.78	131.19	1069.54	<.0001
	70	Model	4	646.42	161.6	1361.85	<.0001
		Error	10	1.18	0.11		
		Corrected total	14	647.6			
		Inoculation	4	646.42	161.6	1361.85	<.0001

Appendix 18: Analysis of variance showing Soya Bean Shoot Fresh Weight

Parameter	Source	DF	SS	MS	F Value	Pr>F
	Model	4	960.06	240.01	460.98	<.0001
Soya beans	Error	10	5.2	0.52		
Shoot Fresh weight	Corrected total	14	965.26			
	Inoculation	4	960.06	240.01	460.98	<.0001

Appendix 19: Analysis of variance showing Soya Bean Shoot Dry Weight

Parameter	Source	DF	SS	MS	F Value	Pr>F
	Model	4	239.61	59.9	336.54	<.0001
Soya bean	Error	10	1.78	0.17		
Shoot Dry weight	Corrected total	14	241.39			
	Inoculation	4	239.61	59.9	336.54	<.0001

Appendix 20: Analysis of variance showing Soya Bean Root Fresh Weight

Parameter	Source	DF	SS	MS	F Value	Pr>F
Root Fresh weight	Model	4	104.01	26	239.3	<.0001
	Error	10	1.08	0.108		
	Corrected total	14	105.1			
	Inoculation	4	104.01	26	239.3	<.0001

Appendix 21: Analysis of variance showing Soya Bean Root Dry Weight

Parameter	Source	DF	SS	MS	F Value	Pr>F
Root Dry weight	Model	4	25.83	6.45	149.05	<.0001
	Error	10	0.43	0.043		
	Corrected total	14				
	Inoculation	4	25.83	6.45	149.05	<.0001

Appendix 22: Analysis of variance showing Soya Bean Chlorophyll concentration

Parameter	Day	Source	DF	SS	MS	F Value	Pr>F
Chlorophyll Concentration Soya Bean mgg^{-1}	**14**	Model	4	63.72	15.93	2172.14	<.0001
		Error	10	0.07	0.007		
		Corrected total	14	63.79			
		Inoculation	4	63.72	15.93	2172.14	<.0001
	28	Model	4	73.11	18.28	3108.98	<.0001
		Error	10	0.09	0.009		
		Corrected total	14	73.19			
		Inoculation	4	73.11	18.28	3108.98	<.0001
	42	Model	4	55.09	13.77	2951.21	<.0001
		Error	10	0.05	0.005		
		Corrected total	14	55.14			
		Inoculation	4	55.09	13.77	2951.21	<.0001
	56	Model	4	36.79	9.19	1970.86	<.0001
		Error	10	0.05	0.005		
		Corrected total	14	36.84			
		Inoculation	4	36.79	9.19	1970.86	<.0001
	70	Model	4	45.01	11.25	1125.43	<.0001
		Error	10	0.1	0.01		
		Corrected total	14	45.12			
		Inoculation	4	45.01	11.25	1125.43	<.0001

Appendix 23: Analysis of variance showing Soya bean nodules number

Parameter	**Source**	**DF**	**SS**	**MS**	**F Value**	**Pr>F**
Soya bean nodules number	Model	4	218.93	54.73	117.29	<.0001
	Error	10	4.67	0.47		
	Corrected total	14	223.6			
	Inoculation	4	218.93	54.73	117.29	<.0001

Appendix 24: Analysis of variance showing Soya Bean pods number

Parameter	**Source**	**DF**	**SS**	**MS**	**F Value**	**Pr>F**
Soya bean pods number	Model	4	210.27	52.57	112.64	<.0001
	Error	10	4.67	0.47		
	Corrected total	14	214.93			
	Inoculation	4	210.27	52.57	112.64	<.0001