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BIO - PROSPECTING FOR EFFECTIVE RHIZOBIA ISOLATES FOR SOYBEAN PRODUCTION IN GHANA

BY

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BIO - PROSPECTING FOR EFFECTIVE RHIZOBIA ISOLATES FOR SOYBEAN PRODUCTION IN GHANA

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BY

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BSc. AGRICULTURE (HONS)

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DECLARATION

I hereby declare that, except for references to other peoples' work which have been duly acknowledged, this submission is the results of my own original research towards MSc degree and that it has not been presented for any degree elsewhere.

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ABSTRACT

Biological nitrogen fixation is considered an inexpensive means of soil fertility replenishment and as such needs to be exploited. Wild – legume rhizobia have been reported to form successful symbiosis with some important grain legumes like soybean. Hence this study sought to obtain wild – legume rhizobia (indigenous isolates) from wild and uncultivated legumes such as *Desmodium* spp, *Centrosema* spp, *Mimosa* spp, *Crotolaria* spp, *Calopogonium* spp and *Ceasalpinea* spp. A bio - prospecting activity was carried out to collect nodules from uncultivated legumes in parts of Ashanti and Northern regions of Ghana. After culturing and characterization of the indigenous isolates in the microbiology laboratory (SRI), 85 isolates showed characteristics similar to rhizobia on YMA (CR and BTB) media: 65 isolates showed fast growing character while 20 were slow growers. The 85 indigenous isolates were further tested for their infectivity (ability to nodulate legumes) on soybean in sterile river sand and non – sterile soil media. Eleven of the isolates were infective. Symbiotic effectiveness index, SEI showed that isolate NAG 218 was highly effective (SEI > 80), while NAG 150, NAG 155, NAG 180 and NAG 181 were effective (SEI between 50 – 80%) and NAG 152, NAG 168, NAG 170, NAG 171, NAG 173 and NAG 211 were classified as lowly effective (SEI between 35 – 50%). The symbiotic potential of the isolates on the promiscuous soybean varieties in non – sterile soil showed that isolate NAG 152 performed relatively better (11%) than the Legumefix strain. NAG 171 on the other hand produced statistically similar nodule dry weight as Legumefix strain. Shoot dry weight produced by NAG 168 and NAG 211 were statistically at par with the shoot dry biomass of Legumefix strain and USDA 110. Effective isolates at high cell concentration levels (10^8 and 10^4 –CFU) were not competitive. Isolates NAG 152, NAG 168, NAG 171 and NAG 211 showed

potentials for fixing nitrogen thus increasing the possibility of obtaining local strains for soybean production.

DEDICATION

This thesis is dedicated to Prof. R. C. Abaidoo and Dr. Nana Ewusi – Mensah for their constant encouragement and support during the entire period of study.

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CHAPTER ONE

1.0 INTRODUCTION

Smallholder farmers in Africa rely largely on the addition of inorganic fertilizers to replenish N in agricultural soils. The cost of mineral fertilizer, for instance, at the farm gate in Africa is about two to six times higher than in Europe or North America (Donovan, 1996). In 1994, the removal of subsidies on these fertilizers in Ghana, led to a decline in mineral fertilizer use by 60% (Drechsel and Gyiele, 1999). Thus mineral fertilizer in sufficient quantities is beyond the financial reach of most smallholder farmers. Yet, the use of inorganic fertilizers causes increase in crop productivity (Ridley *et al*., 2004) even though it also results in release of nitrogenous greenhouse gases into the atmosphere when not taken up by plants (Flechard *et al.,* 2007). Leaching of these fertilizers into ground water (Trindade *et al.,* 2001) also results in environmental implications. Interest in the use of crop residues (organic inputs) is increasingly arising in tropical agricultural systems for improved soil productivity which can reduce the use of external inputs of inorganic fertilizer (Tetteh, 2004; Fening *et al*., 2005). Thus organic inputs are often proposed as alternatives to mineral fertilizers. However, they have been under-utilized in the humid tropics partly due to low nutrient content in some of the materials and the huge quantities required in order to satisfy the nutritional needs of crops (Ayoole, 2006).

Conversely, biological N fixation, the key means of recycling nitrogen (N) in the biosphere, is an economically justifiable and ecologically safe and ready source of N to agriculture. It is a relatively low-cost source of N for small-holder farmers in developing countries where chemical N input is neither available nor affordable (Amanuel *et al*., 2000). Graham and Vance (2000) suggested that as far as

sustainable agriculture is concerned, effective management of nitrogen in the environment is very important. Contribution of legumes to fixed nitrogen cannot be overemphasized as indicated by Herridge *et al.* (2008) who reported that, legumes can produce over 20 million tons fixed nitrogen each year but this requires an effective symbiosis between the legume and the microsymbiont. Brockwell *et al.* (1995) reported that a common approach to obtain an effective symbiosis leading to improved BNF has been the reliance on superior exotic rhizobia strains, but this has failed to achieve the desired responses in a lot of environments. Streeter (1994) has earlier attributed this failure to poor nodulation competitiveness of the introduced rhizobia since inoculant rhizobia as reported by Vlassak and Vanderleyden (1997) often fail to occupy a significant proportion of nodules due to their inability to compete with indigenous often ineffective rhizobia populations in tropical soils. Thus, to improve the response of tropical legumes to inoculation, it is important to identify native rhizobia with superior symbiotic and saprophytic competence from legumes and soils and to use them in large doses within inoculants to build upon the biodiversity and sizes of indigenous rhizobial population and hence improve Biological Nitrogen Fixation (BNF). Alves *et al.* (2003) reported that in well-managed fields, 70–85% of the N required for soybean (*Glycine max* (L.) Merr.) crop could be derived from symbiotic fixation.

In an attempt to match legume crops with effective strains adapted to prevailing environmental conditions and with good competitive ability against the local less effective strains, many challenges have been faced in agriculture. Abaidoo *et al.* (2007) indicated that, cross inoculation of strains compatible with any given legume may exist; nevertheless, a more precise matching of the wide diversity of rhizobia in symbiosis is necessary. Specificity of the symbiotic association enables maximisation

of biological nitrogen fixation. Musiyiwa *et al*. (2005) also demonstrated that a wide diversity of soybean nodulating rhizobia existed in Zimbabwean soils. However, results obtained from a study on rhizobia indicated that only 2.3 percent of the 129 isolates had higher nitrogen fixation efficiency than the standard commercial strain, MAR 1491.

Promiscuous soybean varieties released by the International Institute of tropical agriculture have the ability to nodulate freely with native *Bradyrhizobium* spp obviating the need for inoculation (Mpepereki *et al*., 2000; Sanginga *et al*., 2003) but studies have shown that this could not fully provide the legumes with the amount of nitrogen they required (Okogun and Sanginga, 2003). The need for inoculation with effective and competitive rhizobia thus remains paramount. Nevertheless, continual importation of inoculants and the inability of exotic strains to outcompete native strains is likely to increase farmers' cost of legume production, especially soybean. On the other hand, closer matching of strain and crop for improved symbiotic efficiency by careful strain selection from wild legumes is not widespread in the context of Ghanaian agriculture. Effective rhizobia strains have been speculated to be associated with wild legumes but have not been exploited in the light of obtaining strains that can effectively nodulate cultivated legumes particularly promiscuous soybean varieties in Ghana.

Appunu *et al.* (2006) and Zengeni *et al.* (2006) have indicated the necessity to continuously identify new, elite isolates adapted to the prevailing environmental conditions that will offer the opportunity to improve BNF.

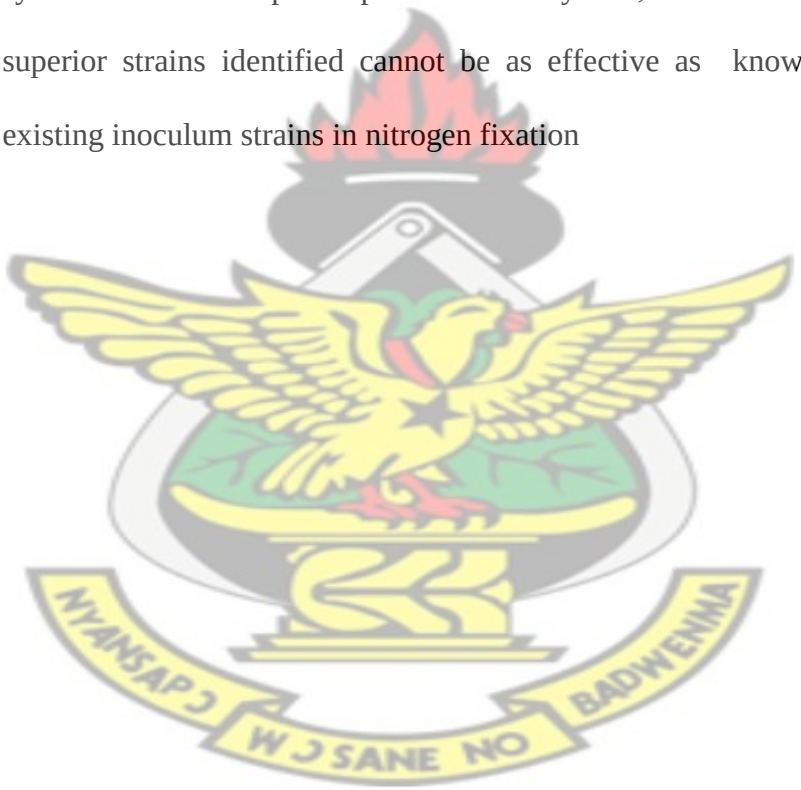
Hence, the overall objective of this study was to identify novel strains of rhizobia that are acclimatized to the prevailing environmental conditions from wild legumes for enhanced BNF in promiscuous soybean.

The specific objectives were to:

i. isolate and evaluate the symbiotic effectiveness potential of rhizobia from wild legumes on promiscuous soybeans under greenhouse conditions;
ii. identify superior strains for future field testing leading to production of inoculum from local strains.

The above specific objectives were based on the null hypothesis that:

i. rhizobia strains that are associated with wild legumes cannot form effective symbiotic relationship with promiscuous soybean;
ii. superior strains identified cannot be as effective as known standard and existing inoculum strains in nitrogen fixation



CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Contribution of Legumes to Nitrogen Fixation

A well-established characteristic of legumes is their ability to develop root nodules that fix N in symbiosis with compatible rhizobia; a process known as biological nitrogen fixation (BNF). Establishment of the symbiosis involves a complex interplay between host (legume) and rhizobia (micro-symbiont) (Giordano and Hirsch, 2004). For sustained nitrogen input into an agro-ecosystem, biological nitrogen fixation is the most important way. Indeed, BNF supplies approximately two-thirds of the nitrogen fixed globally, while the rest of the nitrogen is industrially synthesized by the Haber–Bosch process (Rubio and Ludden, 2008).

Legumes that form nodules have the potential to provide the entire N required for their growth thus influencing the N balance of the soil and its availability to accompanying or subsequent crops. Legumes reduce the cost of production and the potential for N contamination of water resources; in the case of pulses, grains of high nutritional value are produced (Hardarson and Atkins, 2003). Legume crops are identified as globally important because they grow in the tropical, subtropical and temperate climates.

Soybean (*Glycine max* (L.) Merrill), belongs to the large botanical family, *Leguminosae*, in the subfamily *Papilionideae* and performs biological nitrogen fixation in symbiosis with the bacterium, *Bradyrhizobium japonicum* (Salvucci *et al*., 2011). It is successfully cultivated in climates with hot summers, with optimum growing conditions and mean temperatures of 20 to 30^{o}C (Bohner, 2009). Growth of soybean is significantly retarded below or above these temperatures. The crop is

nutritionally beneficial to man and livestock. It has other industrial and commercial uses as well. Soybean is classified as an oilseed, containing significant amounts of all the essential amino acids, minerals and vitamins for human nutrition. It is therefore an imperative source of human dietary protein which is approximately of 40%, and 20% oil content (FAO, 2009). The nutritional and economic value of the soybean crop is being promoted in Ghana by the Ministry of Food and Agriculture hence the need to expand its production (Sarkodie - Addo *et. al*., 2006). The crop also converts atmospheric nitrogen for its own use and also for the benefit of subsequent crops in rotation thus improving the poor soil fertility status of most African countries including Ghana and also benefiting African farmers who cannot access or afford inorganic fertilizer (MoFA and CSIR, 2005; IITA, 2009).

2.2 Biological Nitrogen Fixation and its Contribution to Soil Fertility Replenishment

Nutrients are lost through natural processes, such as leaching or volatilization and therefore, in sustainable ecosystems these nutrients must be replaced either by fertilizers or through natural processes (Boddey *et al.*, 2000). Studies on nitrogen fixing associations have been shown to be of significant ecological and agricultural importance (Denarie *et al.*, 1996). A great importance of the rhizobia - legume association (BNF) has been reported in a number of environments such as terrestrial, fresh water, marine and arctic (Salisbury and Ross, 1992). All living organisms have been reported to depend on the nitrogen fixation process (Raven *et al.,* 1992). Freiberg *et al.* (1997) reported a decline in plant growth as a result of deficiency in mineral nitrogen, thus symbiotic relationships between plants and a variety of nitrogen - fixing organisms is important to obviate this deficiency. Viera – Vargas *et al.* (1995) have indicated that the symbioses between *rhizobia* and legumes are a

cheaper and usually more effective agronomic practice for ensuring an adequate supply of N for legume-based crop and pasture production systems than the application of fertilizer - N.

Biological Nitrogen Fixation (BNF) is defined as the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3), a plant usable form (Franche *et al.,* 2009) through symbiotic association between a legume and the root nodule bacteria (rhizobia). The formation of a novel N fixing organ, the nodule, on the roots and in some cases on the stems of a legume is an indication of a BNF process. Legume nodules represent the most effective system for providing reduced N in agricultural production systems. This symbiosis occurs in forage legumes, grain legumes and some leguminous trees. The dry matter produced by a legume is positively correlated with the amount of N_2 fixed under conditions where yield is limited by soil nitrogen. In the same way, the number and mass of legume nodules may provide a rough estimate of the levels of N_2 fixation (Hardarson and Atkins, 2003). Currently, the annual nitrogen fixation inputs by crop legumes have been reported to be 21.45 Tg, and the inputs of pasture and fodder, 12 - 25 Tg (Herridge *et al*., 2008). Introducing more legumes into farming systems helps to reduce soil erosion losses. Increased plant protein levels and reduced depletion of soil N reserves are also obvious consequences of legume N_2 fixation (Zahran, 1999).

Biological Nitrogen Fixation is ecologically benign and has received aggregated interest in ecology since greater exploitation can reduce the use of fossil fuels and can be helpful in reforestation as well as the restoration of misused lands to productivity (Burris, 1994). The notable key factors that affect BNF include: the number and competitive ability of the compatible rhizobia, factors that alter the supply of photosynthates to nodules such as drought or water-logging and inadequate

soil fertility or unfavourable pH and temperature (Hardarson and Atkins, 2003). Cultivar variation on the other hand, has been reported to affect levels of nitrogen fixation in many legume crop species, and in some crops, particular combinations of strain and cultivar have been shown to be especially efficient at fixing nitrogen (Graham, 2000).

The use of elite strains in rhizobial inoculants combined with specific legume host/cultivar offers a means of establishing well nodulated pasture legumes in many situations (Drew and Ballard, 2010). Nevertheless, introduction of elite strains into the soil does not always insure higher BNF hence higher yields (Lupwayi *et al.,* 2000). For example, introduced strains should be able to compete with native rhizobia for nodulation in the absence of all other factors in order to achieve high nitrogen fixation.

Legumes are similar to all other plants in that they can utilise mineral (inorganic) N in soil such as ammonium and nitrate for growth but this delays the formation of nodules and the onset of N_2 fixation and thus may reduce the amount of N_2 fixed (Unkovich *et al.,* 1996).

2.2.1 The Nodulation Process

Nodulation is defined as the number of nodules formed and the total nodule mass produced by a plant; which is often related to soil rhizobial population size, with high nodulation occurring where compatible rhizobial population is high (Patrick and Lowther, 1995). The symbiosis between legume - rhizobia is highly specific (Cleyet Marel *et al*., 1996; Denarie *et al.,* 1992) with complex signaling processes between the host plant and rhizobia partner. Legumes release flavonoid compounds into the rhizosphere which is the beginning of the signaling process. The compounds trigger soil inhabiting rhizobia to also release highly specific reverse signal molecules, nod

factors that is only useful by specific legume species (Cooper, 2004) for initiating nodule formation. Denarie *et al*. (1992) reported that legumes select for specific rhizobia partner species which is in line with the ability of rhizobia to nodulate with a defined group of legumes species, or host range.

Nodules are referred to as specialized structures whose development forms a key aspect of the legume - rhizobia symbiosis. Nitrogen gas in the atmosphere is reduced to ammonia by differentiated bacteria (bateroids) housed in the cells of nodules (Oldroyd and Downie, 2008). Nodulation is an energy driven process which uses a large percentage of the legume plants energy for nitrogen fixation. Thus excessive nodule formation without an associated increased N_2 fixation is detrimental to the plant growth. Hence, most legume hosts therefore control the number of nodules and zone of nodule development through mechanisms known as autoregulation of nodulation (Oka-Kira and Kawaguchi, 2006). Legume hosts that lack this regulatory system are regarded as excessive nodulators and are said to be hypernodulating mutants (Ferguson *et al*., 2010; van Brussel *et al*., 2002).

For efficient BNF, competent nodulation is required which occurs in the plant - derived root organs called nodules. Furthermore, attempts to increase N_2 fixation activity by selecting efficient host legume cultivars that result in greater nodulation have achieved only limited success. However, nitrogen fixation activity is related to whole plant activity and greater nodulation does not necessarily always mean higher symbiotic N_2 fixation or yield (Pracht *et al*., 1994).

2.3 Constraints to Nodulation and Biological Nitrogen Fixation

The growth and activities of N_2 - fixing plants are limited by several environmental conditions. A principle of limiting factors states that "the level of crop production

can be no higher than that allowed by the maximum limiting factor" (Brockwell *et al.*, 1995). In the N_2 - fixing system, the process of N_2 fixation is strongly related to the physiological state of the host plant.

Soil properties and environmental factors have been shown to affect legume nodulation and symbiotic nitrogen fixation either by impacting rhizobia population sizes, survival and diversity, or interfering directly with the process of nodule formation. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation in the presence of limiting factors that enact limitations on the vigor of the host plant (Thies *et al.*, 1995). In several legumes, nodulation and N_2 fixation are limited by temperature (Niste *et al*., 2013), water stress (Tate, 1995) and deficiencies in soil nutrients such as N, P, and micronutrients (Sanginga *et al*., 1995).

2.3.1 Temperature

Niste *et al*. (2013) reported that temperature is one of the major factors affecting rhizobial growth, survival in the soil and the symbiotic process itself. Biological Nitrogen Fixation is affected by high soil temperatures in the tropics and subtropical areas (Michiels *et al.*, 1994). Bacterial infection and N_2 fixation in various legumes species including soybean are also affected by high root temperatures and the critical temperature for N_2 fixation ranges between 35 and 40^{o}C for soybean (Michiels *et al.*, 1994). Root hair infection and early nodule development processes are very sensitive to sub-optimal temperatures, which can impede nodule development, and thereby delay the onset of nodulation (Lira *et al*., 2005). The growth of plants dependent on nitrogen fixation is more hindered by low soil temperature than that of plants receiving mineral nitrogen. Soil temperatures may reach up to 40 - 60°C in the tropics and sub – tropics. Understandably, such high temperatures in the surface soils

(5 - 6 cm upper layer) will partially or wholly reduce rhizobia activity, particularly if the exposure period is prolonged (Yadav and Nehra, 2012). High temperatures may also lead to fast senescence and decay of soybean nodules (Bordeleau and Prevost, 1994).

2.3.2 Water Stress

Population density of rhizobia has been indicated by Tate (1995) to be lowest under the most desiccated conditions and tend to increase as the moisture stress is relieved. Soil water deficiency also highly affects symbiotic nitrogen fixation of legumes. Tropical legumes such as soybean exhibit a reduction in nitrogen fixation when subject to soil moisture deficit (Zahran, 1999). Nodule initiation, growth, and activities leading to nitrogen fixation are all more sensitive to water stress than are general root and shoot metabolism and as such have been reported to be highly affected by soil moisture deficiency (Albrecht *et al.*, 1994). Nodulation and nitrogen fixation response to water stress depends on the growth stage of the legume plants. Pena-Cabriles and Castellanos (1993) reported that water stress imposed during vegetative growth was more detrimental to nodulation and nitrogen fixation than that imposed during the reproductive stage. Sellstedt *et al.* (1993) found a 26% decrease in the amount of N obtained through N_2 fixation as a consequence of water stress when estimated by the acetylene reduction method. Excessive moisture content at the early stages of soybean growth may cause more extensive yellowing in leaves hence reduced nodulation is not always the cause of yellowing (PHII, 2001). Serraj and Sinclair (1998) indicated that soil water shortage resulted in a decreased nodule number and weight of soybean. The magnitude of the effects depends on the severity of the stress. Potassium is known to improve the resistance of plants to

environmental stress (Zahran, 1999) and can apparently alleviate the effects of water shortage on symbiotic N_2 fixation of *V. faba* and *P. vulgaris*.

2.3.3 Nitrogen

Keyser and Li (1992) indicated that high soil mineral nitrogen (N) levels in the rhizosphere suppress nodule formation and functioning. When nodulation is reduced, nitrogen deficiency symptoms including yellowing and stunted growth of legume crops are observed especially if residual nitrogen is not available in soils. The amount of N fixed in soils with low mineral N but containing sufficient water and enough of other nutrients capable of supporting plant growth is often high (Unkovich *et al.,* 2008). A higher prospect exists for obtaining a positive response to inoculation when soil nitrate is low with high potential for growth while in contrast, high soil nitrate can potentially hinder N_2 fixation (Peoples *et al*., 1995).

2.3.4 Phosphorus

Phosphorus deficiency in soils prevents nodulation and also affects the survival of rhizobia in soils (Giller, 2001). Phosphorus (P) plays a vital role for legumes, because there is a substantial need for P in N_2 fixation. The high requirement for P in legumes is consistent with the involvement of P in whole plant growth as well as the high rates of energy requirement for symbiotic nitrogen fixation and nitrogen assimilation in the nodule (Israel, 1987). Thus phosphorus fertilization improves nodulation and plant growth in soils deficient in P (Giller, 2001). Under low or high pH, phosphorus becomes fixed in the soil and unavailable to plants (Chen, 2006). Ahmed (2007) reported an inverse relationship between root growth and P deficiency, root growth is stimulated as a strategy to improve the phosphorus

nutrition. According to Weber *et al.* (1996) legumes require about 30 kg P ha^{-1} for optimal growth and N_2 fixation.

2.4 Types of Nodules Formed by Legumes

Nodules can be classified into two main groups (Indeterminate and determinate) according to their mode of development (Maunoury *et al*., 2008). Legumes form either indeterminate or determinate types of nodules which also differ widely in morphology and also in the number of bacteroids they contain.

The indeterminate types of nodules generally originate from cell divisions in the inner cortex of the root and have a persistent meristem. Matured nodules tend to be cylindrical and branched in shape. Indeterminate nodules also exhibit a gradient of developmental states of infecting rhizobia and plant cells that are generally categorized into five distinct zones (Timmers *et al*., 2000) as follows: Zone I, (the growing tip of the nodule or meristem); Zone II, (the area where plant cells are infected by bacteria exiting from growing infection threads); Zone III, (the region where plant cells are occupied by N_2-fixing bacteroids); Zone IV, (the region where older infected plant cells and bacteroids are senescing); and Zone V, (the area where rhizobia from older parts of the infection threads can re-infect the senesced nodule tissue as intracellular saprophytes). In the indeterminate types of nodules, the symbiosomes contain single swollen bacteroids that can be pleomorphic in shape (Lodwig *et al*., 2005; Oono *et al*., 2010).

Determinate nodules on the other hand develop from cell divisions within the outer or middle cortex of the root, lack a persistent meristem, and tend to be spherical in shape. These types of nodules do not show any clear developmental gradient; infected plant cells enlarge to accommodate the invading and dividing bacteria, with

N_2 fixation commencing simultaneously throughout the infected plant cells, leading to one homogenous N_2 - fixing zone (Maunoury *et al*., 2008). Symbiosomes from determinate nodules usually contain two or more bacteroids of similar size to free-living bacteria.

2.5 Rhizobia Biodiversity

Rhizobia are defined as Gram-negative, saprophytic soil or water microorganisms that can form N_2-fixing symbioses with legumes by eliciting nodules on the roots or stems of their hosts (Masson-Boivin *et al*., 2009; Sprent, 2007). Within the nodule, the free-living form of the microsymbiont differentiates into bacteroids that convert atmospheric N_2 to ammonia (Jones *et al*., 2007).

Naturally, rhizobia are found in soils of a given locality and they are referred to as the indigenous or native rhizobia. Diverse types of these indigenous rhizobia exist in most soils and their populations are enhanced where compatible legumes are grown and soil fertility is also high (Zengeni *et al*., 2006). According to Bala *et al.* (2001), a particular soil may contain different types of species and various strains within a species, while similar isolates may also be found in distant places (Abaidoo *et al*., 2007). The bacterium forms association with various types of legumes ranging from field grain annual legumes such as soybean to perennial trees such as *Sesbania.* Lalani Wijesundra *et al*. (2000) reported that the cross-inoculation of grain legumes of agricultural importance such as soybean with rhizobial isolates from wild legumes resulted in an increase in dry matter and total nitrogen contents of cross infected plants.

Sinorhizobium fredii and *S. xinjiangense* have presently been identified as strains of fast - growing soybean rhizobia (Peng *et al*., 2002). Furthermore, there are 4

recognized strains of slow-growing soybean rhizobia: *Bradyrhizobium elkanii, B. japonicum, B. liaonin-gense,* and the recently-discovered *B. yuanmingense* biovar that nodulate soybeans (Appunu *et al*., 2008). Wei *et al*. (2002) hitherto have described 40 species and 7 genera in α-subclass of the proteobacteria, which included *Allorhizobium, Azorhizobium, Bradyrhizobium, mesorhizobium, Rhizobium* and *Sinorhizobium* and a species in the genus *Methylobacterium* (Sy *et al*., 2001). The species within the β-subclass have been defined by two genera: *Burkholderia* and *Cupriavidus* (Wei *et al*., 2002). γ - proteobacteria members have also been found to be involved in nodulation (Benhezia *et al*., 2004).

2.6 Characterization of Rhizobia

Rhizobia are bacteria that selectively infect roots of legumes and have a characteristic rod shape. They are also gram negative heterotropic (Somasegaran and Hoben, 1994; Prescott *et al*., 1996) soil bacteria with the ability to build a symbiotic relationship and also fix nitrogen. Manifestation of this symbiosis is observed through the formation of nodules and subsequent nitrogen fixation (Riely *et al*., 2004). Rhizobia are associated extensively to crops and wild legumes alike.

Root nodule bacteria generally grow under a temperature of 25 - 30 °C (optimum) and a pH range of 6-7 (Vincent, 1970; Somasegaran and Hoben, 1994). Rhizobia show a typical translucent, viscid, slimy growth on Yeast Mannitol Agar (YMA) media with individual colonies having domed shape, elevated feature with entire margins. (Gupta *et al.,* 2007). According to Somasegaran and Hoben (1994), fast-growing rhizobia change colour of the bromothymol blue indicator dye from green to yellow while slow-growing rhizobia turned the indicator dye to blue. Normally rhizobium growth occurs under aerobic conditions. In nitrogen fixing roots of

legumes, the oxygen transporter, leghemoglobin, ensures oxygen transport for respiration machinery while maintaining a low partial pressure of free oxygen that could otherwise damage the nitrogenase (Lindstrom, 2001). Hence, rhizobia are able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994). These bacteria take dual forms, i.e. free-living in soils and symbiotic inside of host legumes differentiating them from most other soil microorganisms. The ability to form symbiotic relationships with members of the plant family *Fabaceae* is a unique feature associated with bacteria belonging to the family *Rhizobiaceae* (Pepper and Upchurch, 1991). Interestingly, not all members of this family nodulate; estimates have shown that 90% of *Papilionoideae* and *Mimosoideae* and 25% *Caesalpinoideae* are known to nodulate.

2.7 Specificity and Effectiveness in Rhizobia - Legume Symbiosis

A fundamental aspect of the legume - rhizobia relationship is the effectiveness of the symbiosis, that is, the amount of N_2 fixed by the rhizobia and made available to the plant. A wide variation in the effectiveness of symbiotic interactions can exist (Sprent, 2007; Thrall *et al.*, 2000). Based on this variation, Howieson *et al.* (2005) have defined four categories of symbiotic interaction: no symbiotic interaction, i.e. plants do not nodulate; an ineffective or parasitic interaction, where nodules form but there is no N_2 fixation; a partially effective symbiosis, where fixation produces 20–75% of the biomass achieved by a nitrogen - fed control and an effective symbiosis, where nodulated plants produce > 75% of the biomass achieved by a nitrogen - fed control.

A greater understanding of the factors that govern specificity and effectiveness in legume-rhizobia symbiosis will be required if agricultural systems are to provide the sustainable increases in productivity needed to cope with an increasing world

population, higher nitrogen fertilizer prices and other pressures (Howieson *et al.*, 2008). Perret *et al*. (2000) reported that the specificity between symbiotic partners minimizes the formation of ineffective, non-fixing nodules by the host plant. Rhizobia differ in their response to different signal molecules produced by legumes thus some have a narrow host range and form nodules with a limited number of legumes. On the contrary, legumes may also be host to a particular kind of symbiont or form symbioses with a wide range of rhizobia. Meanwhile, *Bradyrhizobium elkanii*, *Sinorhizobium fredii* and *Bradyrhizobium japonicum* that are distantly related rhizobia can nodulate *Glycine max* (a single host). Maximization of N is enhanced by the specificity of an association. According to Giller (2001), grain legumes can yield up to about 300 kg/ha/year whilst some tree legumes fix as much as 600 kg/ha/year when well matched with their symbionts.

2.8 Access to Indigenous Rhizobia

Endemic populations of soil rhizobia may arise from two sources: native strains and naturalized strains. The naturalized populations of rhizobia are strains that originate from previously grown legume crops treated with commercial inoculant and the native rhizobia are the indigenous strains which nodulate wild legumes and can also infect introduced legumes (Vessey, 2004). The occurrence of a wide diversity of strains increases the opportunity of a legume host finding a compatible rhizobium in any particular soil. Some smallholder farmers prefer using promiscuous varieties of soybean as opposed to the higher yielding specific varieties because of the challenges they face in getting access to inoculants (Mpepereki *et al*., 2000; Musiyiwa *et al*., 2005). Many developing countries do not have inoculant factories and therefore indigenous rhizobia become an important resource in their natural state. Exploitation of indigenous rhizobia by farmers with no access to inoculants has been reported as

successful (Lindstrom *et al*., 2010). Nonetheless, the heterogeneous nature of native rhizobia populations makes it difficult to access this resource in its natural condition and lack of knowledge regarding the genetics of symbiotic effectiveness hampers development. Population sizes of indigenous rhizobia compatible with the legume crop of choice are often very low in economically significant soils. Recent reports indicated that the wild-legume rhizobia formed successful symbioses with some grain legumes, rendering these bacteria very important from both economic and environmental points of view (Zahran, 2001).

2.9 Need for Inoculation

According to Herridge *et al.* (2002) when a legume is being cultivated in a particular location for the first time; where indigenous rhizobia are ineffective in fixing nitrogen; where compatible rhizobia do not exist and where the population of these bacteria is insignificant, inoculation becomes necessary. Nazih and Weaver (1994) reported that a population of rhizobia >1000/g soil is required for optimum nodulation and N_2 fixation. Thus the primary aim of inoculation is to increase the number of desirable strains of rhizobia at the rhizosphere (Lupwayi *et al.,* 2000) and consequently increase both biological nitrogen fixation and grain yield. Sometimes inoculation is applied as a form of indemnity against crop failure (Deaker *et al.,* 2006) since far less problems are associated with inoculation when not needed (over inoculation) than not applying inoculants (Herridge *et al*., 2002) and producing N deficient crops.

2.10 Competitive Ability of Rhizobial Strains

Under most soil conditions, odds are stacked against inoculant strains; Vlassak and Vanderleyden (1997) reported that inoculant strains have a limited gene pool for

adapting to local conditions as opposed to indigenous strains which are well adjusted to prevailing conditions at the site and, moreover, can adapt to just about any change in conditions. It is therefore of utmost importance that inoculant rhizobia have strong nodulation effectiveness for the host being grown as well as well adapted to the relevant soil conditions. Competitiveness has been indicated by Tripplet and Sadowsky (1992) as a complex phenomenon which is influenced by interaction between soil factors and genetic traits of the host and rhizobium symbiont. There is therefore the need to consider not only the N_2 - fixing capacity, but also competitive ability of rhizobia in selecting for superior strains against native rhizobia which are frequently ineffective in N_2 fixation (Rengel, 2002). The term `competition', when used for the *Rhizobium* spp., generally implies competition for nodule formation between the various *Rhizobium* strains from the moment these strains are present in the same environment, until the moment of their presence inside the nodules (Simon *et al.*, 1996). Superior N_2 - fixing strains therefore have to out compete native rhizobia and occupy a significant proportion of the nodules. This can be achieved when rhizobia are selected under natural conditions in competition with the native rhizobia. Better rhizobial symbiosis can be achieved by more effective rhizobia that will establish well in the soil and the rhizosphere (saprophytic competence), will cause greater nodulation, occupy a greater proportion of nodules, and have greater activity of nitrogenase and associated enzymes (Rengel, 2002). Rupela and Sudarshana (1990) have indicated that, native rhizobia form the highest number of nodules. It is thus important to select isolates that are effective from native rhizobia populations because the population consists of diverse, effective and ineffective strains.

2.11 Estimating the Amount of N_2 Fixed

Estimation of nitrogen fixation is the measurement of the amount of nitrogen derived from the atmosphere as a result of the legume- rhizobia symbiotic association. According to Unkovich and Pate (2000) various methods to estimate N_2 fixation under field conditions have been established over more than a century since research in this area begun. The methods include: Acetylene Reduction Assay (ARA) technique, Isotope based method (^{15}N lebelled compounds), Total Nitrogen Difference (TND) method and the Ureide method (xylem- solute) (Unkovich *et al.,* 2008). However, under conditions where yield is limited by soil N, the dry matter produced by a legume is positively correlated with the amount of N_2 fixed. In the same way the number and mass of legume nodules may provide a rough guide to the level of N_2 fixation. Visual appraisal of the leghaemoglobin content (degree of red pigmentation) of nodules can provide a relative indication of their effectiveness and thus used as an estimate of N_2 fixed (Hardarson and Atkins, 2003).

2.12 Summary of Literature Review

Legumes are well noted for their ability to develop root nodules and to fix atmospheric nitrogen in symbiosis with compatible rhizobia through biological nitrogen fixation. Legumes are considered important crops because of their potential to provide the nitrogen (N) they need for their growth as well as for the growth of accompanying or subsequent crops. The BNF process is affected by key factors such as the number and competitive ability of compatible rhizobia, temperature, soil water stress, soil nutrient deficiencies as well as cultivar variations. The legumes soybean is an imperative source of human dietary protein and also has industrial and commercial uses. To enhance the BNF process in soybean, the use of superior exotic rhizobia strains as inoculants has been recommended where the crop is being cultivated for the first time or where native rhizobia are ineffective and incompatible. This approach however has failed to achieve the desired results since the introduced strain(s) are not able to out-compete the native rhizobia for nodule occupancy. Promiscuous soybean varieties with the ability to nodulate freely with native rhizobia were released by IITA as an alternative to improve BNF, but studies have shown that this could not fully provide legumes with the optimum amount of N they required. However, cross – inoculating important grain legumes such as soybean with rhizobia isolates from wild legumes results in increased dry matter accumulation and total N content of cross infected plants. It is thus of utmost importance that new, elite isolates associated with wild legumes and that are adapted to the tropical soil conditions are identified to improve BNF.

CHAPTER THREE

3.0 MATERIALS AND METHODS

Three sets of experiments were performed during this study; sampling of nodules from roots of uncultivated legumes for isolation of rhizobia followed by authentication and effectiveness screening of isolates in potted sterile river sand under greenhouse conditions, evaluation of best performing isolates for effectiveness in non - sterile potted soil and lastly assessment of competitiveness of selected effective isolates in non - sterile potted soil all under greenhouse conditions.

3.1 Sampling Sites and Nodule Collection

Sampling of nodules from uncultivated legumes (*Centrosema pubescens*, *Desmodium* spp., *Crotolaria* spp, *Mimosa* spp, *Caesalpinia* spp and *Calopognium* spp) was carried out in parts of Ashanti, Eastern and Northern regions of Ghana (Figure 3.1). In each location, sampling was done at 200 m interval and documenting the geographical position of the plant using a Geographical Positioning System (GPS). Nodules from the legumes were detached and put into vials containing desiccated silica gel and cotton wool to prevent decomposition and invasion by other soil microorganisms. The vials were kept in boxes under a cooled environment and transported to the laboratory for rhizobia isolation.

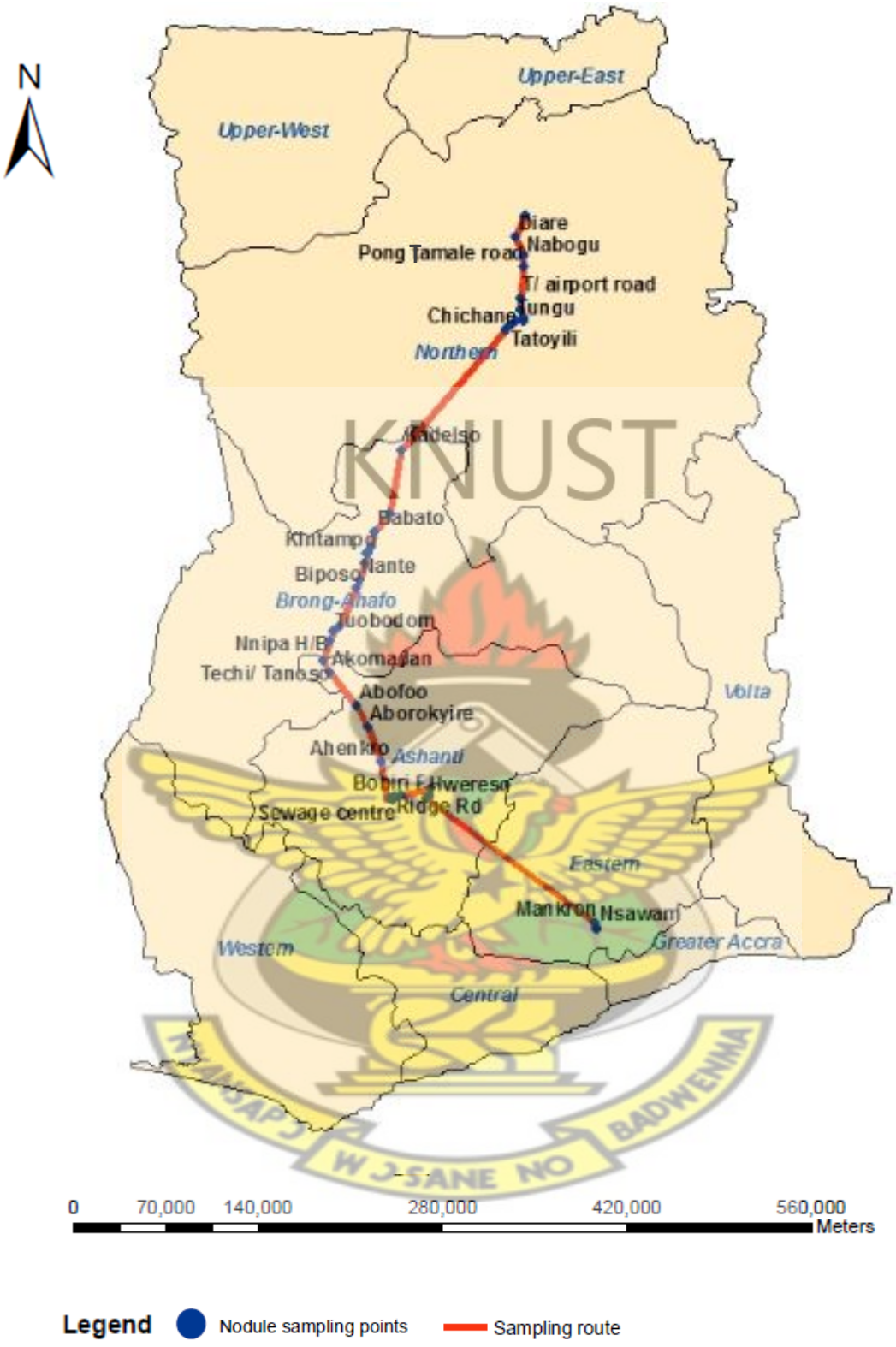


Figure 3.1. Map of Ghana Showing Nodule Sampling Sites

3.2 Media Preparation

Congo Red Yeast Mannitol Agar media was prepared by weighing the precise amount of each of the chemicals (Appendix 1) into a conical flask containing 300 ml of distilled water. The mixture was placed on magnetic stirrer with a heater to ensure thorough mixing and then topped up to 1 litre. The heating was to prevent the media from solidifying because of the agar. One milliliter congo red was added to the resulting mixture and the pH adjusted to 6.8 with a drop wise addition of 1*N* HCl. To prepare Bromothymol Blue (BTB) Yeast Mannitol Agar, 5 ml bromothyml blue / litre was added as the indicator instead of congo red. The conical flask was covered with cotton wool and aluminum foil, and autoclaved at a temperature of 121 ^{o}C for 30 minutes.

3.3 Isolation and Culturing of Strains from Nodules

Undamaged nodules were sterilized using 95% ethanol for 10 seconds and transferred to a 3% solution of sodium hypochlorite for 3 minutes. Then, the nodules were rinsed in six changes of sterilized distilled water and crushed in normal saline solution (0.85% NaCl) as described by Somasegaran and Hoben (1994). The suspension was then streaked on yeast extract mannitol agar (YEMA) (Appendix 1). The isolation process was asceptically carried out under a laminar flow in the microbiology laboratory of the Soil Research Institute, Kwadaso - Ghana. The first isolation from nodules typically produced a mixture of colony types and contaminants. The colonies typical of rhizobia were repeatedly streaked on YEM agar medium (Jordan, 1984) to obtain pure cultures.

Plated cultures were labeled with the isolates ID and date, sealed with parafilm and incubated at 30 ^{o}C for 10 days. The pure cultures obtained were later transferred onto agar tube slants and stored at 4 ^{o}C in a refrigerator.

3.3.1 Acid-Base Production Test (Bromothyml Blue reaction)

The ability of isolates to produce acid or alkaline products were evaluated by streaking strains on YMA with bromothymol blue (BTB) (5 ml) as indicator (Appendix 1) (Somasegaran and Hoben 1994). Culture for each isolate was incubated on the medium for 3 - 7 days and observed for color changes (Alberton *et al*., 2005).

3.4 Authentication and Evaluation of Effectiveness of Test Isolates

This experiment was carried out at the Greenhouse of the Department of Horticulture, Faculty of Agriculture in pots. Nutrient solution was prepared as described by Broughton and Dilworth, (1971). The required quantities of each stock solution (Appendix 2) was pipetted into 5 litres of distilled water and shaken vigorously after which it was topped up to 10 litres. One normality HCl was used to adjust the pH of the solution to between 6.6 and 6.8. Pots were sterilized by cleaning with 70% ethanol and filled with 4 kg acid washed sand and autoclaved. The pots were then arranged on stands in the greenhouse. The river sand was collected from Sewua in the Ashanti region of Ghana, acid washed with concentrated sulphuric acid and rinsed severally under running water after which it was autoclaved at 121 ^{o}C for 1 hour (Lupwayi and Haque, 1994). The sand in the pots was ponded using half strength N-free nutrient solution (Broughton and Dilworth, 1971). Five hundred millilitres of the nutrient solution was added to each pot and left for a day to drain to field capacity. For the plus N control treatments, KNO_3 (0.05% and 0.1%) was added. The two different rates of N were used with the aim of identifying the best N

concentration to use for an authentication study since in a previous study 0.05% KNO_3 had proved apparently insufficient

3.4.1 Planting and Inoculation of Seeds

Soybean seeds (Anidaso) were surface sterilized with 95% ethanol for 1 minute and rinsed in several changes of distilled water after which it was planted in the potted sand. Six seeds were sown per pot and thinned to two after a week of planting. A 300ml portion of nitrogen - free nutrient solution was used to water the pots after a week of sowing. This was repeated weekly and distilled water was used to water intermittently (every two days) for 8 weeks. Addition of nutrient solution was always targeted to achieve 75% field capacity of the plant growth medium.

Broth cultures of the indigenous rhizobia isolates and reference strains (USDA 110, USDA 136, and USDA 138) were prepared by inoculating Yeast Mannitol Broth (YMB) with the isolates and incubated at 28 ^{o}C until the broth became turbid. Two millilitres broth (10^{8} CFU) of each isolate or reference strains were used to inoculate each seedling one week after planting. The pots were then labelled with the identification numbers for each of the isolates and reference strains.

3.5 Treatments and Experimental Design

There were eighty - nine treatments comprising 85 indigenous isolates, USDA 110, + 0.05% KNO_3, + 0.1% KNO_3 and negative control (No inoculation, No mineral fertilizer). The treatments were arranged in a completely randomized design with three replications. A total of 267 experimental units were obtained which were monitored for 8 weeks in a Greenhouse environment of average minimum and maximum temperatures of 21 ^{o}C and 45 ^{o}C respectively.

3.6 Harvesting and Data collection

After eight weeks, plants were harvested by carefully uprooting them so that no nodules were left in the growth media (river sand). The nodules were collected, enumerated and recorded accordingly. Parameters such as shoot and nodule dry weight were also recorded. The values obtained for each parameter measured were means for the two plants in each replicate pot. Shoot dry weight value of each isolate was compared with reference strain USDA 110 treated control and the positive control (KNO_3 at 0.05%) to assess the symbiotic effectiveness index and relative effectiveness, respectively. Relative effectiveness of the isolates was calculated using the formular proposed by Purchino *et al.* (2000) and as follows:

$$Relative\ Effectiveness = \frac{Test\ strain\ inoculated\ plant\ dry\ weight}{Nitrogen\ treated\ plant\ dry\ weight} x\ 100$$

$$Symbiotic\ Effectiveness\ index = \frac{Test\ strain\ inoculated\ plant\ dry\ weight}{Reference\ strain\ inoculated\ \ plant\ dry\ weight} x\ 100$$

With nitrogen fixing potential, effectiveness was classified as ineffective <35%; lowly-effective, 35 to 50%; effective, 50 to 80%; and highly effective, >80%. Isolates that produced effective and highly effective nodules were reisolated to obtain pure cultures and used to replace the original isolates.

3.6.1 Screening of Effective Indigenous Rhizobia Isolates for their performance on two soybean varieties in Non- Sterile Soil under Greenhouse Conditions

This experiment was setup in the Greenhouse of the Department of Horticulture, Faculty of Agriculture. Soils were collected from the plantation section of the Crop and Soil Sciences Department of KNUST. The land was previously cropped to

eggplant in 2013. Physical, chemical and biological properties of the soil determined were as follows: pH (1: 1 H_2O); total nitrogen - Kjeldahl digestion and distillation procedure (Bremner and Mulvaney, 1982); available phosphorus - Bray-P1 method (Olsen and Sommers, 1982); total carbon - modified Walkley-Black wet oxidation method (Nelson and Sommers, 1982) and Cation Exchange Capacity (C.E.C.) – Atomic Absorption Spectrophotometer, flame photometer and by titration (Helmke and Sparks, 1996). Field capacity of the soil was determined using the cylinder method (Somasegaran and Hoben, 1994). Most Probable Number (MPN) was also carried out to determine the population of indigenous rhizobia present in the soil (Somasegaran and Hoben 1994). The treatment consisted of eleven isolates that constituted 13% of the 85 authenticated isolates, 3 inoculum strains, a positive N control and a negative N (uninoculated and unfertilized control). Six litres capacity pots were perforated at the base and filled with 5 kg of soil. The pots were watered to 75% field capacity. Potassium dihydrogen phosphate (KH_2PO_4) with the active element phosphorus (P) was added to the soil in each pot as a basal nutrient at a rate of 30 kg P /ha of soil or 0.33g /5 kg soil. The basal application of phosphorus was done to eliminate its deficiency effects on nitrogen fixation. Early and late maturing soybean seeds (Nangbaar and Jenguma, respectively) were surface sterilized with 95% ethanol and subsequently rinsed in several changes of distilled water. Six seeds were planted per pot and thinned to two after a week of planting. Broth cultures of the indigenous rhizobia isolates and inoculum strains (USDA 110, Biofix and Legumfix) were prepared by inoculating Yeast Mannitol Broth (YMB) with the strains and incubated at 28^{o}C until they became turbid.

One millilitre broth (of rate 10^8 CFU) of each isolate or inoculum strain was used to inoculate each seed at planting. Nitrogen at 100 kg N/ha (applied as 0.54 g/ 5 kg soil)

in the form of urea was split applied (25% at planting and 75% three weeks after planting) to the soils which was used as the positive (N- treated) control treatments. A negative N control which was neither inoculated nor fertilized was included.

3.6.2 Physical, Chemical and Biological Properties of Soil Used for Potted Experiment and Estimated Native Rhizobia Population

Table 3.1 shows the physic – chemical properties of non- sterile soils used for effectiveness screening in the greenhouse. After rhizobia enumeration from non – sterile soils, rhizobia population was estimated to be 81.3 cells/ g soil (Table 3.1)

Table 3.1. Physical and Chemical Properties, and Rhizobia Population of Non - Sterile Soil

Properties	Description	Remarks
pH (1:1) (H_2O)	5.88	*Moderately acidic
Organic Carbon (%)	0.61	^Low
Total N (%)	0.06	**Very low
Available P (mg kg^{-1})	37.3	^Medium
Exchangeable Ca (cmol $_{(+)}$ kg^{-1})	4.41	#Low
Exchangeable Mg (cmol $_{(+)}$ kg^{-1})	1.34	#Medium
Exchangeable Na (cmol $_{(+)}$ kg^{-1})	0.08	#Very low
Exchangeable K(cmol $_{(+)}$ kg^{-1})	0.11	#Very low
MPN (Rhizobia cells g^{-1} of soil)	81.3	

*, Bruce and Rayment (1982); **^,** Boerma *et al.* (1995); ******, Landon (1996); #Metson(1961)

3.7 Treatments and Experimental Design

There were a total of 16 treatments as follows; 11 local strains (NAG 150, NAG 152, NAG 155, NAG 168, NAG 170, NAG 171, NAG 173, NAG 180, NAG 181, NAG 211, and NAG 218); three reference strains (USDA 110, Biofix, and Legumfix); Nitrogen positive N control and a Negative control N (uninoculated and unfertilized) which were arranged in split plot in a completely randomized design with soybean varieties being the main plot factor and the various isolates, the sub plot factor. All the treatments were replicated three times resulting in a total of 96 experimental units.

3.8 Assessment of Competitiveness of Selected Effective Isolates

The competitive ability of effective isolates selected based on their nodulation ability and symbiotic effectiveness index were tested in the presence of native rhizobia assessed using non-sterile soil as described in section 3.5. This was done in parallel with the effectiveness study in non – sterile soil. Three inoculum rates of 10^8, 10^6 and 10^4 - CFU were applied to soybean (Nangbaar) at planting. The broth inoculants comprising three of the isolates that had effectiveness index between 50 and 80% were selected alongside USDA 110 reference strain for the evaluation. An uninoculated and an N – fertilized (100 kg N /ha) as negative and positive controls, respectively were included. In all, the total of 14 treatments (Table 3.2) were arranged in a completely randomized design and replicated three times.

Table 3.2 Competitive Study Treatments

Isolates / N source	Cell Concentrations / N rate
NAG 218	10^8
	10^6
	10^4
NAG 150	10^8
	10^6
	10^4
NAG 181	10^8
	10^6
	10^4
USDA 110	10^8
	10^6
	10^4
-N	-
+N	100 kg N

3.9 Estimating Indigenous Rhizobia Population in Non-Sterile Soil

This was done following the most - probable number (MPN) procedure, as described by (Somasegaran and Hoben, 1994). Cowpea seeds (Asontem) used as trap host for the native rhizobia in the soil were surface sterilized with 95% of ethanol and in 3% (v/v) solution of sodium hypochlorite. The seeds were successively rinsed in

sterilized distilled water, and incubated at 28+ 2 ^{o}C in sterilized petri plates containing moistened filter paper to aid germination. Healthy well-grown seedlings with similar size and radicle length were transferred aseptically into growth pouches containing N – free nutrient solution (Broughton and Dilworth, 1971). After 5 - 7 days old, the pouches were reorganized on the racks. Five – fold serial dilutions of the soil used for the potted experiment was made. One hundred grammes of soil was weighed into 400 ml sterile distilled water to make the 5^{-1} soil: water solution. The resulting solution was mixed thoroughly using a vortex mixer. A series of soil dilutions from 5^{-2} to 5^{-6} was then prepared by pipetting 5 ml of diluent into 20 ml of sterile distilled water. Each soil dilution was mixed using a vortex mixer and used to inoculate the plants. There were four replications of each soil dilution used to inoculate each plant to enable determination of the most probable number of bacteria in soil sample using existing tables. Plants were watered with nutrient solution as and when necessary. The inoculated seedlings in growth pouches were monitored for 4 weeks after which they were harvested and carefully examined for the presence or absence of nodules. Those with nodules were recorded as positive and those without nodules as negative. Population estimates were assigned to the results using MPNES software (Woomer *et al.*, 1990).

3.10 Harvesting and Data Collected

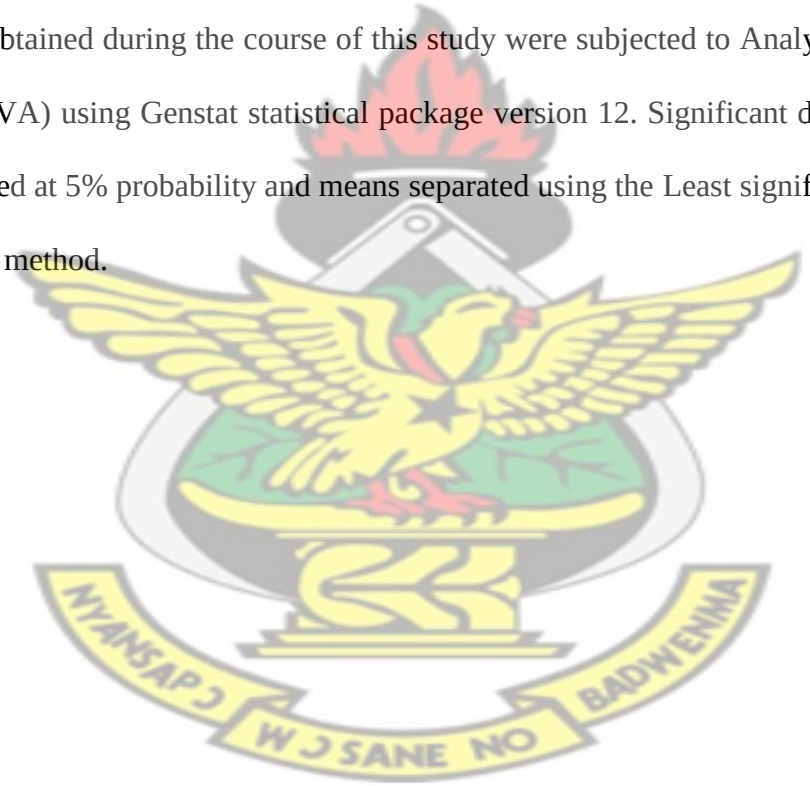
Growth parameters and nodulation were assessed eight weeks after planting. Soybean plants were carefully cut at the first node point from the soil level and over dried at 60^{o}C for 72 hours and dry weights recorded. Roots were carefully recovered from the soil and put in a 1 mm sieve and washed gently under running tap water to get rid of soils attached to it. Nodules were then detached, counted and oven dried to estimate the nodule dry weight. Shoot and root biomass accumulated was also estimated. The

values recorded for all the parameters measured represented means of two plants per pot.

Dry shoots were milled and used for analysis of total N and P in the laboratory. The macro Kjeldahl method involving digestion and distillation as described by Soil Laboratory Staff (1984) was used in the determination of %N while total phosphorus in plant samples was determined using the spectrophotometric vanadium phosphomolybdate method.

3.11 Data Analysis

Data obtained during the course of this study were subjected to Analysis of Variance (ANOVA) using Genstat statistical package version 12. Significant differences were accessed at 5% probability and means separated using the Least significant difference (LSD) method.



CHAPTER FOUR

4.0 RESULTS

4.1 Growth Characteristics of Isolates

Two hundred isolates were obtained after isolation from the nodules sampled during the bio – prospecting activity. Eighty - five out of the isolates showed characteristics of rhizobia after culturing on Congo Red Yeast Extract Mannitol agar (CR YEMA) (Table 4.1.) Out of the 85 isolates, 83% did not absorb the congo red indicator, 10% absorbed it partially and 7% showed centre absorption of the congo red YEMA media. When the isolates were cultured on bromothymol blue (BTB) YEMA media, 77% turned the indicator from green to yellow corroborating their characteristics as fast growing rhizobia while 23% turned it blue; a characteristic of slow growing rhizobia (Table 4.1).

Table 4.1. Growth Characteristics of Isolates Cultured on Congo Red and Bromothyml Blue YEMA Media

Isolate	Legume Host Genus	YMA Reaction		Isolate	Legume Host Genus	YMA Reaction	
		CR	BTB			CR	BTB
NAG 130	Caesalpinia	NA	+	NAG 155	Desmodium	NA	-
NAG 131	Mimosa	NA	-	NAG 156	Mimosa	NA	+
NAG 133	Centrosema	NA	+	NAG 157	Mimosa	NA	+
NAG 134	Centrosema	NA	+	NAG 158	Mimosa	NA	+
NAG 135	Centrosema	NA	+	NAG 159	Mimosa	NA	+
NAG 137	Crotolaria	NA	+	NAG 160	Centrosema	NA	+
NAG 138	Desmodium	NA	+	NAG 161	Mimosa	NA	+
NAG 139	Desmodium	NA	+	NAG 162	Mimosa	NA	+
NAG 140	Centrosema	NA	+	NAG 163	Centrosema	NA	+
NAG 141	Centrosema	NA	+	NAG 164	Centrosema	NA	+
NAG 142	Centrosema	NA	+	NAG 165	Desmodium	NA	+
NAG 143	Centrosema	NA	+	NAG 166	Desmodium	NA	+
NAG 144	Centrosema	NA	+	NAG 167	Desmodium	NA	-
NAG 145	Crotolaria	NA	+	NAG 168	Desmodium	NA	-
NAG 146	Crotolaria	NA	+	NAG 169	Mimosa	NA	+
NAG 148	Centrosema	NA	+	NAG 170	Mimosa	PA	+
NAG 149	Centrosema	NA	+	NAG 171	Mimosa	PA	+
NAG 150	Mimosa	NA	-	NAG 172	Desmodium	NA	+
NAG 151	Mimosa	NA	-	NAG 173	Calopogonium	NA	-
NAG 152	Mimosa	NA	-	NAG 174	Calopogonium	NA	-
NAG 153	Centrosema	NA	+	NAG 175	Desmodium	NA	-
NAG 154	Centrosema	NA	+	NAG 176	Crotolaria	NA	+

Table 4.1. Growth Characteristics of Isolates cultured on Congo Red and Bromothymol Blue YEMA Media (Continued)

Isolate	Legume Host Genus	YMA Reaction		Isolate	Legume Host Genus	YMA Reaction	
		CR	BTB			CR	BTB
NAG 177	Crotolaria	NA	+	NAG 198	Crotolaria	NA	+
NAG 178	Crotolaria	NA	+	NAG 199	Centrosema	NA	+
NAG 179	Desmodium	NA	+	NAG 200	Centrosema	NA	-
NAG 180	Desmodium	NA	-	NAG 201	Calopogonium	NA	-
NAG 181	Desmodium	NA	-	NAG 202	Calopogonium	NA	+
NAG 182	Desmodium	NA	+	NAG 203	Calopogonium	NA	+
NAG 183	Calopogonium	PA	+	NAG 204	Calopogonium	NA	+
NAG 184	Calopogonium	NA	-	NAG 205	Crotolaria	NA	+
NAG 185	Calopogonium	PA	-	NAG 206	Mimosa	CA	+
NAG 186	Calopogonium	NA	+	NAG 207	Mimosa	CA	+
NAG 187	Desmodium	NA	+	NAG 208	Mimosa	CA	+
NAG 188	Desmodium	NA	-	NAG 209	Mimosa	CA	+
NAG 189	Desmodium	NA	+	NAG 210	Mimosa	CA	-
NAG 190	Mimosa	NA	+	NAG 211	Crotolaria	NA	+
NAG 191	Desmodium	NA	+	NAG 212	Centrosema	NA	+
NAG 192	Desmodium	CA	+	NAG 213	Mimosa	PA	+
NAG 193	Desmodium	NA	+	NAG 214	Mimosa	NA	+
NAG 194	Desmodium	PA	+	NAG 215	Mimosa	PA	+
NAG 195	Centrosema	NA	+	NAG 216	Mimosa	PA	+
NAG 196	Centrosema	PA	+	NAG 217	Mimosa	NA	+
NAG 197	Crotolaria	NA	-	NAG 218	Desmodium	NA	-

NAG = N_2 Africa Ghana, NA= Not Absorbed, PA= Partially absorbed, CA= Centre Absorbed, + = Yellow, - = Blue

CR = Congo red BTB = Bromothymol blue

4.2 Authentication and Evaluation of Effectiveness of Isolates from Uncultivated Legumes Using Sterile River Sand

4.2.1 Symbiotic Nitrogen Fixing Potential of Isolates in Sterile River Sand

The eighty-five isolates that showed characteristics of rhizobia on the YEMA were further tested on soybean (Anidaso variety) for nodulation and effectiveness in fixing nitrogen.

4.2.1.1 Nodulation and Shoot Biomass Yield

Eleven isolates representing 13% (NAG 150, NAG 152, NAG 155, NAG 168, NAG 170, NAG 171, NAG 173, NAG 180, NAG 181, NAG 211, and NAG 218) out of the 85 isolates formed nodules with the test crop. The remaining isolates did not nodulate with the test crop and were thus eliminated from further consideration. Nodule numbers formed by the isolates ranged from 3.7 to 55 per pot with isolates NAG 150 and NAG 218 producing nodule numbers that were not significantly different ($p > 0.05$) from the reference strain USDA 110 (Figure 4.1). No nodulation was observed in the positive N and negative N treated plants.

Table 4.2 shows that the nodule dry weight recorded for the isolates ranged from 17 to 200 mg/pot with no significant ($p > 0.05$) differences among rhizobia isolates. Meanwhile, isolate NAG 218 produced nodule dry weight of 200 mg/pot which was 5.3% more than the dry weight of the nodules produced by the USDA 110 strain. The least nodule dry weight was recorded by isolate NAG 155. Significant differences were observed between the shoot dry weights with the -N control recording 1.4 g/pot (the least) and the +N control recording 4.8 g/pot (highest) (Table 4.2). The USDA 110 strain produced shoot dry weight that was significantly ($p < 0.05$) different from that of isolate NAG 150. The variations in the shoot dry weight of the rhizobia isolates showed a threefold difference between the highest and lowest means.

Multiple regression analysis between shoot dry weight and nodulation showed that 59% of the variation in shoot dry weight could be attributed to its association with nodule number and nodule dry weight (Table 4.3)

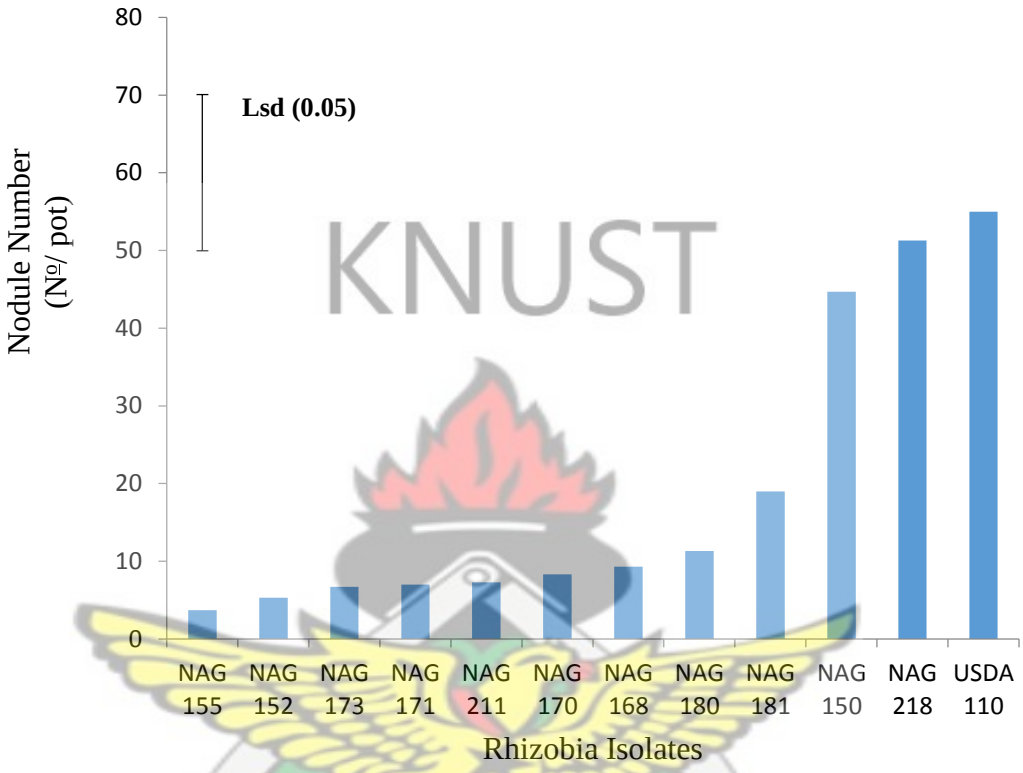


Figure 4.1. Nodule Numbers of Isolates and USDA 110 Strain

Table 4.2. Shoot and Nodule Dry Weight as Affected by Inoculation with Indigenous Isolates

Isolates	Nodule Dry Weight (mg/pot)	Shoot dry weight (g/pot)
NAG 150	140	2.6
NAG 152	100	1.5
NAG 155	17	1.7
NAG 168	133	1.7
NAG 170	117	1.6
NAG 171	147	1.4
NAG 173	117	1.6
NAG 180	130	1.8
NAG 181	157	2.3
NAG 211	100	1.8
NAG 218	200	3.4
USDA 110	190	3.7
+N	-	4.8
-N	-	1.4
F.pr(0.05)	0.202	< 0.001
LSD	106.6	1.0
CV (%)	47.7	28.0

Table 4.3. Multiple Regression of Shoot Dry Weight with Nodulation

	Coefficients	Standard Error	P-value
Intercept	1.08	0.2	< 0.001
Nodule number	0.025	0.005	< 0.001
Nodule dry weight	0.004	0.002	< 0.001

$$\text{Shoot dry weight} = 1.08\ (\pm 0.2) + 0.025\ (\pm 0.005)\text{NN} + 0.004\ (\pm 0.002)\ \text{NDW}$$

($R^2 = 0.59$, $p < 0.001$)

Where NN = Nodule number NDW = Nodule dry weight

4.2.1.2 Symbiotic Effectiveness Index of Isolates

Effectiveness of the eleven isolates that were authenticated as rhizobia were classified based on their performance compared to the USDA 110 inoculum strain expressed as a percentage (Purchino *et al.,* 2000). The data showed that, 9% of the

isolates were classified as highly effective; 36% were effective while 55% were lowly effective (Figure 4.3). The highest score of 94.5% was obtained for isolate NAG 218, performance of which was considered not different from the performance of the USDA 110.

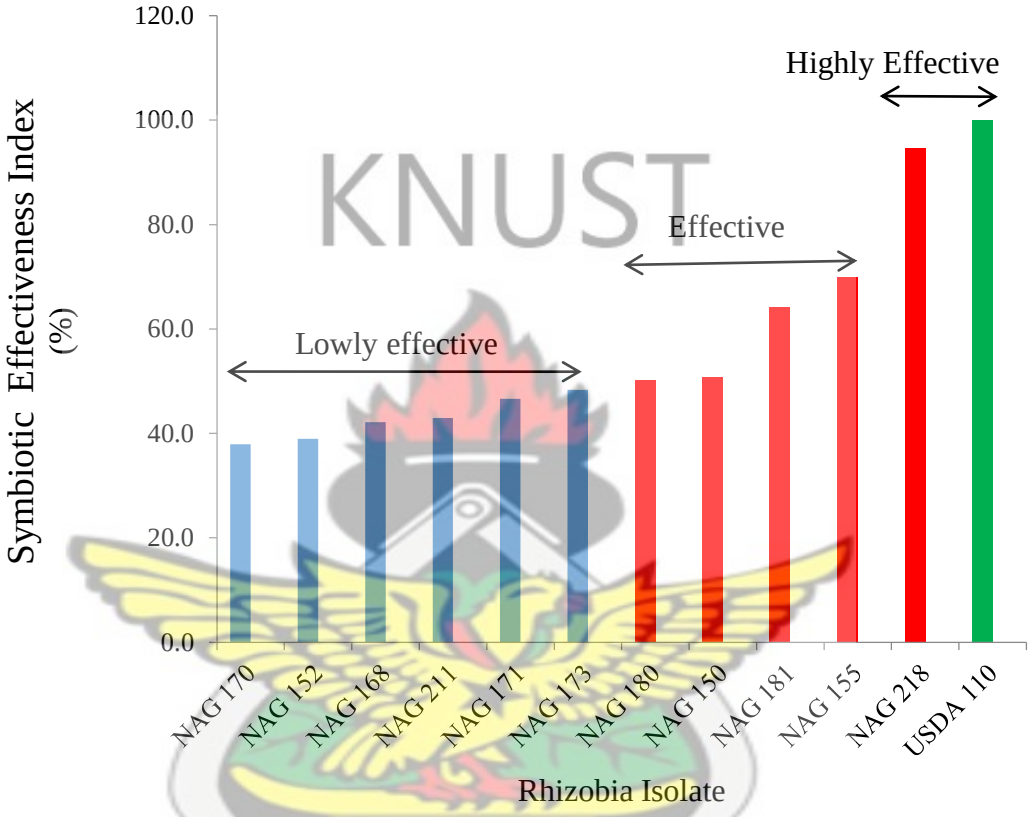


Figure 4.2. Symbiotic Effectiveness Index of Indigenous Isolates and USDA 110

4.3 Screening for Symbiotic Potential of Rhizobia Isolates Using Two Soybean Varieties Grown in Non - Sterile Soil

Two soybean varieties, Jenguma and Nangbaar, were evaluated for their response to the eleven rhizobia isolates. All the isolates were shown to induce nodule formation on the test varieties (Table 4.3). Soybean variety and rhizobia isolate interaction varied significantly for nodule dry weight but not significant for nodule number.

Jenguma produced higher nodule numbers (136.0) which was 8% more than the nodule number produced by Nangbaar variety (Table 4.3). The results of the study

further showed that, isolates NAG 152 and NAG 170 induced high nodule numbers that was not significantly ($p < 0.05$) different from the nodule numbers produced by the Legumefix inoculum strain. The lowest nodule number was produced by the positive N treated plants which was not different statistically from the nodule numbers recorded for negative N control and isolate NAG 173. Soybean variety and rhizobia isolate interaction as a result of inoculation produced no significant ($p > 0.05$) differences in nodule numbers. Notwithstanding, Jenguma and rhizobia isolate interactions produced more nodules than Nangbaar and rhizobia isolate interaction. The number of nodules (120.7) estimated in the negative N control plants for Jenguma variety was comparatively higher than the numbers (85.3) recorded for Nangbaar variety.

The highest nodule dry weight (1423 mg/pot) was obtained by Jenguma representing 20% more weight than the weight recorded for nodules of Nangbaar (1137 mg/pot). Performance of the rhizobia isolates were observed to vary significantly ($p < 0.05$) with regards to nodule dry weight with isolate NAG 152 giving the highest weight (Table 4.3). Isolate, NAG 171 produced nodule dry weight that was statistically similar to the weight produced by NAG 152. All the commercial rhizobia strains except Legumefix, produced significantly ($p > 0.05$) lower nodule dry weights compared to isolates NAG 152 and NAG 171. The nodule dry weight obtained by the Legumefix inoculum strain was statistically at par with that of isolate NAG 171. The lowest nodule dry weight was obtained by the positive N treated plants.

In Table 4.4, the interactions observed between soybean variety and rhizobia isolates in terms of nodule dry weight were highly significant ($p < 0.05$). Eighty – one percent of the association between Jenguma and isolates yielded high nodule dry weights than the Nangbaar and isolate association. Jenguma and native rhizobia

symbiosis (negative N control) produced nodules with dry weights (1617 mg/pot) that was significantly ($p < 0.05$) higher than the Nangbaar and native rhizobia symbiosis (1027 mg/pot). Isolates NAG 152, NAG 155, NAG 168, NAG 171, NAG 173 and NAG 180 and their association with Jenguma resulted in nodule dry weights that were significantly ($p < 0.05$) higher than the weights obtained from their association with Nangbaar. The symbiosis between Jenguma and USDA 110 produced significantly higher nodule dry weight than the symbiosis between Nangbaar and USDA 110. The positive N control plants produced low nodule dry weight.

Table 4.4. Symbiotic Performance of Rhizobia Isolates on Soybean Varieties

		Nodule number (N^{o}/pot)	Nodule dry weight (mg/pot)
Variety			
Jenguma		136.0	1423
Nangbaar		125.9	1137
Isolates			
NAG 150		129.8	1343
NAG 152		147.7	1412
NAG 155		140.7	1353
NAG 168		132.8	1373
NAG 170		147.3	1312
NAG 171		140.0	1400
NAG 173		99.8	1325
NAG 180		138.3	1395
NAG 181		133.2	1267
NAG 211		139.8	1342
NAG 218		137.8	1290
USDA 110		121.3	1252
BIOFIX		131.0	1032
LEGUMEFIX		166.3	1265
N -		103.0	1322
100 kg N		86.3	797
Fpr	Variety	0.028	< 0.001
	Isolates	< 0.001	< 0.001
	Interactions (variety and isolate)	0.318	< 0.001
LSD	Variety	9.0	50.8
	Isolates	25.4	143.6
	Interactions (variety and isolate)	35.9	203.1
CV (%)		16.8	9.7

Table 4.5. Effect of Soybean Varieties and Rhizobia Isolates Interaction on Nodule Dry Weight

	Nodule dry weight (mg/pot)	
Isolates	Jenguma	Nangbaar
NAG 150	1513	1173
NAG 152	1563	1260
NAG 155	1570	1137
NAG 168	1540	1207
NAG 170	1443	1180
NAG 171	1557	1243
NAG 173	1563	1087
NAG 180	1590	1200
NAG 181	1497	1037
NAG 211	1357	1327
NAG 218	1407	1173
USDA 110	1463	1040
BIOFIX	983	1080
LEGUMEFIX	1297	1233
N -	1617	1027
100 kg N	810	783
LSD	203.1	203.1

4.3.1 Evaluation of Effectiveness of Isolates on Growth Parameters under Greenhouse Conditions

4.3.1.1 Shoot Biomass Yield

Table 4.5 shows the shoot biomass produced by the soybean varieties following inoculation. Nangbaar produced higher (22.60 g/pot) shoot dry weight which was significantly ($p < 0.05$) greater than the quantity produced by Jenguma (22.1 g/pot). Effect of rhizobia isolate inoculation showed that the positive N control produced the highest shoot biomass yield of 29.2 g/pot.

Isolates NAG 168 and NAG 211 inoculated plants produced shoot dry matter that was comparable statistically to the amount produced by the Legumefix inoculum strain and USDA 110. The shoot biomass yield produced by the Biofix inoculum

strain did not differ significantly from that of the Legumefix strain. The interaction between soybean variety and rhizobia isolates did not result in any significant ($p > 0.05$) differences in shoot biomass.

4.3.1.2 Root Biomass Yield

The root dry weight produced by the two varieties varied significantly with Jenguma producing more root dry weight (6.0 g/pot) than Nangbaar (5.4 g/pot) (Table 4.5). Inoculation with rhizobia isolates resulted in significant differences in root dry weight. Isolates NAG 173 and NAG 211 produced high root dry matter that were statistically at par with the root dry matter recorded for Biofix and Legumefix inoculant strains. The highest root dry matter was produced by the positive N (100 kg N/ ha) treated plants which was significantly higher than the root dry matter recorded for all the isolates. The USDA 110 inoculum strain produced root biomass that was statistically lower than the biomass produced by Biofix but similar to the performance of the Legumefix strain.

The interaction between soybean variety and rhizobia isolates was significantly different in terms of root dry biomass (Table 4.6). Jenguma variety and rhizobia isolate interaction produced root dry matter that was significantly higher than Nangbaar variety and rhizobia isolate interaction. The highest root biomass was produced by the positive N (100 kg N/ha) treated plants for Jenguma which was statistically higher than the root dry matter accumulated by the positive N (100 kg N/ha) for Nangbaar. Isolates NAG 180 and NAG 211 and their interactions with Jenguma produced significantly ($p < 0.05$) higher root dry matter than their interactions with Nangbaar. The root dry matter produced by Biofix strain and Jenguma interaction was significantly ($p < 0.05$) higher than the Biofix strain and Nangbaar interaction.

Table 4.6. Shoot and Root Biomass Yield as Affected by Rhizobia Inoculation

		Shoot dry weight	Root dry weight
		(g/pot)	
Variety			
Jenguma		22.1	6.0
Nangbaar		22.6	5.4
Isolates			
NAG 150		21.5	5.4
NAG 152		21.4	5.6
NAG 155		21.8	5.4
NAG 168		22.2	5.4
NAG 170		21.5	5.2
NAG 171		21.1	5.4
NAG 173		21.2	6.0
NAG 180		21.1	5.3
NAG 181		21.2	5.2
NAG 211		22.6	6.0
NAG 218		21.5	5.5
USDA 110		22.3	5.3
BIOFIX		23.8	6.1
LEGUMEFIX		23.2	5.8
N -		22.3	5.5
100 kg N		29.2	7.7
Fpr	Variety	0.038	< 0.001
	Isolates	< 0.001	< 0.001
	Interactions (Variety and Isolate)	0.93	0.014
LSD	Variety	0.4	0.3
	Isolates	1.2	0.8
	Interactions (Variety and Isolate)	1.8	1.1
CV (%)		4.8	11.9

Table 4.7. Root Dry Matter Yield of Soybean Inoculated with Rhizobia Isolates

	Root dry weight (g/pot)	
Isolates	Jenguma	Nangbaar
NAG 150	5.093	5.703
NAG 152	5.55	5.59
NAG 155	5.41	5.363
NAG 168	5.51	5.287
NAG 170	5.263	5.08
NAG 171	5.387	5.41
NAG 173	6.453	5.523
NAG 180	6.173	4.47
NAG 181	5.297	5.177
NAG 211	6.68	5.36
NAG 218	5.873	5.193
USDA 110	5.723	4.853
BIOFIX	6.91	5.313
LEGUMEFIX	6.203	5.443
N -	5.793	5.227
100 kg N	8.373	7.05
LSD	1.1	1.1

4.3.2 Shoot Nitrogen and Phosphorus Partitioning in Soybean Varieties

4.3.2.1 Shoot Nitrogen Uptake

Table 4.7 shows the nitrogen (N) and phosphorus (P) partitioning in shoots of soybean after inoculation and basal application of P. Shoot N uptake showed no significant ($p > 0.05$) differences between soybean varieties neither was there significance ($p > 0.05$) between soybean variety and rhizobia isolate. Inoculation with rhizobia isolates significantly influenced shoot N uptake with Biofix inoculum strain inducing the highest shoot N uptake (46.9 g/pot). The positive N control obtained shoot N that was not statistically different from the uptake recorded for Biofix strain. Isolates NAG 155 and NAG 168 induced shoot N uptake that was

statistically similar to the shoot N uptake induced by the positive N control treatment, Legumefix inoculum strain and the USDA 110.

4.3.2.2 Shoot Phosphorus Uptake

Total shoot P uptake was significantly ($p < 0.05$) different in terms of soybean varieties, rhizobia isolates and their interactions as shown in Table 4.7. Nangbaar recorded total shoot P uptake of 3.1 g/pot which was significantly ($p < 0.05$) higher than the uptake for Jenguma (2.4 g/pot). Effect of rhizobia inoculation produced significant ($p < 0.05$) differences in P contents of soybeans with isolate NAG 211 inoculated plants recording the highest P uptake which was not different statistically from the uptake in the positive N treated plants. All the inoculant strains except USDA 110 produced shoot P uptake that were statistically at par with the uptake of the positive N treated plants and isolate NAG 211. Table 4.8 shows shoot P uptake by soybean and rhizobia isolate inoculation. Soybean variety and rhizobia isolate interaction showed significant ($p < 0.05$) differences in shoot P uptake. The highest P uptake was produced by Nangbaar and NAG 181 symbiosis and was statistically similar to the uptake recorded by the positive N treated plants for Nangbaar.

Table 4.8. Effect of Inoculation and Basal Phosphorus Application on Shoot N and P Uptake

		Shoot N uptake (g/pot)	Shoot P uptake (g/pot)
Variety			
Jenguma		40.8	2.4
Nangbaar		40.9	3.1
Isolates			
NAG 150		37.9	1.8
NAG 152		40.7	1.7
NAG 155		41.5	2.2
NAG 168		41.6	3.3
NAG 170		39.4	2.2
NAG 171		37.9	1.9
NAG 173		40.9	1.9
NAG 180		39.0	2.8
NAG 181		39.8	3.4
NAG 211		40.2	3.6
NAG 218		39.8	3.1
USDA 110		41.6	2.8
BIOFIX		46.9	3.0
LEGUMEFIX		39.8	3.3
N -		40.6	3.3
100 kg N		46.3	3.6
Fpr	Variety	0.42	< 0.001
	Isolates	0.01	< 0.001
	Interactions (Variety and Isolate)	0.92	< 0.001
LSD	Variety	1.6	0.3
	Isolates	4.9	0.8
	Interactions (Variety and Isolate)	6.3	1.1
CV (%)		9.4	24.5

Table 4.9. Effect of Soybean Variety and Rhizobia Isolate Interactions on Shoot P Uptake

	Shoot P uptake (g/pot)	
Isolates	Jenguma	Nangbaar
NAG 150	0.8	2.9
NAG 152	1.7	1.7
NAG 155	1.2	3.2
NAG 168	2.9	3.7
NAG 170	2.0	2.4
NAG 171	1.9	1.9
NAG 173	1.2	2.6
NAG 180	2.3	3.3
NAG 181	2.8	4.0
NAG 211	3.4	3.8
NAG 218	3.1	3.1
USDA 110	2.7	3.0
BIOFIX	3.7	2.4
LEGUMEFIX	3.5	3.1
N -	3.6	3.0
100 kg N	2.3	4.9
LSD	1.1	1.1

4.4 Assessment of Competitiveness of Some Selected Effective Isolates

4.4.1 Nodulation of Nangbaar as Induced by Selected Effective Rhizobia at Three Different Cell Concentration Levels

Table 4.9 shows that the nodule numbers produced by the various isolates at different cell concentration levels did not vary significantly ($p > 0.05$). Nevertheless USDA 110 and isolate 218 at the 10^4 - CFU concentration produced relatively higher nodule numbers than their corresponding 10^6 and 10^8 cell concentrations. The positive N treated plants produced the least number of nodules. The negative N control plants produced relatively more nodules than some inoculated plants

Nodule dry weight, showed significant variations as a result of inoculation with selected effective rhizobia at different cell concentration levels. The USDA 110 at 10^4 CFU produced the highest nodule dry weight which was only significantly ($p < 0.05$) higher than the nodule dry weights recorded for isolates NAG 211 at 10^4 – CFU, NAG 150 at 10^4 – CFU levels and the positive N treated plants. Isolate NAG 181 at all the three cell concentration levels obtained nodule dry weights that was statistically similar to the nodule dry weight obtained by USDA 110 at 10^4 – CFU level. The lowest nodule dry weight was produced by the positive N control.

Table 4.10. Nodulation Performance of Selected Effective Rhizobia Isolates

Isolates	Cell Concentration levels (CFU)	Nodule Numbers (Nº / pot)	Nodule Dry weight (mg/ pot)
NAG 218	10^8	66.3	983
	10^6	69.3	1040
	10^4	84.3	927
NAG 150	10^8	94.0	957
	10^6	82.3	1073
	10^4	78.3	893
NAG 181	10^8	86.7	1133
	10^6	90.0	1107
	10^4	83.7	1093
USDA 110	10^8	84.3	1033
	10^6	75.0	993
	10^4	95.7	1153
+ N	+ 100 kg N	49.0	477
- N	-	80.0	1013
Fpr		0.078	< 0.001
LSD		25.67	207.8

4.4.2 Effect of Inoculation on Shoot and Root Dry Weights

Table 4.10 shows the mean shoot and root dry weights of inoculated and uninoculated soybean. Inoculation with USDA 110 at 10^4 – CFU level produced significantly ($p < 0.05$) higher shoot biomass than all the other treatments except USDA 110 at 10^6 – CFU level. Isolate NAG 181 at 10^4 and 10^6 – CFU levels produced shoot dry matter that were not statistically different from the biomass recorded for USDA 110 at 10^6 – CFU level; the lowest shoot dry matter was obtained by isolate NAG 150 at 10^8 CFU concentration. Significant ($p < 0.05$) differences were observed between the various treatments in terms of root dry matter with the positive N control producing the

highest root dry matter. NAG 150 at 10^8 concentration produced root biomass that was statistically the least among the treatments (Table 4.9).

Table 4.11. Rhizobia Isolate Inoculation and Its Effect on Shoot and Root Biomass Yield

Isolates	Cell Concentration levels	Shoot dry weight (g / pot)	Root dry weight (g / pot)
NAG 218	10^8	17.09	5.76
	10^6	17.07	5.42
	10^4	17.05	4.81
NAG 150	10^8	15.10	4.25
	10^6	16.36	4.83
	10^4	16.80	5.09
NAG 181	10^8	17.00	4.83
	10^6	17.51	4.55
	10^4	16.07	4.72
USDA 110	10^8	16.61	4.59
	10^6	18.65	4.73
	10^4	19.44	5.53
+ N	+ 100 kg N	26.18	7.51
- N	-	16.95	5.17
Fpr		< 0.001	< 0.001
LSD		1.89	0.83

CHAPTER FIVE

5.0 DISCUSSION

5.1 Growth Characteristics of Isolates after Culturing

Rhizobia have been reported to show characteristics such as typically translucent, viscid and slimy growth on yeast mannitol agar media with individual colonies having domed shape and elevated features with entire margins (Gupta *et al.*, 2007) and also not absorbing congo red indicator. The large proportion of isolates that did not absorb congo red (Table 4.1) in this study and gave a good indication that the isolates obtained could be rhizobia. Notwithstanding, a few of the isolates also absorbed the congo red indicator which according to Somasegaran and Hoben (1994) is a characteristic of some *Sinorhizobium meliloti* strains.

Growth characteristics of isolates cultured on BTB yeast mannitol agar media showed that 77% of the isolates were fast growers while 23% were slow growers (Table 4.1). Maria Cristina and Norma (1997) reported the occurrence of both fast and slow growing strains for many genera of tropical legumes.

5.2 Authentication and Evaluation of Isolates from Uncultivated Legumes under Greenhouse Conditions

For a total of 85 isolates assessed for their symbiotic potential, eleven exhibited capacities to induce nodule formation on soybean indicating that they were infective (Figure 4.1). Infectivity (the ability to form nodules) and symbiotic effectiveness (capacity to fix nitrogen) are the two features commonly used to assess the ecological and evolutionary relationship between rhizobia and their host (Brockwell, 1998). The remaining 74 isolates though with similar characteristics to that of the 11 failed to nodulate the test crop probably because of limited compatibility with the soybean

variety used in the authentication experiment. It can thus be said that the isolates that were not infective may be rhizobia that are not compatible with the soybean variety used. Rhizobia lacking genes for infecting legumes are common in rhizosphere of some suitable host legumes (Sullivan *et al*., 1996). Figure 4.1 shows that infective isolates formed nodules (between 3.7 and 55/ pot) on the test crop. The difference in numbers can be attributed to the considerable variation in infectivity, nodulation and possibly effectiveness of the test isolates. According to Mpepereki *et al. (*1996) tropical rhizobia are diverse with sub-groups of varied symbiotic specificity and effectiveness.

Significant differences were also observed between the shoot dry weights (Table 4.2) of the various isolates which is indicative of a wide variation in effectiveness of symbiotic interactions. A significant positive linear relationship existed between nodule number, nodule dry weight and shoot dry weight (Table 4.3).It was however noted from the coefficients that, nodule number influenced shoot dry weight more than nodule dry weight. This observation was in disparity with the reports of Hefny *et al.* (2001) who found that nodule number was not an appropriate trait for selection of the most effective N_2 fixing association. Shoot dry weight has been reported severally as a good indicator of relative strain effectiveness; there is also a good correlation between shoot dry matter production and nitrogen fixation capacity of legumes grown under low soil N conditions (Somasegaran and Hoben 1994; Peoples *et al.*, 2002).

The symbiotic effectiveness index (SEI) estimated in this study showed that, 9% of the isolates were highly effective while 36% were effective and 55% were lowly effective (Figure 4.3). The highest SEI of 94.5% recorded for isolate NAG 218 isolated from *Desmodium* spp in Tamale in the Northern region of Ghana was ranked as highly effective as commercial strain USDA 110. This observation affirms the

statement made by Terpolilli *et al.* (2008) that the efficiency in nitrogen fixing symbioses can vary from those that fix little nitrogen to those that fix nitrogen levels equivalent to or even greater than plants treated with mineral N. The result of this study generally suggests the possibility of obtaining elite rhizobia from uncultivated legumes for soybean production.

5.3 Performance of Indigenous Rhizobia Isolates on Two Soybean Varieties Grown in Potted Non – Sterile Soils

Nodulation capacity according to Hansen (1994), is known to vary between and within legume species. In Table 4.3 Jenguma (110 -120 days to maturity) was observed to produce significantly ($p < 0.05$) more nodule number and a relatively higher nodule dry weights of nodules than Nangbaar (90 days to maturity). This observation corroborates with the findings of Balatti and Pueppke (1992) who while studying the efficiency of soybean genotypes to nodulate with fast growing *Ensifer* found that the length of genotype life cycle was correlated with its ability to develop nitrogen fixing nodules or inefficient nodules.

Rhizobial inoculation elicited significant differences in nodule number and nodule dry weight (Table 4.4) which is in line with the findings of Katulande (2011). According to Amerger and Lobreau (1982), a legume may favour a number of rhizobia strains to form nodules leading to difference in nodulation competitiveness. The choice made by the host is not dependent on the N-fixing ability of the strain. In this study, introduced indigenous isolates that produced higher number of nodules were not relatively more effective than those that produced less number of nodules (Table 4.3). Abd El-Maksoud and Keyser (2010) reported similar findings stating that, a great number of nodules may be formed by a strain fixing little or no nitrogen even in the presence of effective strains. Nodule dry weight estimated in the negative N control

treatment (uninoculated and unfertilized) as a result of the presence of native rhizobia for both varieties was significantly different ($p < 0.05$) with Jenguma producing relatively more nodule dry weights than Nangbaar. This signifies that, competition between introduced rhizobia (isolates) and the native rhizobia in the inoculated treatments could be intense. The greater number of nodules and the subsequent high nodule dry weights produced by the soybean variety, Jenguma in the negative control treatment suggests that it is more promiscuous and compatible with the native rhizobia (Musiyiwa *et al.*, 2005) than Nangbaar variety. Rhizobia population of the experimental soil was estimated to be 81.3 cells/ g of soil. According to Thies *et al.* (1991) positive response to rhizobia inoculation and the ability of the introduced strain to compete with and overcome the indigenous rhizobia is inversely related to the number of indigenous rhizobia. The nitrogen fertilized control recorded significantly low nodule number and nodule dry weight. Availability of sufficient mineral N in the positve control caused a reduction in the symbiosis between soybean variety and native rhizobia which led to a subsequent reduction in nodulation. Similar findings have been reported by Uddin *et al.* (2008) who stated that, the application of mineral nitrogen fertilizer (urea) significantly inhibited nodule numbers.

Soybean variety and rhizobia isolate interaction produced significant difference ($p < 0.05$) in nodule dry weight (Table 4.4) but not in nodule number (Table 4.3) which suggest that rhizobia inoculation may not be the explanation for the significantly ($p < 0.05$) different number of nodules produced by the varieties . This further confirms that the ability of the plant to nodulate is defined by the plant genotype (Salvucci *et al*., 2011). Nodule dry weight showed significant ($p < 0.05$) differences between the two varieties after inoculation. Studies by Bourion *et al.* (2007) showed that variation in plant physiology and in root and nodule morphogenesis between pea genotypes

may result in variation of signal exchange between the bacteria and plant partners which may lead to differences in efficiency of interaction with rhizobia species. Rengel (2002) reported that there is generally a gene(s) – for – gene interaction between rhizobia and the host. Isolates NAG 152, NAG 155, NAG 168, NAG 171, NAG 173 and NAG 180 and their symbiosis with Jenguma produced significant differences in nodule dry weight (Table 4.4). This could be explained by the specificity between soybean lines and rhizobia isolates as reported by (Gandanegara *et al*., 1992).

Nangbaar recorded 2.3% more shoot dry matter than Jenguma (Table 4.5). Genetic variations in biomass and seed yield of varieties have been reported by Hungaria and Bohrer (2000). Significant differences between rhizobial strains for nodule dry weight and shoot biomass have been reported for soybean under growth room, greenhouse and phytotron conditions (Appunu and Dhar, 2006). Though no significant ($p > 0.05$) difference was observed in shoot biomass yield for soybean variety and rhizobia isolate interaction, most of the association between Nangbaar and rhizobia isolates produced relatively more shoot biomass than Jenguma. The positive N treated plants produced the highest shoot dry weights which was not significantly different from the biomass obtained by Biofix. Abayomi *et al.* (2008) reported a significant increase in dry matter due to mineral nitrogen application to cowpea.

Table 4.5 shows that, Nangbaar variety recorded significantly ($p < 0.05$) lower root dry matter that was 11% less than the root dry weight produced by Jenguma. Significant differences were also observed between the performances of the isolates and soybean variety and isolate interaction on root biomass yield. According to Vessey and Layzell (1987), high levels of nitrogen in the root zone can increase yield

through more favourable partitioning of dry matter to the shoots than to the roots. This is affirmed by the high shoot dry matter recorded for Nangbaar.

Nutrient uptake results showed no significant ($p > 0.05$) differences between the soybean varieties and also their interaction with the indigenous isolates, notwithstanding, Nangbaar (90 days to maturity) partitioned relatively more of its N into shoot than Jenguma (105 – 110 days to maturity). This finding is in contrast with the report made by Abaidoo *et al.* (1999) that N_2 fixation increases with increasing crop duration (days to maturity), because longer growth duration allows for longer period of N_2 fixation. The main effect of inoculation induced significant differences in nitrogen uptake with isolates NAG 155 and NAG 168 resulting in a high shoot N uptake that was not statistically different ($p > 0.05$) from the uptake recorded in the positive N control, legumefix and biofix (Table 4.8). Date (2000) explained that inorganic fertilizer treated plants (positive controls) are included in an experiment to show that the legume has a potential to grow well with adequate amounts of nitrogen and that the growth is limited by factors other than N.

According to Weber *et al.* (1996), the required amount of phosphorus for optimal growth and N_2 fixation of legumes is 30 kg P ha^{-1}. The physical and chemical properties of the soil used for this experiment showed that 37.3 mg / kg P was available for plant growth which according to (Boerma *et al.*, 1995) was moderate. This notwithstanding, 30 kg P ha^{-1} (0.075 g/ 5 kg KH_2PO_4) was applied as basal fertilizer to the soybean varieties, to eliminate any possible P deficiency situation. Nangbaar recorded 29% more shoot P uptake with subsequent increased shoot biomass than Jenguma which indicates that different varieties responded differently to the additional basal phosphorus applied. Increased soil phosphorus supply has been

noted to increase whole plant growth and plant nitrogen concentrations in several leguminous species including soybean (Israel, 1993).

5.4 Competitiveness of Selected Effective Isolates in Non – Sterile Soil

According to Brockwell *et al.* (1995), the best way to establish a new strain of rhizobia amongst a naturally – occurring population is to apply a heavy rate of effective, persistent inoculum. Based on this, broth cultures of the selected effective isolates were prepared at three different cell concentrations levels; 10^8 - CFU (the heavy rate), 10^6 - CFU (recommend rate) and 10^4 - CFU (below recommended rate) (Table 3.2)

The term `competition', when used for the *Rhizobium* spp., generally implies competition for nodule formation between the various *Rhizobium* strains from the moment these strains are present in the same environment, until the moment of their presence inside the nodules (Simon *et al*., 1996). For inoculant rhizobia to dominate nodulation, it is of utmost importance that they have strong nodulation effectiveness for the host being grown and also well adapted to the relevant soil conditions. Rengel (2002) indicated that better rhizobial symbiosis can be achieved by more effective rhizobia that will establish well in the soil and the rhizosphere (saprophytic competence), cause greater nodulation, occupy a greater proportion of nodules, and have greater activity of nitrogenase and associated enzymes. Comparism between the different cell concentration rates for nodule numbers showed no significant ($p > 0.05$) differences (Table 4.9). Notwithstanding, the USDA 110 at 10^4 - CFU and isolate NAG 218 at 10^4 - CFU produced more nodules than their corresponding increased rates (10^6 - CFU and 10^8 - CFU). This observation is in disparity with the report made by Brockwell *et al.* (1995) that, linear relationships exist between the rhizosphere population of rhizobia and nodulation as well as plant growth. Nodule number

recorded for the negative N control was not significantly ($p > 0.05$) different from the plants inoculated with effective isolates; indicating that a high competition occurred between the native rhizobia in the soil and the introduced isolates albeit the large numbers of the introduced isolates. Amarger (1981) also demonstrated that competitive success in forming nodules was a characteristic of each strain and was independent of the level of effectiveness. Table 4.9 showed that nodule dry weight recorded varied significantly ($p < 0.05$) between nitrogen sources (isolates) but followed the same trend as nodule number which further confirms the strong competition imposed by the native rhizobia in the experimental soil

Table 4.10 shows the significant differences in shoot dry weights as influenced by the different nitrogen sources with the positive control recording the highest. It can thus be said that the rhizobia isolates population and nodulation as well as plant growth are inversely related. According to McLoughlin *et al*. (1990) the number of inoculant bacteria in comparison with native rhizobia is not the most important parameter of competitiveness because other more qualitative criteria (rhizobia mobility) may influence their capacity to nodulate lateral roots in addition to the crown where the frequency of nodulation is the greatest due to inoculum most frequently being applied to seed. According to Olsen *et al.* (1996), 10^6 rhizobia per seed is considered to be the minimum standard because only a portion of the applied inoculum will still be associated with the seed after planting. This could mean that the proportion of cells in the broth culture of 10^4 - CFU retained on the seeds were not different from the amount retained from 10^6 and 10^8 - CFU.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Out of the 85 indigenous isolates obtained, thirteen percent (13%) were infective. Isolates obtained from *Desmodium* (NAG 155, NAG 180, and NAG 181) and *Mimosa* (NAG 150, NAG 218) species were effective; isolate NAG 218 was as highly effective as USDA 110 strain on the basis of their symbiotic effectiveness index. Testing the effectiveness of the indigenous isolates in the presence of native rhizobia in non – sterile soil revealed significant ($p < 0.05$) differences in the performance of the test isolates on the two promiscuous soybean varieties used. Isolates NAG 152 produced nodule dry weight which was 11.6% more than that of Legumefix strain while NAG 171 induced nodule dry weight that was statistically at par with the dry weight produced by the Legumefix inoculant strain. In terms of shoot dry weight, NAG 168 and NAG 211 performed statitistically similar to Legumefix inoculum strain and USDA 110. Jenguma and Nangbaar showed significant variations in nodulation. This study has demonstrated based on nodule dry weight and shoot dry matter yields that isolates NAG 152, NAG 168, NAG171 and NAG 211 obtained from *Mimosa, Desmodium and Crotolaria* spp, formed effective symbiosis with promiscuous soybeans contradicting the null hypothesis that rhizobia strains that are associated with wild legumes cannot form effective symbiosis with promiscuous soybean varieties. The competitiveness study showed that highly effective isolates at high cell concentration levels were not necessarily competitive. Effectiveness assessment also proved that, isolates NAG 152, NAG 168, NAG 171 and NAG 211 have the potential to nodulate promiscuous soybean and their performance was at par with some existing commercial inoculant strains (Legumefix, Biofix and USDA 110)

6.2 Recommendation

Field testing of the best performing isolates under a wide spectrum of environmental conditions, which is the last stage in the rhizobia selection process should be conducted to obtain candidate strains for local inoculum production

Further research such as molecular characterization of the identified and potential *Brady rhizobium* isolates (NAG 152, NAG 168, NAG 171 and NAG 211) should be carried out to establish their identities.

Bio – prospecting for effective native rhizobia should be intensified in order to increase the chances of obtaining local strains with superior nitrogen fixing potential.

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APPENDICES

Appendix 1. Components of Yeast Mannitol Media

Chemicals	**Yeast Mannitol Agar**	**Yeast Mannitol Broth**
	Quantity Measured g/L	
K_2HPO_4	0.5	0.5
$MgSO_4.7H_2O$	0.2	0.2
NaCl	0.1	0.1
Yeast extract	0.5	0.5
Mannitol	10	10
Agar	15	-
Distilled water	1000 L	1000 L

(Vincent, 1970)

Appendix 2. Broughton and Dilworth N - free Nutrient Solution

Stock Solutions	Element	M	Form	g/L	M
1	Ca	1000	$CaCl_2 \cdot 2H_2O$	294.1	2.0
2	P	500	KH_2PO_4	136.1	1.0
3	Fe	10	Fe-citrate	6.7	0.02
	Mg	250	$MgSo_4 \cdot 7H_2O$	123.3	0.5
	K	250	K_2SO_4	87.0	0.5
	Mn	1	$MnSO_4 \cdot H_2O$	0.338	0.002
4	B	2	H_3BO_3	0.247	0.004
	Zn	0.5	$ZnSO_4 \cdot 7H_2O$	0.288	0.001
	Cu	0.2	$CuSO_4 \cdot 5H_2O$	0.100	0.0004
	Co	0.1	$CoSO_4 \cdot 7H_2O$	0.056	0.0002
	Mo	0.1	$Na_2MoO_2 \cdot 2H_2O$	0.048	0.0002