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NODULATION AND NITROGEN FIXATION OF INDIGENOUS RHIZOBIA IN SOILS CULTIVATED AND UNCULTIVATED WITH AFRICAN YAM BEAN (*SPHENOSTYLIS STENOCARPA*)

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN SOIL SCIENCE.

INTEGRI PROCEDAMUS

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OCTOBER, 2015

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DECLARATION

I hereby declare that, except for references to other works which have duly been cited this work is as a result of my own research and it has not been presented either in whole or part to any University for the award of a degree.

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DEDICATION

I dedicate this work to my former supervisor, Prof. S. K. A. Danso (late) who took a special interest in my MPhil education. Prof., may your soul rest in perfect peace.

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Ebenezer, that is how far the Lord has brought me and I thank Him for a successful completion of this work. I am sincerely grateful and appreciative to my principal supervisor, Dr. (Mrs.) Stella Asuming-Brempong for her love, encouragement, patience and research directions for the successful completion of this work. I am also thankful to my co-supervisor, Dr. I. Y. D. Lawson for his support, encouragement and research direction for the completion of this work.

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ABSTRACT

The association between leguminous plants and rhizobia helps to meet the limiting nitrogen in the soil by producing about 40 to 60 million metric tons (Mt) per hectare per year of nitrogen. However, the focus of legume-*Rhizobium* symbiosis has been on biomass productivity, nodulation and nitrogen fixation of the major and domesticated food legumes whereas little research has been conducted on the indigenous legumes which hold promise in retaining agricultural diversity and achieving food security. Among these are the underutilized, neglected and unknown ones which include African yam bean (*Sphenostylis stenocarpa*). Further research into this crop is necessary to explore its biological nitrogen fixation and nutritional capabilities. This study was carried out to investigate the nodulation and nitrogen fixing capacity of the indigenous rhizobia in African yam bean (AYB) from some Ghanaian soils (Adenta series, Bekwai series, Nzima series and soils from Akorviefe, Dzolokpuita and Dzologbogame also known as Kuli series) that have been cultivated and uncultivated with African yam bean. Secondly, the study was to assess the response of AYB to inoculation and nitrogen fertilization. Results from Most probable number estimates showed that Bekwai series did not record any African yam bean nodulating rhizobia (0 cells g^{-1} soil), whereas the other five soils recorded variable numbers of rhizobia. Soils from Akorviefe (Kuli series) recorded the highest African yam bean nodulating rhizobia (290 cells g^{-1} soil), followed by soil from Dzolokpuita (Kuli series, 140 cells g^{-1} soil), Adenta series (72 cells g^{-1} soil) with soil from Dzologbogame (Kuli series) and Nzima series recording the least number (52 cells g^{-1} soil). The results of symbiotic effectiveness studies showed that there were no highly effective rhizobia present in the soils tested but moderate and ineffective rhizobia strains were observed. Soils not cultivated with African yam bean (Adenta, Toje, Akuse and Haatso) recorded 87% ineffective and 13%

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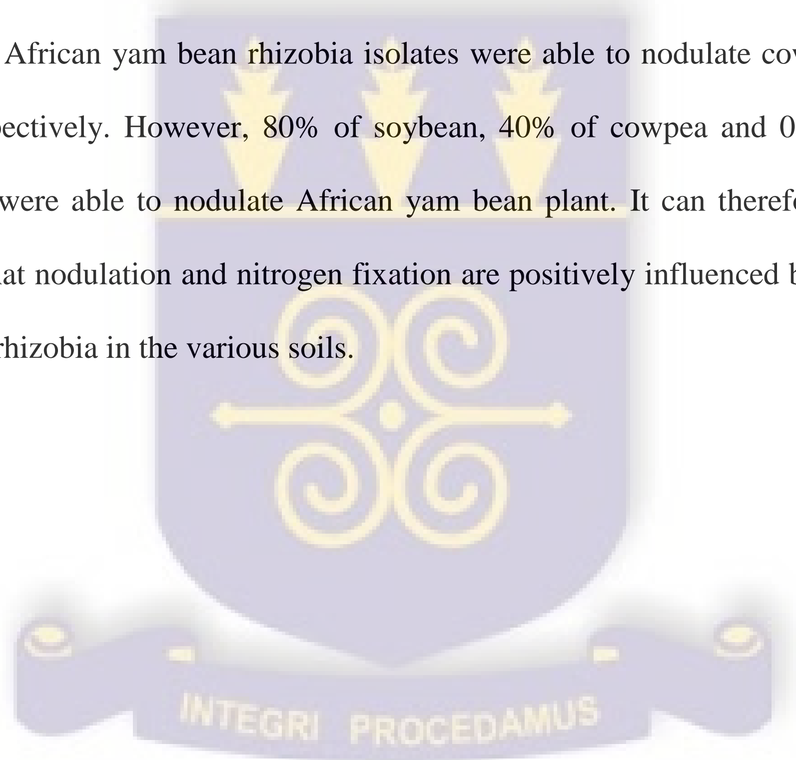
moderately effective rhizobia whereas soils cultivated with African yam bean (soils from Akorviefe, Dzolokpuita and Dzologbogame [Kuli series]) recorded 97% ineffective and 3% moderately effective rhizobia. Nitrogen application at the rate of 70 kg N ha^{-1} had a negative effect on nodule numbers, nodule dry weight and N_2 fixation whereas positive effect was observed for total N accumulation and shoot biomass in all the soils. In contrast, inoculum application had a positive effect on nodule numbers, nodule dry weight, N_2 fixation, N accumulation and shoot biomass in all the soils used. Cross inoculation results showed that 56%, 33% and 11% of African yam bean rhizobia isolates were able to nodulate cowpea, pigeon pea and soybean respectively. However, 80% of soybean, 40% of cowpea and 0% of pigeon pea rhizobia isolates were able to nodulate African yam bean plant. It can therefore be concluded from this study that nodulation and nitrogen fixation are positively influenced by the indigenous Africa yam bean rhizobia in the various soils.



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CHAPTER ONE

1.0 INTRODUCTION

The third largest flowering plants are legumes (Singh *et al.,* 2007) and they belong to the family *Fabaceae* or *Leguminosae*. The family includes the major grain legumes, oilseed, forage crops, ornamentals, medicinal and agroforestry species (Lewis *et al.,* 2005). Singh *et al.* (2007) documented that legumes are important and form an integral part of human civilization. Grain legumes play important roles in the nutrition of many people and the protein content ranges from 17% to 40% in contrast to that of cereals and meat being 7-13% and 18-25%, respectively (Genovese and Lajolo, 2001). These legumes are widely grown in Africa as multipurpose crops where the leaves are eaten raw or cooked, the grains eaten green or dried and the haulms used as forage (Sprent *et al.,* 2009).

Legumes are unique in that they have the ability to nodulate and fix atmospheric nitrogen with compatible bacteria (rhizobia) aside its high protein content (Graham and Vance, 2003). Nitrogen is a primary limiting plant nutrient in the soil but by the association between legumes and root nodule bacteria (rhizobia) the nitrogen requirement is met to an extent (Vitousek *et al.,* 1997). This symbiotic association produces free available nitrogen which is estimated as 40 to 60 million metric tons (Mt) per hectare per year (Smil, 1999). The legume-*Rhizobium* symbiosis emphasis has been on biomass productivity, nodulation and nitrogen fixation of major and domesticated food legumes. However, for the indigenous legumes, that are vast in Africa ranging from large rainforest trees to small herbs that produce edible seeds and tubers and are capable of expanding the food basket of Africans, there has been little research focus on them (Mapfumo *et al*., 1999; Lock, 1989). Indigenous legumes have several uses and parts of the plant can be consumed thus making them

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promising with further development in retaining agricultural diversity and achieving food security which is important in a world that is dominated by few crops such as soybean (Sprent *et al.,* 2009). The indigenous legumes also often grow and establish in well-drained and low-fertile soils (Schrire, 2005) where crops such as maize have failed to give good yields (Mapfumo *et al.,* 2005). Presently underutilized and underexploited indigenous legumes are also on the increase based on their nutritional and agronomic importance (Graham and Vance, 2003). These include African yam bean *(Sphenostylis stenocarpa*), pigeon pea *(Cajanus cajan)*, bambara groundnut *(Vigna subterranea)* (Klu *et al.,* 2001), marama bean (*Tylosema esculentum*) (Dakora *et al.,* 1999), sword beans (*Canavalia gladiata*) (Ekanayake *et al.,* 2000), and *Desmanthus illinoesis* (Howieson *et al.,* 1995) etc.

African yam bean (AYB) is an underutilized, neglected and little known crop (Adewale and Odoh, 2012; Bioversity, 2009) that has the potential of averting the crisis of food insecurity due to the crop's great nutritional potentials such as its richness in essential amino acids, potassium and phosphorus (Uguru and Madukaife, 2001) and its promise of reducing malnutrition in Africans (Adewale, 2010)). According to Adewale (2010), less research and less acceptability by middle aged farmers could attribute to the poor awareness of this crop with respect to nodulation, nitrogen fixation, inoculation response, nutritional value and agronomic importance.

Further research into this underutilized and underexploited crop is however essential for survival of humans in a world dominated by few major crops. African yam bean however needs to be explored and developed in the areas of its high nutritional value compared to other major legumes (Adewale and Odoh, 2012), its biological nitrogen fixation ability and adaptability to a variety of soils (Klu *et al.,* 2001). Dogbe *et al*. (2000) documented that screening for highly effective native *Rhizobium* in

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soils and inoculating with these isolates could help explore this legume with respect to biological nitrogen fixation as a substitute for nitrogen fertilization.

Hence, this study was conducted to examine the nodulation and nitrogen fixation of indigenous rhizobia of African yam bean from some Ghanaian soils that have been cultivated and uncultivated with African yam bean.

Specific Objectives:

1. Enumeration of the population of indigenous African yam bean rhizobia in some soils cultivated and uncultivated with African yam bean.
2. Screening for highly effective rhizobia of African yam bean capable of being used as inoculant.
3. Assessing the effect of inoculating highly effective rhizobial strains and nitrogen application on nodulation, nitrogen fixation and growth of African yam bean.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Nitrogen in the environment

The growth of all organisms depends on the availability of mineral nutrients, and none is more important than nitrogen, which is required in large amounts as an essential component of proteins and other cellular constituents such as chlorophyll (Barsanti and Gualtieri, 2014). Air is the major reservoir of nitrogen that consists of 78% nitrogen gas (N_2). This nitrogen gas is in the unavailable form because of the strong triple bonds between the N atoms making it relatively inert. N_2 gas must be converted to either ammonium (NH_4^+) or nitrate (NO_3^-) or urea $(NH_3)_2CO$ in order for plants and animals to use atmospheric nitrogen (Kormondy, 1996). Organisms, both living and dead, and soil act as the reservoir for unstable nitrogen in organic and inorganic forms (Kormondy, 1996). Stable nitrogen such as nitrogen gas (N_2) moves from the atmosphere to other forms such as nitrate (NO_3^-) and ammonium (NH_4^+) and back to the atmosphere through a process known as nitrogen cycle (Hofman and van Cleemput, 2004). Nitrogen cycle is summarized below:

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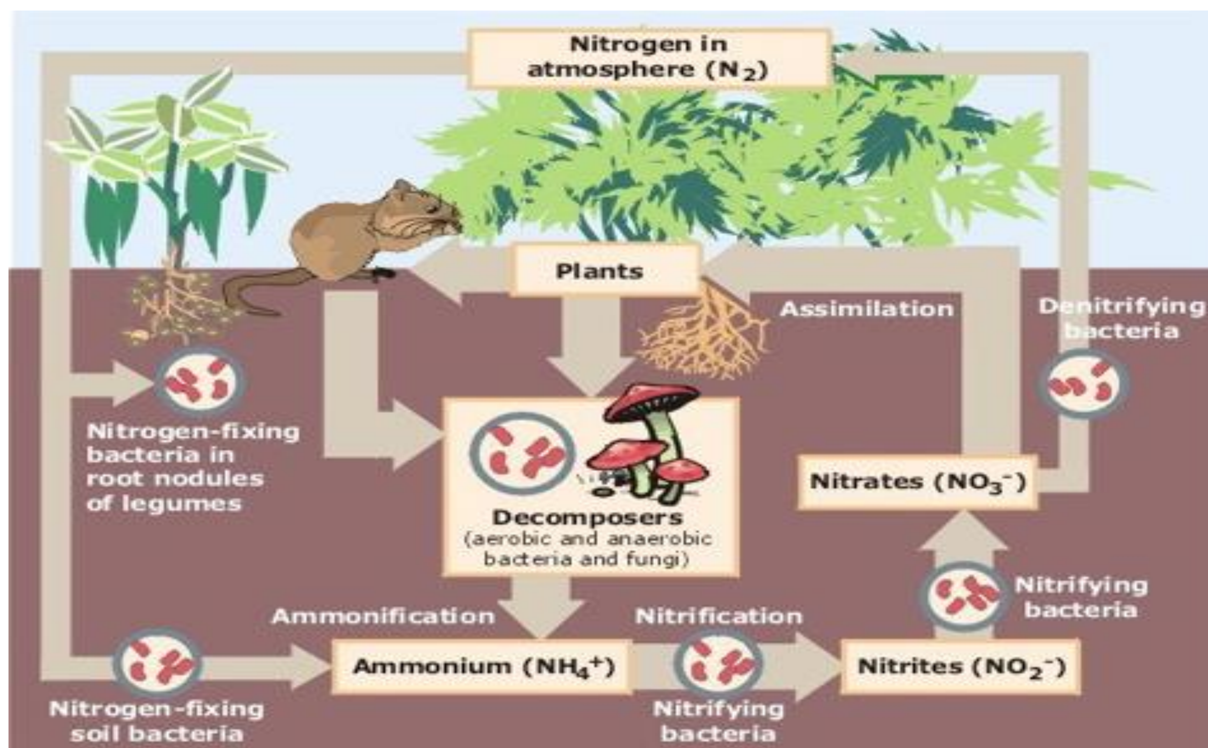

Fig. 2.1: The Nitrogen Cycle
Source: (www.macroevolution.net/nitrogen-cycle-diagram)

2.2 The concept of nitrogen fixation

Nitrogen is one of the macronutrients required by plants aside phosphorus and potassium and is a limiting factor for plant growth (Crawford, 1995). The continual loss of nitrogen from the soil by the processes of denitrification, soil erosion, leaching, chemical volatilization, and most importantly the removal of nitrogen by crop from the land surface make nitrogen limiting for plant to use. However through nitrogen fixation, available nitrogen is added to the soil.

Non-biological nitrogen fixation occurs through the effects of lightning, and industrially by the Haber-Bosch process. However the energy required for the industrial process is needed in a large quantity (Wagner, 2012). Lightening is estimated to fix 10 million metric tons of nitrogen while Haber-Bosch process of nitrogen fixation has been estimated to fix about 50 million tons per

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year (Bezdicek and Kennedy, 1998). Biological nitrogen fixation is an essential natural process that supports life on earth and it involves the use of microbes that have the enzyme nitrogenase (Salisbury and Ross, 1992). Biological nitrogen fixation is estimated to fix 40 to 60 million tons per hectare per year of nitrogen annually (Smil, 1999).

2.2.1 The Haber-Bosch process

The industrial conversion of the stable N_2 in the atmosphere into usable forms (also known as Haber-Bosch process) requires high input of energy i.e. high temperatures (600-800°C) and pressures (5.06×10^7 Pa) to rapture the triple bond of molecular nitrogen (N≡N) (Cheng, 2008; Giller and Wilson, 1991). Atmospheric nitrogen and hydrogen are combined chemically to form ammonia (NH_3) at a temperature of about 600°C. Usually, iron based catalysts are added to speed up the reaction. On commercial basis this process is widely used for the production of ammonia.

$$N_{2(g)} + 3H_{2(g)} \rightleftharpoons 2NH_{3(g)} \quad \Delta H = -92\ kJ\ mol^{-1}$$

The table below shows the amount of nitrogen fixed on the global scale.

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Table 2.1: Amount of nitrogen fixed (biological and non-biological) on the global scale.

Type of fixation	N_2 fixed (10^{12} g per year)
Non-biological	
Industrial	about 50
Combustion	about 20
Lighting	about 10
Total	about 80
Biological	
Agricultural land	about 90
Forest and non-agricultural land	about 50
Sea	about 35
Total	about 175

Source: Bezdicek and Kennedy (1998)

2.2.2 Biological Nitrogen Fixation (BNF)

The biological form of fixing atmospheric nitrogen is known as Biological Nitrogen Fixation (BNF) which involves the fixing of atmospheric nitrogen by microorganisms that possess the enzyme nitrogenase into NH_3 (Lindermann and Glover, 2003). This process is very important since it provides nitrogen to higher plants and animals and can relieve the requirements for added nitrogenous fertilizer during the growth of leguminous crops. Biological nitrogen fixers may be symbiotic or non-symbiotic (Tilak *et al.,* 2005).

Many bacteria, fungi, protozoa and nematodes colonize the rhizosphere of plants which leads to these microbes living in close association with the plants than further away from the rhizosphere (Bowen and Rovira, 1999) due to the availability of carbon sources to such organisms (Katznelson *et al.,* 1956). Plants release high levels of nutrients from their roots such as amino

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acid, organic acids, sugars, aromatics and secondary metabolites such as polysaccharides and proteins (Marschner, 1995). However, the amount of nitrogen available in the rhizosphere is often limiting for growth and therefore bacteria that fix N_2 would be expected to have a competitive advantage (Giller, 2001). These microbes may exist as free living organisms in the soil or attached to root surfaces (Bowen and Rovira, 1999).

2.2.2.1 Non-symbiotic Nitrogen fixation

Non-symbiotic or asymbiotic nitrogen fixations have been associated with many microorganisms. They range from those that fix N_2 completely independent of plants or other organisms (by free living bacteria) to those that fix N_2 when associated with the roots of certain plants, without the formation of any recognisable anatomical structures, such as nodules of legumes (Hardarson *et al*., 1987). Some of these organisms include species of bacteria. Examples of this type of N_2-fixing bacteria include species of *Azotobacter*, *Bacillus*, *Clostridium*, *Klebsiella, Thiobacillus, Azospirillium, Desulfovibrio, Aerobacter, Methanobacterium, Spirillum, Chlorobium, Pseudomanas, etc.* Fixation of nitrogen by these organisms are dependent upon the presence of substantial amounts of readily oxidizable organic molecule in the soil (such as organic matter) (Hardarson *et al.,* 1987). These organisms obtain energy by oxidizing these organic molecules (Wagner, 2012). Other free-living organisms are chemoautotrophs and can utilize inorganic compounds as energy source to fix nitrogen (Wagner, 2012). This could be the reason for Hardarson *et al.* (1987) documenting that many unfertilized soils of low N status continued to support and sustain moderate grain yields in the absence of symbiotic N_2 fixation. According to a report by Greenland (1977) it is not unusual to observe 20-50 kg $Nha^{-1}yr^{-1}$ which shows a high contribution of non-symbiotic N_2 fixation by free-living organisms. However the limitation with this type of N_2 fixation is the lack of suitable carbon and

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energy sources for the organisms involved (Wagner, 2012) making their contribution to global nitrogen fixation rates being considered minor.

Many species of blue-green algae have also been established to fix N_2. They include species of *Anabena, Nostoc* etc. They are photoautotrophic and do not need any external supply of available organic matter since sunlight provides the needed energy for nitrogen fixation (Hardarson *et al.,* 1987). These organisms are abundant in paddy soils and overlying floodwaters since that provides an ideal environment for them to grow and fix atmospheric N_2 (Giller, 2001). The blue green algae contribute 10-30 kg N/ha/year or more to the soil (Hardarson *et al.,* 1987). In the paddy field is found *Azolla*, a floating aquatic fern which is in symbiotic relationship with cyanobacterial species, *Anabaena azollae* (Giller, 2001). The *Anabaena azollae* colonizes the dorsal lobe cavity of the *Azolla* and nitrogen fixation occurs in the blue green algae's specialized cells called heterocyst (Hardarson *et al.,* 1987).

2.2.2.2 Associative nitrogen fixation

Another form of non-symbiotic nitrogen fixation is the Associative nitrogen fixation. Species of *Azospirillium* which form associations with grasses and cereals were discovered to be nitrogen fixing when these organisms isolated from rhizosphere of these plants fixed appreciable amount of nitrogen (Patriquin *et al.,* 1983). The amount of nitrogen fixed by this association is reported to be affected by many factors which include low oxygen around the rhizosphere of host plant, competitiveness of bacteria (Steenhoudt and Vanderleyden 2000), the effectiveness of nitrogenase (Vlassak and Reynders, 1979) and the availability of nutrients (Sadasivan and Neyra, 1987).

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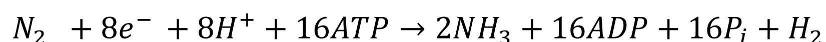
2.2.2.3 Symbiotic nitrogen fixation.

The oldest and most studied of all biological N_2 systems is the symbiotic relationship between legumes and *Rhizobium* (bacteria in soil) through which atmospheric nitrogen is fixed (Hardarson *et al.,* 1987 and Lui *et al.,* 2010). The *Rhizobium* infects the plant tissue and ultimately inhabits plant cells within the root. Once inside the plant cell, the *Rhizobium* fixes N_2 from air into forms that are useful for the plant. The *Rhizobium* benefits from the association by obtaining sugars from photosynthesis and a favourable environment (sheltered from desiccation and predation) from the plant (Sachs *et al.,* 2013). Nitrogen fixed and exported from the nodule is taken up by the host plant for protein synthesis, growth and development (Sessitsch *et al.,* 2002). This forms the basis of importance of legumes in the environment and through it offers great promise for alleviating world protein and food shortage (Hardarson *et al.,* 1987). Biological and non-biological nitrogen fixation is known to fix the largest amount of N_2 in nature where fixation is estimated to be 175 million metric tons annually (Table 2.1).

Extensive studies of Biological nitrogen fixation has been undertaken (Leigh, 2002) but however Franche *et al.* (2009) noticed that the nitrogen reduction process was a complex mechanism which has not yet been completely understood. Reduction of molecular nitrogen to ammonia is mostly simplified by the equation below (Vance, 2002);



$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + 16ADP + 16P_i + H_2$$

The reaction above needs nitrogenase as a catalyst to split the dinitrogen molecule and transform it into ammonia with the supply of energy and electrons (Postgate, 1982). Nitrogenase has two distinct metalloproteins, MoFe Protein (component I or dinitrogenase) and Fe protein (Component II or dinitrogen reductase) (Peters *et al.,* 1995; Howard and Rees, 1996). The

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molybdenum nitrogenase (Mo nitrogenase) is the most common type of nitrogenase found in root nodule bacteria (Fisher and Newton, 2002). The two requirements for nitrogenase to split dinitrogen molecule are an anaerobic environment and the presence of adenosine triphosphate (ATP). When exposed to oxygen, nitrogenase is inactivated because oxygen reacts with the iron component of the protein (White, 1995). Various mechanisms to overcome this problem have been devised by aerobes. Root nodules contain leghaemoglobin that combines with O_2 facilitating its diffusion through the bacteriods at a low and buffered concentration (Burris and Haas, 1994).

Another form of symbiotic nitrogen fixation is the relationship between *Frankia* and non-leguminous tree plants. *Frankia* are gram positive bacteria (nitrogen fixing actinomycetes) that form root nodules on more than 280 species of non-leguminous tree plants (Schwintzer and Tjepkema., 1990). Species of *Alnus* and *Casuarina* are globally known to form effective symbiosis with Frankia (Huss-Danell, 1990). Other non-legumes that form symbiosis with *Frankia* are *Casuarina, Cercocarpus, Coriaria, Discaria, Dryas, Eleagnus, Myrica, Purshia, Shepherdia* and *Podacarpus* (Hardarson e*t al.,* 1987). This type of symbiosis is a major contributor of nitrogen inputs in forests, wetlands and disturbed sites of temperate and tropical regions (Tate, 1995).

2.2.3 Importance of Biological Nitrogen Fixation

Biological nitrogen fixation is an important source of nitrogen in plants. It has been estimated that about 70 million metric tons of N fixed per year has been contributed by legume-*Rhizobium* symbiosis in terrestrial ecosystem (Brockwell *et al.,* 1995). Paul and Clark (1996) therefore reported that the amount of N fixed by this symbiosis accounts for up to 40% of the N fixed globally. Herridge *et al*. (2008) recorded about 1×10^6 tons N per year by crop and pasture

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legume is equivalent to $1 billion in fertilizer N application in Australia. Similar economic evaluations of the benefits of BNF have not been performed for other developing countries, although it is likely that comparable benefits could be expected. Importance of using nitrogen fixing crops in cropping systems, include reducing groundwater pollution in comparison to crops that have been fertilized with chemical fertilizers, enhancing protein production due to their high protein content, contributing nitrogen to succeeding crops and building up soil fertility in some instances (Hardarson, 1993; Lupwayi *et al.,* 2004). Total grain and plant nitrogen can often be increased by intercropping legumes and non-legumes together through N transfer from the legumes (Fujita *et al.,* 1990). Similarly, Waghware *et al.* (1982) observed an increase in grain yield of cereal (e.g. rice) when planted with groundnut, cowpea or soybean.

2.3 The legume Component

2.3.1 Origin of legumes

Legumes are flowering plants that are characterised by their podded fruit, ability to form root tubers in some species (Morel *et al.,* 2012) and the ability of 88% of the species examined to form nodules with rhizobia (de Faria *et al.,* 1989). Legumes grow in wide agro-ecological conditions and have been found in most of the archaeological records of plants (Singh *et al.,* 2007). Being part of early civilization, legumes were grown as crops for over 6000 years (Singleton *et al.,* 1990). The use of legumes in agriculture as pastures and for soil improvement dated back to the Romans where Varro (37 BC; cited by Fred *et al.,* [1932]) recognized the importance of legumes in intercropping production. Cohen (1977) reported the domestication of lentils (*Lens esculenta*) at a site in Iran dating to 9,500 to 8,000 BC and its paste being discovered in Egyptian tombs of Thebes dating 2,300 BC (Singleton *et al.,* 1990). The domestication of bean (*Phaseolus vulgaris*) and soybean (*Glycine max*), staple crops in the

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Americas and Asia, respectively, were more than 3,000 years ago (Hymowitz and Singh, 1987; Kaplan and Lynch, 1999). Faba bean (*Vicia faba*) which originated from the West or Central Asia is mentioned in the Hittite texts and in the Bible (Singleton *et al.,* 1990).

2.3.2 Classification of legumes

Legumes belong to the family of plants classified as *Leguminosae or Fabaceae* (Hardarson *et al.*, 1987) and are second only to the *Gramineae* (cereals and grasses) in their importance to humans (Graham and Vance, 2003). *Leguminosae* or *Fabaceae* are among the three largest families of flowering plants (Morel *et al.,* 2012) with 670 to 750 genera and 18,000 to 19,000 species which include important grain, forage and agro-forestry species (Polhill *et al.,* 1981). The *Leguminosae* is divided into subfamilies namely *Mimosoideae, Caesalpinoideae, Swarzioideae* and *Papilionoideae* but economically, the *Swarzioideae* subfamily is small consisting of about 80 species which renders it unimportant (Singleton *et al.,* 1990). *Papilionoideae* consists of 476 genera and about 1400 species mainly herbaceous though some are trees and shrubs, the *Caesalpinoideae* also consists of 162 genera and about 3000 species mainly tropical and subtropical trees and shrubs whiles the Mimosoideae consists of 77 genera and approximately 3000 species also including tree and shrubs (International Legume Database and Information Service [ILDIS], 2007). The discovery of nodulation in some legumes created an interest among scientists to investigate the extent of nodulation in the *Leguminoseae* family. Among the legumes screened for nodulation so far, 23% of the species in *Caesalpinoideae,* 90% in *Mimosoideae*, and 97% in *Papilionoideae* have been found to nodulate (de Faria *et al.,* 1989). This indicates that the highest incidence of nodulation is encountered in the *Papilionoideae.*

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2.3.3 Uses and importance of legumes

Legumes form important components in agriculture and food systems throughout the world (Katungi *et al.,* 2010). In terms of agriculture, only few species are cultivated on a large scale where it contributes more than 25% of global production, including food staples, fodder for livestock, cover crops and emerging biofuels (Ferguson *et al.,* 2010). Additionally, they are used as pulp for paper production, fuel-woods, timber, oil production, sources of chemicals and medicines, and are also cultivated as ornamental, used as living fences and firebreaks among others (Lewis *et al.,* 2005). Forage legumes play an important role in dairy and meat production being sources of protein, fibre and energy (Morel *et al.,* 2012) as they are grown as pastures to feed animals. They include *Medicago spp.*, *Trifolium spp.*, *Centrocema spp., Pueraria spp., Vicia spp.*, *Stylosanthes spp.*, *Desmodium spp.*, *Calopogonium spp.*, *Chamaecrita spp., Lotonomis spp., Macrotyloma spp., Macroptilum spp.* and *Zornia spp.* (Giller, 2001). Pulses (grain legumes) play an important role in the nutrition of many people due to their high protein content in the seeds (Morel *et al.,* 2012). However these pulses are limited in sulphur, contain anti-nutritional factors including lectins and flatulence factors, and are commonly hard to cook (Graham and Vance, 2003). Examples are groundnut (*Arachis hypogaea*), soybean (*Glycine max*), chickpea (*Cicer arietinum*), cowpea (*Vigna unguiculata*), faba bean (*Vicia faba*), pigeon pea (*Cajanus cajan*), lima bean (*Phaseolus lunatus*) etc.

Legumes provide many benefits to the soil so they are usually utilized as cover crop, intercropped with cereals and other staple foods. They increase organic nitrogen, soil organic matter, diversify microorganisms and mitigate disease problems (U.S Department of Agriculture [USDA], 1998).The legumes of genera *Canavalia*, *Mucuna* and *Crotalaria* are used as cover crops or green manure and are grown for use as an organic manure maximizing the amount of N

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from the legumes to the subsequent crop (Giller, 2001). Legumes provide a source of slow release nitrogen that contributes to sustainable cropping systems when grown in rotation with cereals and this leads to improved human nutrition and soil health (Popelka *et al.,* 2004).

2.3.4 Legumes and BNF

A hallmark trait of legumes is their ability to develop root nodules and to fix N_2 in symbiosis with compatible rhizobia (Graham and Vance, 2003). This symbiosis has several advantages including improved agricultural productivity, restoring and maintaining soil fertility, economy of expensive fertilizers and limitation of groundwater pollution by nitrates (Morel *et al.,* 2012). As biofertilizer, nitrogen fixing microorganisms can be applied to seed, root or soil to colonize the rhizosphere, or the interior of the plant, and promote growth by increasing the nitrogen supply to the host plant (Morel *et al.,* 2012). These biofertilizers are economical, sustainable and environmentally friendly resource to guarantee the nitrogen requirement of an agro-ecosystem (Morel *et al.,* 2012).

BNF benefits not only the legumes but also any intercropped or succeeding crop, therefore reducing or removing the need for nitrogen fertilization. Soils with low mineral nitrogen content tend to provide ammonium into the legume biomass by nitrogen fixing microorganisms allowing faster growth than the other plant competitors (Morel *et al.,* 2012). In contrast, with soils with high mineral nitrogen, nitrogen fixing microorganisms tend to be competitively excluded by non-fixing species because the nitrogen fixation process is bio-energetically costly (Houlton *et al.,* 2008).

BNF can be increased by the use of well adapted and efficient nitrogen fixing microorganisms and/or genetic modified plant species to ensure high levels of productivity in the legume (Morel

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et al., 2012). Legumes have different nodulation potential and these are often translated into difference in nitrogen fixed. Danso (1992) reported that some legumes are poor fixers, example being common bean (*Phaseolus vulgaris*) for which N derived from fixation varies from 20-40% of its nitrogen requirement compared to the more than 70% for Faba bean (*Vicia faba*).

2.4 African Yam Bean (AYB) (*Sphenostylis stenocarpa*) (Hochst. Ex. A. Rich.) Harms

2.4.1 Origin and taxonomical description of AYB.

African yam bean (AYB) *(Sphenostylis stenocarpa* [Hochst. Ex. A. Rich] Harms) belongs to the legume family which originated from Ethiopia, but both wild and cultivated types now occur in tropical Africa as far north as Egypt and also throughout West Africa from Guinea to Southern Africa (Potter, 1992). It is common in Central and West Africa, particularly in Cameroon, Cote d'Ivoire, Ghana, Nigeria and Togo (Potter, 1992). The African yam bean was introduced into Ghana from Togo in 1958 (Adansi, 1975) and is often cited among the lesser known and underexploited species (Rachie and Roberts, 1974). In Ghana and Nigeria, African yam bean typifies a neglected traditional crop decreasing in size among the farmers (Amoatey *et al.,* 2000). Only few farmers are involved in its cultivation hence, they are the holder of the crop's genetic resources (Adewale *et al.,* 2012).

The generic name *Sphenostylis* was the botanical genus evolved by Harms (1899) to describe a group of distinctive leguminous taxon formerly grouped within the genus *Dolichos* and *Vigna* (Adewale and Odoh, 2012). Like the other tuberous legumes (for example, *Pachyrhizus spp.*), this legume belongs to the *Fabaceae* family and the order *Fabales* which is classified as the most important cultivated species in the genus *Sphenostylis* (Okigbo, 1973; Potter and Doyle, 1994) and is indigenous to tropical Africa. *Sphenostylis stenocarpa* is economically the most important

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specie within the genus *Sphenostylis* (Potter, 1992). The taxonomic profile of African yam bean is presented in Table 2.2.

Table 2.2: The taxonomic profile of African yam bean.

Rank	Scientific Name/Common Name
Kingdom	*Plantae*
Sub kingdom	*Tracheobionta*
Super division	*Spermatophyta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabaceae*
Sub family	*Papilionoideae*
Tribe	*Phaseoleae*
Sub tribe	*Phaseolinae*
Genus	*Sphenostylis* E. Meyer (*Sphenostylis)*
Species	*Sphenostylis stenocarpa* (Hochst. Ex. A. Rich.) Harms.

Source: Adewale and Odoh. (2012)

2.4.2 Morphological description and cultivation of AYB

Sphenostylis stenocarpa is a vigorously climbing herbaceous vine whose height can reach 1.5-3 metres or more depending on the height of the stakes and cultivar. It is found in forests, open and wooded grasslands, rocky fields as well as marshy grounds, occurring both as a weed and a cultivated crop (Duke *et al*., 1977; Potter, 1992). Duke *et al.* (1977) reported that it grows on a wide range of soils as well as acid and highly leached sandy soils at altitudes ranging from sea level to 1,950 m. It forms slightly woody pods which are linear and measure up to 30 cm

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containing 20 to 30 seeds which mature within 170 days. It is cultivated for its seeds and tuberous roots. The seeds are very hard and diverse in colour and shape usually spherical, ellipsoid with dark-brown, creamy-white or brownish yellow colour (Duke *et al.,* 1977; Edem *et al.,* 1990). It is grown mainly as an intercrop with maize *(Zea mays*) and cassava (*Manihot esculentus*) where it twines around their stems for support (Potter, 1992; Klu *et al*., 2001). The climbing habit of the bean is also utilised as it can form a living fence where it is grown on stakes around fields of cocoyam (Potter, 1992). Potter (1992) also documented that the support is very valuable to the plant since better yields for both seeds and tubers have been obtained when planted with such crops as maize and yams. The stem of the plant produces small underground tubers of various sizes and shapes that are very similar to sweet potatoes (Kay, 1987).

2.4.3 Biological nitrogen fixing capacity of AYB.

AYB nodulates profusely and probably has high nitrogen-fixing ability, thereby helping to replenish soil nitrogen (Klu *et al.,* 2001). It has been shown to form nitrogen-fixing nodules if inoculated with slow growing *Bradyrhizobium* bacteria (Assefa and Kleiner, 1997). This ability to fix atmospheric nitrogen means the plant will not require large amounts of nitrogen fertilizer to meet growth demands, thus making its production affordable to the poor farmers living mainly in areas where it grows (Oagile, 2005). Okpara and Omaliko (1995) reported of yield increases of yellow yam *(Dioscorea cayensis*) in an intercrop between AYB and attributed that to the latter's ability to fix atmospheric nitrogen.

2.4.4 AYB as an underexploited and underutilized crop

Despite the ability of the African yam bean to nodulate profusely and fix atmospheric nitrogen, little research attention has been paid to it compared to the major legumes such as cowpea, groundnut and soybean (Klu *et al.,* 2001, Saka *et al.,* 2004). Klu *et al.* (2001) also noted that the

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crop was near extinction thus being classified as a minor, underutilised and underexploited crop (Bioversity, 2009). Adequate attention and breeding efforts should be directed towards its improvement (Saka *et al.,* 2004). The African yam bean requires enhancement genetically for farmer and consumer desired traits such as reduced hardness of the seed coat to make room for easier cooking (Oshodi *et al.,* 1995), disease resistance, drought tolerance (Onyeike and Omubo-Dede, 2002), reduced maturation period (Okpara and Omaliko, 1997) and reduced anti-nutritional factors (secondary metabolites) which are reported to cause discomfort and flatulence when consumed (Machuka *et al*., 2000).

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Fig 2.2: African yam bean plant with some plant parts a.) Africa yam with mature pods ready for harvest. b.) Tubers produced by the African yam bean plant. c.) Raw seeds of African yam bean (Cream coloured) d.) Raw seeds of African yam bean (Black coloured).

2.5 The *Rhizobium* (Nitrogen fixing bacterium)

Rhizobium is a nitrogen-fixing soil bacterium capable of inducing the formation of root or stem nodule on leguminous plants in which nitrogen is reduced to ammonia for the benefit of the plant (Sikora and Redžepović, 2003). *Rhizobium* is gram negative, heterotrophic, non-spore forming, rod-shaped (approximately 0.5-0.9µm in width and 1.2-3.0µm in length), aerobic bacterium,

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having variable number of flagella (or simple sub-polar flagellum as is common in types derived from tropical legumes) and capable of prolonged independent existence in the soil (Somasegaran and Hoben, 1994). They are motile and are able to move in damp soil through the water films surrounding the soil particles, but the rate of movement is slow (Hamdi, 1970). They generally grow at 25°C-30°C (optimum) and in the pH range of 6-7 (Somasegaran and Hoben, 1994). Somasegaran and Hoben (1994) reported that *Rhizobium* grows under aerobic conditions but when fixing nitrogen low levels of oxygen are required to protect the enzyme *nitrogenase* which makes it able to grow in microaerophilic conditions. It is noted to be either in the soil as saprophytes or in nodules as symbiont obtaining all nutrients from the host plant (Fujihara, 2009).

Denarie *et al.* (1996) reported that rhizobia are genetically diverse and physiologically varied group of bacteria that cause nodule formation on legumes. In a free-living state in the rhizosphere of legumes, rhizobia are the abundant part of the soil micro-flora (Allen and Allen, 1981; Somasegaran and Hoben, 1994). A unique feature associated with rhizobia is the ability to form symbiotic relationships with members of the plant family *Fabaceae* (Pepper and Upchurch, 1991). Brockwell *et al*. (1995) reported that rhizobial strains are lacking in many soils despite the general distribution of leguminous crops.

2.5.1 Taxonomy and diversity of *Rhizobium*

Rhizobia have been extensively studied because of their considerable agricultural and environmental significance. The taxonomy of this bacterium has undergone considerable revision in the past two decades and still in state of transition (Graham *et al.,* 1991; Elkan, 1992). Discovery of legume-*Rhizobium* symbiosis has been attributed to the German scientists Hellriegel and Wilfarth in 1887 who identified in their experiment that N_2 fixation was

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associated with nodulated legumes and that the nodules were the site for fixation (Deaker *et al.,* 2004). Beijerinck then isolated this bacterium from the root nodules of legumes and named it *Bacillus radicola* (Perret *et al.,* 2000). Later, Frank changed the name to *Rhizobium* with originally just one species, *R. leguminosarum* (Frank, 1889). The taxonomy of the nodule bacteria was reviewed by Fred *et al*. (1932) which was based on the nodulation host range. This concept was also used in rhizobial taxonomy, but later it was abandoned as an unreliable taxonomic marker due to overlapping host range (Wilson, 1944). In the early 1960s, bacteriologists used more diverse characteristics such as morphological, nutritional and metabolic characters (Graham, 1964; Moffet and Colwell, 1968), as well as serology (Graham, 1964) in taxonomy studies. This led Jordan (1984) to describe two genera, *Rhizobium a*nd *Bradyrhizobium* based on the growth rate, DNA homology, base ratios and organisation, capsular exopolysaccharides, carbohydrate metabolism and their intrinsic resistance to antibiotics. *Rhizobium* strains which infect and fix nitrogen in temperate legumes are fast growing and *Bradyrhizobium* strains typically symbiotic on tropical legumes are slow growing. With the introduction of more genetic characteristics, (DNA-DNA and DNA-rRNA hybridizations, rRNA catalogues, rDNA sequencing) more diversity was discovered among the rhizobia and their relationships with other groups of bacteria (Willems, 2006). Trinick (1980) discovered that cowpea *Rhizobium* was capable of nodulating a non-leguminous tropical plant *Parasponia* otherwise *Leguminosae* have so far been known to be the sole hosts for rhizobia.

Strains of rhizobia inhabiting a particular soil may be diverse in symbiotic characters as well as in phenotypic and genotypic characters. Cropping history, the degree of disturbance of an environment and the range of legume species of an area can influence the diversity of rhizobial strains of an area (Brockwell *et al.,* 1995). Amann *et al*. (1995) reported that assessment of

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diversity within the rhizobial natural populations has received increased attention. It is possible to determine the composition and characteristics of the indigenous strains isolated from different cultivated legumes. Handley *et al.* (1998) reported that genetic methods generally have higher discriminatory power and are faster than most phenotypic methods. Prior to this, rhizobial strain diversity studies were mainly based on cross-inoculation and phenotypic characters (Schwinghamer and Dudman, 1980). Consequently the taxonomy of root nodule bacteria has been deeply changed in current times consisting of several genera including *Azorhizobium, Bradyrhizobium, Burkholderia, Cupriavidus, Devosia, Ensifer, Herbaspirillium, Mesorhizobium, Methylobacterium, Ochrobacterium, Phyllobacterium, Shinella* and *Rhizobium* of alpha and beta proteobacteria (Dudeja and Narula, 2008). The gamma-proteobacteria was found associated with legume nodules, although their presence and role is yet to be defined (Benhizia *et al.,* 2004).

2.5.2 Abundance in soil

Rhizobia are identified to be one of the important plant symbiont studied (Hirsch, 1996) although they are not found in all soils (Diatloff and Brockwell, 1976). Due to this, there has been considerable work done on the size and levels of natural populations in different agricultural soils (Hirsch, 1996). Fening (1999) noted that no defined culture medium exits for selective isolation of rhizobia and reliance has been on legume infection technique. The assumption of this technique is that the microorganisms can multiply in the rhizosphere and infect the plant when soil dilution containing at least one *Rhizobium* cell is used, which is then examined for nodules (Fening, 1999).

Great variation occurs in the population size of rhizobia depending on soil type, environment, appropriate host and its rhizosphere and type of cultivation of soil (Hiltbold *et al.,* 1985, Rupela *et a*l., 1987). In addition, biological factors could also affect rhizobial population size in soil

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which include predation by protozoa and attack by bacteriophages (Danso *et al.,* 1975, Barnet, 1980). Rhizobia consistently comprise a smaller group although they can reach higher numbers in the rhizosphere (Hirsch, 1996). The population of rhizobia in agricultural soil is enhanced by the presence of legume that rhizobia can nodulate (Thies *et al*, 1995).

2.6 Methods of studying Rhizobial diversity

Several methods have been used over the years for studying rhizobial diversity in soils which include:

2.6.1 Cross inoculation group concept

In the twentieth century, testing of nodulation on host legumes by different rhizobia was extensively conducted (Willems, 2006). This led to the establishment of cross inoculation group with rhizobia from plant of the group supposed to nodulate other plants in the group (Fred *et al.,* 1932). Nodulation between the legume host and the *Rhizobium* is carefully matched. Based on this concept rhizobial strains may be classified as highly specific in that each has a definite host range which may be narrow or broad (Perret *et al.,* 2000) This concept led to the classification of *Rhizobium* under six groups namely *B. japonicum*, *R. lupini*, *R. meliloti*, *R. leguminosarum*, *R. phaseoli* and *R. trifolii* (Table 2.3) as classified by Fred *et al*. (1932).

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Table 2.3: Cross-Inoculation Group (C-IG) and Legume-*Rhizobium* Association.

***Rhizobium* Species**	**Compactible Hosts**	**C-IG**
Rhizobium melilotus	*Melilotus spp.*, *Trigonella spp.*	Alfalfa group
R.leguminosarum bv *trifolii*	*Trifolium spp.*	Clovers
R.leguminosarum bv *viciae*	*Pisum spp.*, *Lens culinaris*, *Vicia spp.*, *Vicia faba*	Peas
R.leguminosarum bv *phaseoli*	*Phaseolus vulgaris*, *P. coccineus*	Bean group
Rhizobium lupine	Lupinus	Lupine
Bradyrhizobium japonicum	*Glycine max*	Soybean
Cowpea Miscellany	*Vigna spp.*, *Macroptilium spp.*, *Lablab spp.,* Lima bean, *Stylosanthes spp.,* etc.	

Source: Alexander, (1978).

Wilson (1944) reported that some rhizobia species were able to nodulate plants outside their cross inoculation group, known as promiscuous nodulation. This has made many scientists to question the reliability of the cross inoculation concept for determining similarities among rhizobia strains (Wilson, 1944, Bromfield and Barran, 1990). Its continued usage has been necessary based on their convenience and agronomic significance (Graham *et al.,* 1991). This concept thereby has an importance of being used for selection of rhizobial strains which have a potential to be used as inoculants for some legumes (Mpepereki *et al.,* 1996).

2.6.2 Cultural and metabolic methods

To differentiate, characterise and classify rhizobial species phenotypically, cultural and metabolic tests have been done by researchers (Vincent, 1970). These methods include growth rate, colony characteristics, salt tolerance, pH tolerance, carbon utilisation, utilisation of nitrogen sources, vitamin requirements, tolerance to dyes (e.g. BTB, BCB etc.), tolerance to antibiotics,

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tolerance to pesticides, temperature effects, growth rate in peptone broth, nitrate reduction, production of catalase, production of acid or alkali, etc. (Graham *et al.,* 1991; Somasegaran and Hoben, 1994; Mpepereki *et al.,* 1997; Odee *et al.,* 1997). Rhizobia species differ significantly in carbohydrate metabolism and substrate utilization where the carbohydrates include glucose, sucrose, lactose, fructose, arabinose, and succinate (Somasegaran and Hoben, 1994). These carbohydrates serve as an investigative test in the differentiation of known species of *Rhizobium* (Graham *et al.,* 1991; Somasegaran and Hoben, 1994). Lange (1961) suggested these tests characterised the root nodule bacteria to resolve the taxonomic difficulties within the genus *Rhizobium*. Despite the criticism that these methods were impracticable (Graham and Parker, 1964), they are used for phenotypic characterisation in combination with genotypic analysis.

2.6.3 Serological method

The use of serology in studying rhizobial diversity is based on the principle of antigen- antibody reaction where an animal reacts by producing antibodies when an antigen (composed of basic proteins or polysaccharides) of a particular rhizobial strain is injected into it. The most common serological methods recently used are agglutination (Means *et al*., 1964; Wollum, 1987), fluorescent antibody techniques (Bohlool, 1987) and various forms of enzyme-linked immunosorbent assay (Asanuma *et al*., 1985; Ayanaba *et al*., 1986; Spriggs and Dakora, 2009). Beck *et al*. (1993) disclosed that because an antibody is produced to react with a specific antigen, the antibody is used to identify that particular protein-containing strain when linked with enzymes or dyes that make the reaction visible. A convenient means of investigating rhizobia in ecological and agronomic studies is the specificity that exits between a strain's antigen and antibody in serology (Beck *et al*., 1993). The practical importance of serology is to identify groups that have practical importance to the management of symbiosis apart from using it to

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study rhizobial diversity. Vincent (1941) was the first to make use of serological information in classifying rhizobial strains where he differentiated between flagella and somatic agglutinations and in addition made use of agglutination absorption tests for differentiation between rhizobial strains. Antigenic and constitution within groups of strains and serological groups of a range of isolates of clover, medics and peas was reported subsequently (Vincent, 1942; Hughes and Vincent, 1942). Little or no work was reported using these criteria for tropical type legumes until the 1960's when a number of papers on serology of soybean rhizobia appeared, where two new methods of serological assay were developed including gel immunodiffusion technique (Dudman, 1964) and fluorescent antibody specific staining technique (Trinick, 1969). Other work was also done by Klimmer and Kruger (1914) who divided 18 cultures from many different legumes into nine groups using agglutination tests and concluded that rhizobia isolated from different species of plants were serologically distinct but related at the same time. Spriggs and Dakora (2009) also used serological indirect ELISA method to detect strains of some selected rhizobia from *Cylopia maculata* under glasshouse and field conditions and absorbance readings showed that there was no cross reaction between antigens of the rhizobia strains and antibodies of New Zealand white rabbits. But one major problem with using serology to characterize rhizobia is the presence of strains that do not react with all antisera tested and the frequency of non-reactive strains is often significant (Mpepereki and Wollum, 1991; Fuhrmann, 1990). Also the involvement of animal in production of antiserum deters scientists from the use of this method (Beck *et al.*, 1993).

2.6.4 Antibiotic resistance method

Antibiotic resistance is one of the methods used in identifying rhizobial strains in nodules and this can be used to identify the ability of rhizobia to grow in media containing antibiotics

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(Spriggs and Dakora, 2009). Antibiotics used in the identification of rhizobia strains include: streptomycin, kanamycin, spectinomycin, chloramphenicol, rifampicin, naladixic acid etc. (Beck *et al.,* 1993). Antibiotic marker technique is applied in ecological studies where strain identification is not possible by serology due to cross reactions of the strains, or because of unavailability of antisera (Somasegaran and Hoben, 1985). This method is simple and requires no specialised equipment (Ramirez *et al*., 1998). Antibiotic resistance method is a good tool in the study of nodulation and N_2 fixation (Danso and Owiredu, 1988) where it is used as a trait for comparing or characterizing strains as per determinant in selecting strains for antigen-antibody studies and as a marker for ecological studies (Beck *et al,* 1993). Spriggs and Dakora (2009) identified two distinct pairs of *Cyclopia* strains with regard to their intrinsic natural resistant to the antibiotic streptomycin and spectinomycin. In the use of antibiotic resistance method in N_2 fixation studies, Danso and Owiredu (1988) noted that antibiotic resistance mutant of soybean *Bradyrhizobium* strains were able to form substantially more nodules than the native soil *Rhizobium*. There was also a record of higher nodules and N fixed when antibiotically-marked *Cyclopia* strains were inoculated in the soil compared to the native strains (Spriggs and Dakora, 2009).

2.6.5 Symbiotic effectiveness

Symbiotic information is not provided on the determination of population size of native rhizobia and their diversity but there exist differences in effectiveness of the native rhizobial strains within and between soils (Singleton and Tavares, 1986). Eaglesham (1985) disclosed that it was necessary to know the extent the native rhizobial strains can nodulate a particular legume effectively since low rate of nitrogen fixation can occur even with normal nodulation

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(Eaglesham, 1985). Singleton and Tavares (1986) observed that native rhizobial effectiveness differed significantly in soil samples.

Ahmad *et al.* (1981) showed that 30% of isolates from 2 sites were effective whiles Ferreira and Marques (1992) found a difference in effectiveness among 170 strains isolated from native clover. Many scientists have observed environmental factors that may affect symbiotic effectiveness of rhizobial strain. Effectiveness of *Rhizobium trifolii* was hindered by base saturation, pH, exchangeable magnesium and calcium in the soil (Holding and King., 1963). Hagedom (1978) related poor effectiveness to phosphorus levels. Brockwell and Katznelson (1976) also observed that soil type did not affect effectiveness of native rhizobial populations.

2.6.6 Competition for nodule occupancy

Competition refers to an interaction between two or more organisms over limited resources such as nutrient, water, light and space in the environment to meet a biological demand (Alexander, 1971). Rhizobial strains differ much in competitiveness and their ability to occupy the nodule. Many studies on rhizobial competitiveness show that different rhizobia have different nodule occupancy ability (Date and Brockwell, 1978; Amarger, 1984; Bottomly, 1992; Triplett, 1990). Danso and Owiredu (1988) showed that three streptomycin resistant cowpea *Bradyrhizobium* strains differed in their competitive ability by showing differences in nodule occupancy. Host plant species and cultivar, initial population size and distribution in the soil and competition from other rhizobia are the environmental factors that affect nodule occupancy (Bottomly, 1992).

2.7 Inoculation in BNF

In most tropical soils many factors affect rhizobia in BNF which include inadequate rhizobial population (Singleton *et al.,* 1992), absence of compatible rhizobia and inefficient rhizobia in

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fixing nitrogen (Catroux *et al.,* 2001). In such soils inoculating with appropriate rhizobia can improve yield provided no other constraints limit growth of the leguminous plant (Turk and Keyser, 1993). However, indigenous populations of rhizobia are abundant in the soil where legumes are cultivated (Sajjad *et al.,* 2008) and these indigenous populations are highly adapted to their local soil environments which may form more effective symbiosis than commercial inoculants isolated from distant and un-related soil environment (Gandee *et al.,* 1999). Inoculation is therefore defined as the introduction of sufficient amount of viable effective rhizobia to induce rapid colonization of the rhizosphere which allows nodulation after germination and produces optimum yield of legumes (Catroux, 1991).

The high competitiveness of the native rhizobia (Shamseldin and Werner, 2004) poses a threat to realizing the full benefit of inoculation. Thies *et al.* (1991) noted that the native rhizobia which are less effective are often rich in tropical soils and a key to overcoming their competitive advantage is through the composition and delivery of legume inoculants. Waswa (2013) suggested that another way to overcome this competitive advantage is to identify native rhizobia with superior symbiotic and competitive abilities and to use them in large amounts within inoculants thus building upon the biodiversity of indigenous rhizobial populations.

2.8 Molecular characterisation method

Powerful techniques have emerged for the screening, characterizing and evaluation of genetic diversity, evolution and phylogeny due to recent advances in molecular biology principally in the development of the polymerase chain reaction (PCR) for amplifying DNA and DNA sequencing (Karp and Keith, 1995). Among these techniques, is the Random amplified polymorphic DNA (RAPD) which is a polymerase chain reaction (PCR) to detect the polymorphisms in genomic

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DNA and widely used due to its simplicity in application and inexpensiveness (Williams *et al.,* 1990).

Also, PCR analysis using primers related to the repetitive sequences from bacterial genomic DNA is useful in the analysis of naturally occurring interspersed repetitive DNA elements (Versalovic *et al.,* 1994) since these elements are highly protected among most bacterial genome (Giongo *et al.,* 2008). These PCR techniques include REP (Repetitive Extragenic Pallindromic), ERIC (Enterobacterial Repetitive Intergenic Consensus) and BOX (Enterobacterial repetitive sequences) (Louws *et al.,* 1994).

The Amplified Ribosomal DNA restriction analysis (ARDRA) which also involves the PCR amplification of the 16S rRNA and the amplified 16S rDNA product is digested with restriction enzymes. The diversity of the conserved region can be assessed (Berrada and Fikri-Benbrahim, 2014; Pandey *et al,* 2004). Restriction Fragment Length Polymorphism (RFLP) analysis is another technique used to distinguish differences in amplified DNA by restriction enzymes (Odee *et al*., 2002).

Other techniques that have been employed in molecular biology include MLSA (Multilocus sequence analysis) and MLST (Multilocus sequence typing) which analyse several core or accessary genes useful in identifying actual groups of rhizobia (Rivas *et al.,* 2009). Sequencing of ribosomal 16S rDNA genes which is another technique have also been widely used for rhizobial genetic variability evaluations useful for classification and diversity studies (Berrada and Fikri-Benbrahim, 2014). Another method includes the FT-IR technique (Fourier-transform infrared spectroscopy) that allows for the study of diversity of bacteria at the intraspecific level (Oberreuter *et al*., 2002).

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2.8.1. The Importance of Molecular biology in *Rhizobium* studies

Prior to the use of molecular techniques, phenotypic techniques were used to study rhizobia diversity, evolution and phylogeny which helped in characterisation and taxonomy (Schwinghamer and Dubman, 1980). However, these phenotypic techniques have been limited in many ways which include high labour intensiveness, time consuming and low discriminatory power (Thies *et al.,* 2001). Classification schemes based on variation in genetic characters of rhizobia are often more informative than schemes based on phenotypic variation (van Berkum and Fuhrmann, 2000). Molecular techniques have been reported to be available since the 1980s and these were mostly based on polymerase chain reaction (PCR) analysis making them very convenient for characterization, because they are rapid, simple and discriminative (Sajjad *et al*., 2008). However, Sato *et al.* (1999) reported that some of the PCR-based techniques have low reproducibility ability, greatly depended on colony age, source and concentration of reagents and also on DNA purity and extraction protocol.

The use of molecular techniques in assessing rhizobia diversity has been reported to be useful in rhizobial collections and has increased contributions of legume-*Rhizobium* nitrogen fixation to agricultural productivity (Ogutcu *et al.,* 2009). Knowledge of the classification, genetic characterization and biodiversity of native rhizobial population has aided in the selection of inoculant strains (Toolarood *et al*., 2012). The high diversity of rhizobia generates effective strain classification methods needed to identify genotypes that display superior nitrogen fixation capacity (Sikora *et al.,* 2002).

The recent development in PCR techniques has enabled genetic analysis of a larger portion of the rhizobial genome providing additional information and making classification of rhizobia more

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definitive (Prevost and Antoun, 2006). The use of PCR and sequencing techniques have resulted in the creation of new species, examples are *Rhizobium gallicum* and *Rhizobium giardinii* (Amarger *et al.,* 1997) and the confirmation of *Rhizobium etli* (Martinez *et al.,* 1985; Segovia *et al.,* 1993) in the *Rhizobium* genus. Also, by the use of the DNA-DNA hybridization, it has been possible to distinguish *R. tropici* types A and B (Martınez-Romero *et al.,* 1991; Geniaux *et al.,* 1995).

Molecular techniques have assisted in developing quick and easy methods to microbial characterization including differentiating genera, species and even strains (Schneider and de Brujin, 1996; Giongo *et al.,* 2008). Genomic DNA Fingerprinting using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between rhizobial strains (Labes *et al*., 1996) where reliable information are provided on the diversity of *Rhizobium* populations in soils (Oliveira *et al.,* 2000). Polymerase chain reaction (PCR) and the use of primers corresponding to the REP, ERIC and BOX PCR can create highly characteristic patterns when distinguished in agarose gels, providing well separation to the strain level (Adiguzel, 2006). Polymerase chain reaction (PCR) has helped in identifying rapid and explicit marker strains among field rhizobial isolates (Shoukry *et al.,* 2013). PCR-RFLP for diversity assessment, have been evidenced as rapid, effective and reliable technique for identifying genetic differences of rhizobia isolates (Dai *et al.,* 2012)

2.9 Methods of assessing nodulation and Biological Nitrogen Fixation

In order to realise the full potential of legume-*Rhizobium* interaction, it is important to assess the nodulation and the nitrogen fixed. Several methods are known which include: nodule number, nodule colour, nodule mass, shoot dry matter yield, total N uptake, % nitrogen derived from air using the N difference and N^{15} method (Beck e*t al.*, 1993).

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2.9.1 Nodulation

Nodule number, nodule mass, nodule colour, longevity of the nodule population and distribution can be used to evaluate the nitrogen fixation abilities of leguminous crops (Unkovich *et al.,* 2008). Nodule colour can be assessed by gauging the degree of pink/red coloration of nitrogen fixing bacteroids tissue inside the nodule (Peoples *et al.,* 1989). This pink/red coloration is indicative of the nitrogen fixing ability of the bacteroids in the nodules (Oagile, 2005).

In legumes, symbiotic nitrogen fixation resides solely within the nodules. The nodule abundance or mass provides simple and indirect evidence for whether legumes fix nitrogen and the magnitude of nitrogen fixed (Graham, 1981). Nodule assessment is inexpensive and does not need highly skilled labour. The validity of this technique rests solely on the assumption that nodules formed on a given variety of legume fix similar amounts of nitrogen.

2.9.2 Dry matter yield

Crop yields are often increased by the provision of available nitrogen (Rennie, 1982) and dry matter is an indirect evidence of nitrogen fixed (Unkovich *et al*, 2008). In soils with depleted N, dry matter production is mainly dependent on nitrogen fixation (Brockwell *et al*., 1982). The relationship between dry matter production and nitrogen fixed is indicated by the significant accumulation of greater dry matter of effectively nodulated plants, compared to the lower dry matter accumulation by ineffectively nodulated or non-nodulated plants (Burton, 1979, Unkovich *et al*, 2008). One limitation of this method is that significant yield responses are sometimes not attained in the presence or absence of nitrogen fixation. This may be due to other limiting factors besides nitrogen that do not permit the nitrogen fixed to be translated into increased yield (Bethlenfalvay and Phillips, 1977).

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2.10 Measurement of Biological Nitrogen Fixation

There are several methods available for estimating nitrogen fixed in plants although these methods have their advantages and disadvantages (Rennie and Rennie, 1983; Danso, 1985).

2.10.1 Total Nitrogen Difference method

This method involves the quantification of total nitrogen in both the fixing plant and the reference crop plant (Hardarson *et al.,* 1987) and is the simplest of all methods (Danso *et al.,* 1992). This is the basis for the use of N-free media (Leonard, 1943) for estimating nitrogen fixation. According to Danso (1993), in addition to nitrogen in the seed the fixing plant takes up soil N and N from air. Therefore to distinguish between the sources of plant N, a non-nitrogen fixing plant is used to provide an estimate of soil N uptake by the nitrogen fixing legume. Analysis for the total N could be done as the difference between the two plants (Danso and Herridge, 1987). Some of the non-fixing plants that have been used are cereals, (Broadbent *et al.,* 1982), a non-fixing nodulating legume isoline (Legg and Sloger, 1975), uninoculated legumes and legumes inoculated with ineffective rhizobia (Rennie *et al.,* 1982). This method however has been found to be useful in screening large numbers of nitrogen fixing plants or microorganisms in N free media or soils low in N (Rennie and Rennie., 1983). However there are limitations associated with this method which include the difficulty in getting a suitable non-fixing plant for the reference and the likelihood that the fixing legume and non-fixing reference plant may be taking up different quantities of soil N, which could be a major source of error (Danso, 1985). The formula popularly used in this method includes:

$$TN_{(f1)} - TN_{(nf1)} = Ndfa$$

Where

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$TN_{(f1)}$= Total Nitrogen in the N_2 fixing isoline

$TN_{(nf1)}$ = Total nitrogen in the non-fixing isoline

$Ndfa$ = Amount of N_2 derived from atmosphere (fixed N_2)

2.10.2 Isotope Dilution Technique

This method involves the growth of N_2 fixing and non-fixing reference plants in soil labelled with ^{15}N enriched inorganic or organic fertilizers. It is based on differential dilution in the plant of ^{15}N labelled fertilizer by soil and fixed nitrogen (Fried and Middleboe, 1977). The use of this method provides an integrated measurement of the amount of fixed N accumulated by a crop over the growing season (Fried *et al.,* 1983). Nitrogen in the atmosphere is virtually all $^{14}N_2$ (99.633%) plus a small amount (0.367) of the natural ^{15}N (Marrioti, 1983). Any substance which has an atom % ^{15}N greater than that of the atmosphere is enriched with ^{15}N and is expressed as atom % ^{15}N excess. Similarly, material depleted in ^{15}N has an atom % ^{15}N below that of the atmosphere is said to be % ^{15}N depleted material (Marrioti, 1983). If a plant is grown under conditions where the sole source of N (apart from inoculum) is ^{15}N, and it fixes $^{14}N_2$ from the atmosphere, then the plant will have an atom % ^{15}N which is less than that of the fertilizer. The difference can be used to calculate the proportion of N derived from N_2 fixation (Giller and Wilson, 1991). This is the underlying principle of the isotope dilution method of measurement of N_2 fixation. However, its use is limited because of the high cost of the^{15}N isotopic fertilizer and the expensive equipment needed for the determination of the $^{15}N/^{14}N$ isotopic ratios especially in developing countries.

The following working formula was established by Fried and Middleboe (1977) for the calculation of % Ndfa in fixing plant.

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$$\%Ndfa = 1 - \frac{\%Nff(fixer)}{\%Ndff(non-fixer)} \times 100$$

Where;

$\%Ndfa$ = percent N derived from the atmosphere by the fixing plant

$\%Nff(fixer)$= percent N derived from the ^{15}N labelled fertilizer by the fixing plant

$\%Ndff(non-fixer)$ = percent N derived from the ^{15}N labelled fertilizer by the non-fixing plant

2.11 Factors Affecting Nodulation and Nitrogen Fixation

Fixation of N (about 15-20 N/ha) by grain legumes has seasonally been recorded in Africa which is an important factor in cropping systems of traditional farmers (Dakora and Keya, 1997). However the use of biological N is constrained by factors in the soil and the environment. Giller (2001) reported that the ability of free living or symbiotic N_2 fixers to actually fix N_2 in the field is strongly influenced by the prevailing environmental conditions. Such factors may be physical, chemical or biological.

2.11.1 Physical Factors influencing biological nitrogen fixation

2.11.1.1 Soil Temperature

Generally, soil temperature inhibits legume BNF through its control on nodulation, nodule establishment, and nitrogenase activity (Roughley and Dart, 1970). Lui *et al.* (2010) reported this control to be located at the root zone. To be able to define the response of N fixation to soil temperatures, the minimum, maximum and the range of temperatures between could be used (Lui *et al.,* 2010). In certain parts of the tropics, the surface soil temperatures can occasionally

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reach 65-70°C and temperatures above 50°C have been found at 5cm depth (Dudeja and Kharana, 1989) which is sufficiently high to inhibit the germination of seeds and kill many bacteria.

Schomberg and Weaver (1992) reported that the nodulation of arrow leaf clover (*Trifolium vesiculosum* Savi.) was accelerated at a root temperature of 25°C compared with that growing at both 18°C and 32°C. Excessive soil temperatures killed most rhizobia in the root zone, though some rhizobia could survive at 70°C in dry soil (Marshall, 1964). High temperatures prevented nodulation and if nodulation occurred at all, the process of N_2 fixation was inhibited in legumes (Day *et al*., 1978). Equally, cool temperatures led to delayed development of plants, including delays in the formation of nodules and thereby decreased the rate of N_2 fixation. Lui *et al.* (2010) also reported that varieties and species of plants differed in terms of nodule establishment due to soil temperatures.

In soybean (*Glycine max* (*L.*) Merr.), more nodules were produced in the early growth stage at 25°C; while 20°C was optimal for nodule size after nodule generation was completed (Lindemann and Ham, 1979). In contrast, nodule establishment is enhanced with increasing temperature in the range of 10-35°C for white clover (*Trifolium repens L.*) regardless of the varieties and the rhizobial strains (Richardson and Syers, 1985). Survival of bacteria in soils at high temperature was improved by the presence of clay particles and soil organic matter, but many of the soils where high temperatures were experienced were sandy. Day *et al.* (1978) disclosed that rhizobial population of only 4-40 cells per gram of soil were found at a depth of 20-25cm below the surface of the soil. Eaglesham and Ayanaba (1984) also demonstrated the differences in environmental adaptation to high temperatures between rhizobia isolated from different climate zones.

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2.11.1.2 Drought

The occurrence of rhizobia in desert soils and the effective nodulation of legumes growing in these soils highlights the fact that rhizobia exist in soils with limiting moisture levels whiles population densities tend to be lowest under the most desiccated conditions (Tate, 1995; Jenkins *et al.,* 1989). Adequate moisture is required for both the normal growth and development of the legume host and the survival of rhizobia (Giller, 2001).

Drought is the commonest stress factor affecting legume development and yield worldwide (Serraj *et al.,* 2003). Zahran (1999) reported that rhizobia are less sensitive to osmotic stress than legumes which are identified to be more sensitive. A decrease in plant water potential is promoted by decrease in water potential and hydraulic conductivity in a dry soil which results in difficulty for plants to absorb water further affecting a number of physiological processes (Kantar *et al.,* 2010). Nodules are also affected by drought by showing retarded growth which results in a partially developed root cortex-embedded organ (Kantar *et al.,* 2010). Drought reduces oxygen permeability and limits nodule ability to carry out oxidative phosphorylation in spite of maintaining relatively high photosynthetic rates (Aguirreolea and Sanchez-Diaz, 1989).

Legume responses to drought also rest on the type of nodule whether it is determinate or indeterminate (Venkateswarlu *et al.,* 1990). Indeterminate nodules for example those in alfafa, clover and faba bean show resistance to low moisture content (Swaraj, 1987) and are more drought tolerant than the determinate ones found in soybean and common bean (Sprent and Zahran, 1988).

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There is also a rapid decline in rhizobial cells as a result of drought (Giller, 2001). However, rhizobia tend to survive better in dry soils which contain amounts of clay and organic matter (Choa and Alexander, 1982).

Rates of N_2 fixation are therefore more sensitive to reduction in soil water content than other processes such as photosynthesis, transpiration, leaf growth rates or nitrate assimilation (Sinclair *et al*., 1987; Serraj *et al.,* 1999). Transient water stress can lead to shedding of nodules by nodulated legumes. Infection of root hairs is restricted because they become short, stubby and inadequate for rhizobial infection in dry soil (Lie, 1981). It also affects the formation and longevity of nodules, synthesis of leghaemoglobin and nodule function (Hungria and Vargas, 2000).

2.11.1.3 Waterlogging

Waterlogging imparts considerable stress on N_2 fixation (Serraj and Adu-Gyamfi, 2004). If temperatures are high and there is much organic substrate present, free oxygen is rapidly used up hence the presence of anaerobic conditions in waterlogged soil (Giller, 2001). Lack of oxygen is also a major problem for root respiration and can rapidly result in loss of nitrogenase activity in waterlogged soils (Sprent and Gallacher, 1976). There is rapid release into the soil solution large concentrations of iron and manganese in waterlogged soils, which are normally present in relatively insoluble oxidized forms in non-waterlogged soils. Toomsan (1990) reported that the size of the populations of rhizobia sampled from the field was more (10^2-10^4 cells g^{-1} soil) when the soil was moist or fully waterlogged than when the soil was dry ($<10^1$-10^3 cells g^{-1} soil). Also excess water was harmful to N_2 fixation since it lowered oxygen diffusion for nodule functions and may lead to CO_2 build-up (which inhibits nodule formation) (Eaglesham and Eaglesham, 1984; Farahani *et al*., 2008).

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2.11.1.4 Salinity

Salinity limits the productivity of agriculture and activity of nitrogen fixing legumes in arid and semi-arid climates (Rao *et al.,* 2002; Serraj and Adu-Gyamfi, 2004). Increasing salt concentrations may have a detrimental effect on soil microbial populations as a result of direct toxicity and through osmotic stress (Tate, 1995). Salinity decreases plant growth and yield, depending upon the plant species, salinity levels, and ionic composition of the salts (Delgado *et al.,* 1994). High salt concentration can directly impair the legume-rhizobia early interactions during nodule formation (Singleton and Bohlool, 1984).

For most cultivated crops, the salinity response of legumes varies greatly and depends on factors as climatic conditions, soil properties, and the stage of growth (Cordovilla *et al.,* 1995). Variability in salt tolerance among crop legumes has been reported (Zahran, 1991) and some legumes, e.g., *Vicia faba*, *Phaseolus vulgaris*, and *Glycine max*, are more salt tolerant than others e.g., *Pisum sativum.* However some legumes are salt tolerant while their rhizobia are not *e.g.* Prosopis, Acacia and *Medicago sativa* (Abdel-Wahab and Zahran, 1983; Zhang *et al.,* 1991; Fagg and Steward, 1994).

The legume-*Rhizobium* symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress and this inhibits the symbioses than the rhizobia involved (El-Shinnawi *et al.,* 1989; Velagaleti *et al.,* 1990). When soybean plants were inoculated with *Bradyrhizobium japonicum,* soybean root hairs showed little curling or deformation in the presence of 170 mM NaCl and nodulation was completely suppressed at 210 mM NaCl (Tu, 1981). The effects of salt stress on nodulation and nitrogen fixation of legumes have been examined in several studies (Abdel-Wahab and Zahran, 1981, 1991; Delgado, 1994). The reduction of N_2-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules (Hunt *et al.,* 1981) and

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a reduction in cytosolic protein production, specifically leghemoglobin, by nodules (Delgado *et al*., 1993).

2.11.2 Chemical Factors

2.11.2.1 Soil pH

The inhibitory effect of low soil pH on R*hizobium* and its legume host can be direct or indirect through nutrient toxicities and deficiencies (Reeve *et al*., 2002). Low pH considerably reduces the survival and hence colonization of soil by rhizobia (Munns, 1977) and therefore this constrains symbiotic N_2 fixation and nodulation in both tropical and temperate soils (Munns, 1986). Aluminium toxicity as a result of low soil pH also reduces rhizobial survival (Keyser and Munns, 1970). Only bacteria with greater capacity to regulate their internal pH show increased survival rate at low pH (O'Hara *et al.,* 1989). However, rhizobial strains differ in their ability to tolerate soil acidity, making it possible for superior acid tolerant strains to be selected for inoculant production (Moawad and Bohlool, 1984). It has however been found that *R. meliloti* multiplied at pH 4.5 but *Bradyrhizobium* strains failed to multiply at that pH (Cooper *et al.,* 1985). The fast-growing strains of rhizobia have generally been considered less tolerant to acid pH than the slowly growing strains of *Bradyrhizobium* (Graham *et al.,* 1994), although some strains of the fast-growing rhizobia, e.g., *R. loti* and *R. tropici*, are highly acid tolerant (Cooper *et al.,* 1985). The failure of legumes to nodulate under acid-soil (pH less than 5.0) is due to the inability of some rhizobia to persist under such conditions (Bayoumi *et al.,* 1995). Aluminium, iron and manganese toxicities which result from low soil pH and the corresponding decrease in availability of phosphorus, molybdenum and calcium severely affect plant growth and inhibit nodulation and N_2 fixation (Hungria and Vargas, 2000). Calcium deficiency due to low pH, also affects attachment of rhizobia to root hairs, and nodulation and nodule development (Alva *et al.,*

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1990). Low pH affects early stages of infection process including the exchange of molecular signals between symbiotic partners and attachment to the roots (Hungria and Vargas, 2000). Graham (1981) also stated that other stages of nodule establishment and function are also impacted by low pH.

2.11.2.2 Nutrient Deficiency and availability

Most of the nutrients essential for growth of plants or bacteria play specific roles in nodulation and or N_2 fixation. Deficiencies in these nutrients, or other essential elements for the growth of bacteria or plants, can cause reduction in the numbers, size of nodules formed and in the amount of N_2 fixed (Giller and Wilson, 1991).

2.11.2.2.1 Phosphorus

Phosphorus is one of several elements which affect N_2 fixation, and, along with N, it is an essential macronutrient for plant growth and function (Pereira and Bliss, 1989). Phosphorus acts on nodulation, nitrogen fixing and growth of legumes (Israel, 1987; Tang *et al.,* 2001; Valverde *et al.,* 2002). The requirements of host plants for optimal growth and symbiotic nitrogen fixation processes for P have been assessed by determination of nodule development and functioning (Sa and Israel, 1991). Nodules are strong sinks for P as a result N_2 fixation-dependent plants will require more of this element than those supplied with combined nitrogen (Cassman *et al.,* 1981a). The increases of plant growth and plant nitrogen concentration in response to increased soil P supply have been identified for several leguminous species (Andrew and Robins, 1969; Israel, 1987; Israel, 1993). Beck and Munns (1984) discovered that there was substantial variation in the abilities of rhizobia to grow in low concentrations of phosphorus which appeared to be due to variation in the efficiency of phosphorus uptake systems. Strains of rhizobia differ

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markedly in tolerance to phosphorus deficiency and that slow-growing strains of rhizobia appeared more tolerant to low P levels than fast-growing ones (Beck and Munns, 1985).

There is high demand of phosphorus by nitrogen fixing plants than nitrogen (Israel, 1987). Acute deficiency of phosphorus can prevent nodulation by legumes and also impair both host plant growth and symbiotic N_2 fixation (Cassman *et al.,* 1981b; Tang *et al.*, 2001). Tsvetkova and Georgiev (2003) stated that decreased specific nitrogenase activity in nodules of P deficient leguminous plants was associated with decreased energy status of host plant cells of nodules therefore phosphorus deficiency reduced growth and nitrogenase activity. Several researchers have obtained improvements in nodulation and N_2 fixation by supplying phosphorus to phosphorus deficient soil (Bethlenfalvay and Yolder, 1981).

2.11.2.2.2 Soil Nitrogen

Mineral N has been documented to increase crop production in Africa (Dakora and Keya, 1997; Kamanu *et al.*, 2012). In the tropics, Borlaug (1991) reported that about 90% of mineral N is found in living plants, with only a small fraction left in soils which is the reverse in the temperate environments.

The differences in soil nitrogen affects nodulation and nitrogen fixation of African soils (Peoples and Herridge, 1990). Increasing levels of mineral N in the rhizosphere of plants inhibit nodule formation and N fixed (Ani *et al.,* 2007; Streeter, 1988). Works done by many researchers have concluded the inhibition of N on legumes (Gentili and Huss-Danell, 2002; Voisin *et al.,* 2002; Otieno *et al.,* 2009; Walsh, 1995) and this inhibitory effect can be related to *Rhizobium* mechanism to avoid wastage of photosynthase on supporting nitrogen fixing symbiosis when it can turn to using available N (Hirsch, 1996). George *et al.* (1988) stated that soil N availability at

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different sites determine the relative contribution of symbiotic N_2 fixation, regardless of crop duration and total N accumulation by different varieties. This relates to report by Atkins (1986) that leguminous crops use an amount of 2.9-6.1g C g^{-1} N in forming nodules with the absence of N compared to an amount of 0.8-2.4g C g^{-1} N with the presence of N. Thus, with abundance of nitrogen, nitrogen fixing microorganisms tend to be competitively excluded by non-fixing species because the nitrogen fixation process is bio-energetically costly (Houlton *et al.,* 2008).

2.11.2.2.3 Other nutrients

Calcium helps the rhizobial cells to attach to the root hair cells of leguminous plants (Caetano-Anolles *et al.,* 1989). In saline soils, reduced attachment and colonization of root hairs of *V. faba* by *R. leguminosarum* was attributed to the effect of salt on calcium availability (Zahran and Sprent, 1986). Low pH and low Ca levels delay nodulation in *Vigna unguiculata* whiles at high Ca level, nodulation was improved (Hohenberg and Munns, 1984). Calcium also increased the *nod* gene induction and expression activities of clover plants 5 to 10 folds at pH 4.5 to 5.2 (Richardson *et al.,* 1988).

Molybdenum is one of the major micro mineral requirements in plant for N_2 fixation. Giller and Wilson (1991) reported that molybdenum is a constituent of the enzymes nitrate reductase (required for the assimilation of nitrate from the soil) and nitrogenase; so deficiency of Mo can affect N_2 fixation greatly.

Under adequate conditions, potassium (K) is known to stimulate infection and N_2 fixation (Vincent, 1962) while sulphur deficiency affects nodule number, size and N_2 fixation (Munns, 1977). Other nutrients such as manganese, magnesium, aluminium etc. are known to affect nodulation and N_2 fixation (Brady *et al.,* 1990).

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2.11.3 Biological factors

2.11.3.1 The Bacteria

The presence of effective and competitive *Rhizobium* in the soil enhance effective nodulation and nitrogen fixation (Danso and Owiredu, 1988). Dudeja and Khurana (1989) reported that the survival and persistence of an adequate number of effective rhizobia in soils is essential to ensure nodulation. It was discovered by O'Hara (2001) that for *Rhizobium* to grow, survive and colonise soil depends on the chemical and physical properties of the soil, whereby mineral and organic nutrients have to be present in the soil to enhance their metabolic processes. Many soils lack effective strains of *Rhizobium* in spite of the natural habitation of the soil and thereby call for the need for inoculation with effective strains in most soils (Dudeja and Khurana, 1989).

2.11.3.2 The Host plant

The host plant controls rhizobia rhizosphere stimulation and is responsible for initial infection (Dudeja and Khurana, 1989) including specific site and potential number of infections as well as nodulation, potential number of nodules, size and patterns of distribution of the nodules on the root system. It is therefore not surprising that some leguminous plants do not nodulate and fix nitrogen (Dudeja and Khurana, 1989).

2.11.3.3 Other organisms

The growth and survival of rhizobia are determined by the presence of other microorganisms through predation, parasitism and competition (Giller, 2001). Some factors that could reduce rhizobial numbers in soil are bacterial parasites such as *Bdellovibrio,* antibiotics produced by other soil microbes and bacteriocins produced by other rhizobia (Hirsch, 1996).

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Also, rhizobial strains may be lysed by bacteriophages resulting in their poor survival (Barnet, 1980). Protozoa has been shown to reduce the populations of rhizobia in soil by grazing on them (Danso *et al.,* 1975). However, Heijnen *et al.* (1991) reported that soils containing more clay protect rhizobia from protozoa where they take refuge in microspores within soil aggregates.

Also damage by pests, disease and grazing animals has indirect deleterious effect on N_2 fixation where damage to root nodule by some insects in soil is a factor (Giller, 2001)

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Soil Sampling and Description

Six (6) soils were used in the study. These were Adenta, Bekwai and Nzima series, and three others sampled from farmer's fields at Dzolokpuita, Dzologbogame and Akorviefe (with GPS 06^0 78' N: 0^0 44' E, 06^0 75 N: 0^0 43' E and 06^0 76' N: 0^0 47' E, respectively) in the Volta Region where AYB is being cultivated. Bekwai and Nzima were sampled from the catena at the Forest and Horticultural Crops Research Centre (FOHCREC), Okumaning-Kade (60 43' N: 10 36' W) whilst Adenta series was sampled from the School of Agriculture Farm at the University of Ghana Campus, Legon (050 39' N: 00 11' W). The soils were taken from a depth of 0-20cm. The sampled soils were then air dried, crushed and passed through a 2mm sieve to remove stones, plant debris and concretions.

3.1.1 Adenta series

Adenta series is an Alfisol (Soil Survey Staff, 1998) and forms part of the soil series along a catena down the Legon hill. It is found in the Coastal Savannah agro-ecological zone of Ghana and has total annual rainfall of about 800 mm which is bimodally distributed (Dowuona *et al.,* 2012). Adenta series has been classified as Typic Kandiustalf (Eze, 2008) according to USDA classification system. It occurs on the middle slope with site gradient of 1-2% and the dominant vegetation include neem tree and *Panicum maximum* (Eze, 2008). The soil has a parent material of sandy tertiary deposits underlain by Togo quartzite schist (Eze, 2008). It is well drained with a 122 cm deep profile and consists of dark reddish brown sandy clay loam throughout its depth with weak fine granular structure.

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3.1.2 Bekwai and Nzima series

Bekwai and Nzima series form part of the soil series along a catena and are classified as Ferric Acrisol and Haplic Acrisol, respectively (Owusu-Bennoah *et al*., 2000) according to the FAO (1988). Bekwai series is found on the summit of the slope whilst Nzima is at the upper and middle slope but sometimes extends to the summit (Council for Scientific and Industrial Research [CSIR], 2013). They are found in the semi-deciduous forest agro-ecological zone, with mean annual rainfall ranging from 1500 mm to 2000 mm and experiences bimodal pattern (Asomaning *et al*., 2006). The soil moisture regime is udic with temperature regime being isohyperthermic (van Wambeke, 1982). The soils have a granitic parent material as they are developed from the lower Birrimian phyillites (Adu, 1992). Bekwai series is red, gravelly silty clay and well drained sedentary soil with a slope gradient lying between 3 and 12%. Nzima series however is a paler version of the Bekwai series with quartz gravel content being high and also less well drained (Council for Scientific and Industrial Research [CSIR], 2013). Dominant plant species occurring on these soils are *Chromalaema odorata*, *Mimosa pudisca* and *Centrosema pubescence* (Asomaning *et al*., 2006). The major crops cultivated on these soils are cocoa, oil palm, maize, cassava and plantain (Owusu-Bennoah *et al*., 2000). The soils are known to be identified with aluminum toxicity and phosphorus deficiency which have a constraint on the fertility of the soils (Owusu-Bennoah *et al*., 2000).

3.1.3 Soils from Dzolokpuita, Dzologbogame and Akorviefe (Kuli series)

Soils from Dzolokpuita, Dzologbogame and Akorviefe towns are known as Kuli series and classified as Haplic Acrisol (Soil Research Institute, 1990) according to the FAO-UNESCO (1990). They are located at the moist semi-deciduous agro-ecological zone with two rainfall regimes in a year starting from March to July for the first regime and mid-August to October for

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the second regime. Maximum rainfall is about 2103 mm and minimum is 1168 mm. The soils are developed from gneiss-granifold terrain mainly biotite and biotite-amphibole and occurs on middle to lower slopes where gradients are between 8 and 10%. The profile of Kuli series consists of 5-10cm of greyish brown, humus, light clay overlying 10-25-81cm of greyish brown to strong brown, sandy light loam to sandy light clay. Some profiles contain ironstone concretions below 182cm. The difference between these soils are mainly from their management practices by farmers. The major crops cultivated on these soils are cocoa, oil palm, coffee, avocado, yam, plantain, banana, maize, cassava, legumes and a variety of vegetables (*http://howest.ghanadistricts.gov.gh*, 2006).

3.2 Soil Analysis

Physical and chemical analyses conducted on the soil samples are discussed below.

3.2.1 Chemical Analysis

3.2.1.1 Soil pH

The pH of each soil was measured using electrometric method by Peech (1965) in distilled water at a ratio of 1:1 soil: water, using a Hanna H19017 microprocessor pH meter standardized with standard aqueous solutions of pH 4 and pH 7. Twenty (20) grams of sieved soil was weighed into a beaker and 20 mL distilled water was added to form a suspension. The suspension was then vigorously stirred for about 30 min and allowed to stand for 1 hour to allow for the settlement of the entire suspended particles. The pH of the soil was measured after carefully and gently immersing the glass electrode of the pH meter into the supernatant. The same procedure was repeated for 10g of soil and 20mL of 0.01M $CaCl_2$. The pH was determined as pH in salt in soil.

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3.2.1.2 Soil Organic Carbon Determination

Organic Carbon was determined by the wet combustion method of Walkley and Black (1934). Into an Erlenmeyer flask, 0.5 g of soil samples that had been sieved through 0.5 mm sieve was weighed. Ten milliliters of potassium dichromate was added followed by 20 mL of concentrated H_2SO_4, to provide heat. The flask was swirled to ensure that the solution was in contact with all the soil particles and then allowed to stand for one hour. After that 200 mL of distilled water, 10 mL of H_3PO_4 and 2 ml of barium diphenyl sulphate indicator were added and titrated against 0.25 M ferrous ammonium sulphate solution until the colour changed.

The titre value was used to calculate the organic carbon in g/kg soil as:

$$\mathrm{OC}\ g/kg = \frac{[10 - (\mathrm{XM}) \times 0.003 \times 1.33]}{\mathrm{W}} \times 1000 ------------------[1]$$

Where $\mathrm{OC}\ g/kg$= organic carbon in g per kg soil

X =Titre value (mL)

M=Molarity of ferrous ammonium sulphate [$Fe(NH_4)_2(SO_4)_2$]

W= Weight of soil sample

3.2.1.3 Available Phosphorus Determination

Bray 1 method (Bray and Kurtz, 1945) was used to determine the available phosphorus. Air-dried soil of 5g that had been previously sieved through 2mm sieve was weighed into an extraction bottle and 50 mL of Bray 1 (mixture of 0.03M NH_4F and 0.025M HCl) solution was dispensed onto the sample and shaken on a mechanical shaker for three minutes at 200 rpm. The suspension was filtered through No. 42 Whatman filter paper into a clean 100mL glass bottle. To

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an aliquot (5) mL of the filtrate, P-nitrophenol indicator was added to adjust the pH and neutralized with a few drops of 4M NH_4OH until the solution turned yellow. Eight mL of reagent B (1.056g of ascorbic acid dissolved in 200 mL of reagent A) was added and allowed to stand for 15 minutes for colour development (blue colour). Reagent A was obtained by dissolving ammonium molybdate and antimony potassium tartrate in 250 mL of distilled water. Sulphuric acid was added and thoroughly mixed and made to volume (Watanabe and Olsen, 1965). A blank was also prepared with distilled water and 8mL of reagent B. The spectrophotometer was calibrated using P solution standards ranging from 5, 10, 15, 20 and 25mg $L^{-1}$to obtain a standard curve. Measurements of P in the samples were then made on Pharo 300 spectrophotometer at wavelength of 712 nm. The available P concentration in the soil sample was calculated as shown below relation below:

$$\text{P } mg/kg = \frac{\text{R} \times \text{Vol. of extract}}{\text{Vol. of aliquot} \times \text{Weight of soil}} ------------------- [2]$$

Where R = Spectrophotometer reading in mg L^{-1}

3.2.1.4 Available Nitrogen Determination

Five (5) grams of soil was weighed into 100 mL extraction bottle and 2 M KCl was added. The extraction bottle and its content were shaken for 30 minutes in a mechanical shaker at 200 rpm. The suspension was settled for 10 minutes and then filtered through a Whatman Number 42 filter paper. Five (5) mL aliquot was pipetted and about 0.2 g of MgO was added. The content was distilled for ammonia and the distillate collected in 2% boric acid (containing a methylene blue and methyl red indicator mixture) in a 150 mL conical flask. Boric acid with the distillate was titrated against HCL to obtain the titre value. The distillation flask was then removed from the heating mantle and 1 mL sulphamic acid was added to destroy nitrite in the sample and 0.2 g

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Devarda's alloy was also added and redistilled to reduce the NO_3-N to NH_4^+-N. The distillate was collected in 2% boric acid and titrated against 0.02M HCl to obtain the titre value. The concentration of NO_3^- / NH_4^+ kg^{-1} soil was calculated as follows:

$$\mathrm{NO_3^-/NH_4^+\ kg^{-1}} = \frac{M_{HCl} \times V_{HCl} \times 10^{-3} \times 18 \times V_{KCl} \times 1000mg \times 1000g}{\text{Vol. of Aloquot} \times \text{Weight of soil (g)}} --------[3]$$

Where:

M_{HCl} = Molarity of the HCl

V_{HCl} = Titre of the HCl

V_{KCl} = Volume of KCl extractant

18 = Molecular weight of NH_4^+

3.2.1.5 Cation Exchange Capacity (CEC)

Soil of 10g was weighed into an extraction bottle and 100 mL of 1 M ammonium acetate (NH_4OAc) solution buffered at pH 7.0 was added and the extraction bottle was shaken on a mechanical shaker for 1 hr. at 200 rpm. The solution was filtered through number 42 Whatman filter paper. The filtered samples were washed with 100 mL methanol to wash off the non-adsorbed ammonium ions. The ammonium saturated soil was leached with acidified 1M KCl solution. Into a kjeldahl flask, 5mL of the leachate was transferred and then distilled after adding 5 mL of 1M NaOH. The distillate was collected into 5mL of boric acid. Mixed indicator of three drops containing methyl red and methylene blue were added to the distillate and then titrated with 0.01M HCl from green to violet endpoint to obtain the total CEC. The calculation of the CEC is as follows:

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$$\text{CEC } (cmol_c kg^{-1} \text{ soil}) = \frac{V_{HCl} \times M_{HCl} \times 10^{-3} \times \text{Vol. of Extract.} \times 10^{3} \times 10^{2}\text{cmol}}{\text{Vol. of Aliquot} \times \text{Weight of soil(g)}} ----[4]$$

Where:

V_{HCl}=Titre value of the HCl

M_{HCl}=Molarity of the HCl

3.2.2 Physical Analysis

3.2.2.1 Bulk Density

The core sampler method of Blake and Hartge (1965) was used to determine the bulk density. A core sampler was driven through the soil far enough for all of its volume to be filled with the soil. It was then removed and the soil beyond each end of the core sampler was trimmed with a knife. The soil was transferred from the sampler into a moisture can of a known weight (W_1) and oven dried at 105°C for 48 hours after which its weight was taken as (W_2). The bulk density was calculated using the equation shown below. It was assumed that the volume of the soil sample was equal to the volume of the core sampler.

$$\text{Bulk Density } (gcm^{-3}) = \frac{W_2 - W_1}{V} --------------------------[5]$$

V= Volume of core sampler = $\pi r^2 h$

r= radius of core sampler

h= height of core sampler

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3.2.2.2 Particle Size Analysis

Forty grams (40g) of 2mm sieved soil sample was weighed into a dispersing bottle and 100 mL of 5% Calgon (Sodium Hexametaphosphate) solution was dispensed and shaken on a mechanical shaker for two hours at 200 rpm as demonstrated in the Bouyoucos Hydrometer method modified by Day (1965). The suspension was transferred into a 1 L graduated sedimentation cylinder and was made to volume by adding distilled water. The suspension was stirred vigorously using a plunger after which the first hydrometer reading (i.e. silt plus clay) was taken at exactly 5 minutes. After 5 hours (i.e. clay) readings were taken. The suspension was transferred from the sedimentation cylinder onto a 47-micron sieve under running tap water to obtain the sand fraction. The sand particles left in the sieve was transferred into a moisture can, oven dried for 24 hours, cooled in a desiccator and the dry weight determined. The particle size distributions for the various soil series were then calculated as follows:

Clay content = hydrometer reading at 5 hrs. = Ag

Silt content = hydrometer reading at 5 min- hydrometer reading at 5hrs = Bg

Sand content (weight of oven dried sample) = Cg

$$\%\ \text{clay} = Ag/40g\ \times 100 \text{ --------------------------- } [6]$$

$$\%\ \text{silt} = Bg/40g \times 100 \text{ --------------------------- } [7]$$

$$\%\ \text{sand} = Cg/40g \times 100 \text{ --------------------------- } [8]$$

Where: 40 = weight of soil sample in grams

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The distribution values were used to determine the textural class of the soils using the USDA textural triangle presented in the appendix (Appendix 3).

3.2.3 Biological Analysis

3.2.3.1 Most Probable Number Analysis

The rhizobia population estimated in the soils which are able to nodulate the test legume was determined by the Most Probable Number (MPN) plant infection assay (Vincent, 1970) using a modified Leonard jar assembly (Ferreira and Marques, 1992). The Leonard jars were made up of two plastic cups which fitted together. The cup at the bottom contained the nutrient solution which is sucked into the rooting medium in the cup above (containing acid washed sand) by capillary action aided by a cotton wick. About hundred milliliters (100mL) of N-free nutrient solution was dispensed into the lower cup of each Leonard jar. The whole assembly was sterilized by autoclaving. Legume seeds were surface sterilized with 70% alcohol and 0.1% mercuric chloride for 3 minutes and thoroughly rinsed in several changes of sterilized distilled water (Somasegaran and Hoben, 1994). Seeds were pre-germinated on moist tissue paper in sterilized petri dishes till the radicles were about 1-2 cm long. Seedlings were thereafter transplanted into each sterilized Leonard jar which contained autoclaved acid-washed sand. Transplanting was made by the use of a pair of sterilized forceps. The Leonard jars were arranged randomly in a screenhouse. One milliliter (1mL) of four-fold dilutions (four grams of the soil sample were diluted in twelve mL of sterile distilled water) was used to inoculate each jar (Somasegaran and Hoben, 1994) with four replicates of each dilution step. Nitrogen-free nutrient solution was supplied to the plants when necessary. The presence or absence of nodules were determined six weeks after planting and the most probable number of rhizobial cells per gram of soil per legume determined (Vincent, 1970) using the relation below;

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$$\mathrm{MPN} = \frac{\mathrm{m} \times \mathrm{d}}{\mathrm{v}} ------------------------------[9]$$

Where:

m = most likely number from MPN table,

d = lowest dilution in the series

v = volume of aliquot used for inoculation

3.3 Isolation of Rhizobia

Nodules were taken from each of the MPN assays performed. The nodules were surface sterilized by the method of Somasegaran and Hoben (1994). The nodules were crushed in a drop of sterile distilled water with a sterile rod. A loop was used to pick the suspension and streaked on yeast extract mannitol (YEM) agar (composition in Appendix 1) plates which were incubated at 28°C. A total of 35 *Rhizobium* isolates were obtained.

3.4 Screenhouse experiment

3.4.1 Test crops used

The test crops used were the African yam bean (*Sphenostylis stenocarpa)* as the main crop for the research, whilst soybean (*Glycine max* cultivar Anidaso), pigeon pea (*Cajanus cajan* cultivar ICPL 88034), cowpea (*Vigna unguiculata* cultivar black eye) were used for cross inoculation test and maize (*Zea mays* cultivar Obaatampa) as a reference plant.

3.4.2 Symbiotic effectiveness studies

A total of 65 of African yam bean rhizobial isolates were assessed for their effectiveness on the host plant. With 35 isolates being isolated from nodules formed from soils cultivated with

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African yam (soils from Dzolokpuita, Dzologbogame and Akorviefe) and 30 isolates from previous work by Tettey (2014) which were isolated from nodules formed in soils not cultivated with AYB (Akuse, Toje, Haatso and Adenta). The experiment was carried out in Leonard jars filled with acid washed and dried sea sand freed of any nitrogen. The bases of the Leonard jars were filled with stock solutions devoid of nitrogen (N-free nutrient solution) (Somasegaran and Hoben, 1994). African yam bean seeds were surface sterilized using the method of Somasegaran and Hoben (1994). The seeds were pre-germinated until the radicles were about 1-2 cm long and were transplanted at two seedlings per Leonard jar. Inoculation with 1 mL of 1 week old YEM broth culture of the respective isolates was done close to the base of the germinating plant in the Leonard jar using a calibrated pipette (Somasegaran and Hoben, 1994).

As a control, the seedlings were not inoculated but were supplied with nitrogen fertilizer in the form of KNO_3 at a rate of 100 μg ml^{-1}. In another control, the plants were not inoculated and did not receive nitrogen fertilizer. There were four replicates and the experiment was completely randomised in a screenhouse. The inoculated plants were supplied with N-free nutrient solution while the uninoculated controls received nitrogen in one set and no nitrogen in another set (Somasegaran and Hoben, 1994). The plants were harvested 6 weeks after planting. Shoot dry weight (SDW), number of nodules (NN), and nodule dry weight (NDW) were measured. SDW and NDW were determined from material dried at 70^0C until a constant weight was obtained (Argaw, 2012). The mean shoot dry weights dry weights (X) were used to calculate the index of effectiveness (EJ) defined as:

$$\mathrm{EJ} = \frac{X_J - X_{TO}}{X_{TN} - X_{TO}} \times 100 \quad [10]$$

(Ferreira and Marques, 1992)

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Where:

EJ = Effectiveness index

X_J= Shoot dry weight of inoculated test strain

X_{TO}= shoot dry weight of uninoculated control

X_{TN}= shoot dry weight of N control

3.4.3. Cross Inoculation Studies (Host Range Analysis)

The capability of each isolate to nodulate different leguminous plant species was evaluated using the cross inoculation concept. Cowpea (*Vigna unguiculata*), Soybean (*Glycine max*), Pigeon pea (*Cajanus cajan*) and African yam bean (*Sphenostylis stenocarpa)* were the legumes used as host plants. Seeds of the selected host legumes were surface sterilized and pre-germination was done. Two seedlings were transplanted per Leonard jar that contained sterile nitrogen free nutrient solution. Inoculation with 1 mL of one week old YEM broth culture of the corresponding rhizobial isolate was done close to the base of the emerging seedling (Somasegaran and Hoben, 1994). Uninoculated jars served as control treatment. The jars were completely randomised in the screenhouse. Watering of seedlings with sterile N-free nutrient solution was done when necessary. After six weeks of inoculation, the plants were assessed for nodule formation.

3.4.4 Inoculation and nitrogen response studies

The ability of African yam bean to respond to inoculation and nitrogen was evaluated in plastic pots containing 2 kg soil. The bottom of the pots were perforated to aid in drainage of excess water. Plastic plates were placed under the pots for collection of excess water. African yam bean was used as the test crop and maize served as the reference crop, three seeds were planted per pot

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and thinned to two after emergence. Three days after emergence, pots requiring inoculation treatments were inoculated with 5 mLs of 1 week old YEM broth culture (approximately 10^8 cells ml^{-1}) of two isolates obtained from the initial screening study based on their effectiveness (the two highly effective isolates were selected and these were isolates 29 [Inoculum I_2] and 55 [Inoculum I_1] from Table 4.2a and 4.2b). One week after emergence, nitrogen control pots were supplied with nitrogen in the form of Ammonium sulphate [$(NH_4)_2SO_4$] at the rate of 70 kg N/ha. The experimental design was the complete randomized design with three replicates and watering was done on daily basis to keep the soil moist. After seven weeks the shoots were harvested by carefully cutting the stem near the soil surface. Observation and counting of nodules were carried out after a careful wash of the roots under slow running water. The shoots and nodules were then oven dried at 70°C at 72 hrs. (Argaw, 2012). The dry weight of the shoot was measured and the plant samples were prepared for N analysis by milling (Mwenda *et al.,* 2011).

3.5 Chemical Analysis of Plant Material

3.5.1 Digestion of Plant Material

The dried shoot materials of the plant were ground in a grinding machine and exactly 0.5 g of the milled material was weighed into 25 mL volumetric flask. Five mL of concentrated sulphuric acid (H_2SO_4) was dispensed and mixed thoroughly by swirling the flask and its content. The flask was allowed to stand overnight to facilitate the dissolution of the plant sample in the sulphuric acid. Each solution was then heated for about 10 minutes where Hydrogen peroxide (H_2O_2) was added drop wisely until a clear solution was obtained. The digest was decanted into 100 mL flask and made up to the mark with distilled water.

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3.5.2 Determination of Plant Total Nitrogen

Exactly 5 mL aliquot of the digest (described in section 3.5.1 above) was pipetted into a Kjeldahl flask, 5mL of 40% NaOH solution and about 100 mL of distilled water were added and the mixture distilled. The distillate was collected in 2% boric acid to which about two drops of a mixed indicator (methyl red and methylene blue) were added in an Erlenmeyer flask. The distillate in the boric acid was then titrated against 0.01M HCl acid solution (Bremner, 1965).

The % nitrogen was determined with the equation below:

$$\mathrm{N}(\%) = \frac{\text{Titre value} \times \text{Molarity of HCl} \times \text{Vol. of extract} \times 0.0014 \times 100}{\text{Vol. of aliquot} \times \text{Weight of soil}} ------- [11]$$

Where:

0.0014 = milliequivalent of nitrogen

The shoot total N (mg/plant) was derived as shown below;

$$\text{Shoot Total N } (mg/plant) = SDW\ (g\ /plant) \times \frac{\text{shoot \% N}}{100} \times 1000 -------- [12]$$

Where SDW is the shoot dry weight

The %Ndfa was analyzed using the N difference method and the total N fixed was also analyzed by the following equations:

$$\%Ndfa = \%\ N_{(fixer)} - \%N_{(nonfixer)} ---------------------- [13]$$

$$\text{Total N fixed } \left(\frac{mg}{plant}\right) = \frac{\text{\% Ndfa}}{100} \times SDW_{(fixer)} \left(\frac{g}{plant}\right) \times 1000 ------- [14]$$

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CHAPTER FOUR

4.0 RESULTS

4.1 Soil Characterization

Some chemical, biological and physical characteristics of the soils are shown in Table 4.1. The pH in water for the soils used in the study was in the range of 5.4 to 6.3. According to USDA (1998) classification, soil from Akorviefe was slightly acidic. Adenta series, Nzima series and soils from Dzolokpuita and Dzologbogame were moderately acidic whereas Bekwai series was strongly acidic. The pH determined in 0.01M $CaCl_2$ was lower than those determined in water and ranged from 4.4 to 5.8.

The organic carbon (OC) content ranged from 6.0 g kg^{-1} in soil from Dzolokpuita which recorded the lowest to 32.0 g kg^{-1} in Nzima series recording the highest. According to criteria for classifying the organic matter content in soils by Jones *et al*. (2004), organic carbon content was very low in soil from Dzolokpuita, low in Adenta series and soil from Dzologbogame and moderate in soil from Akorviefe, Bekwai and Nzima.

The soil from Akorviefe recorded the highest available phosphorus followed by that of Dzolokpuita, Dzologbogame, Adenta, Nzima and Bekwai in that order. Available nitrogen for the Bekwai and the Nzima series were higher than that of the other soils.

Cation exchange capacity (CEC) was highest in soil from Akorviefe followed by Nzima and Bekwai series respectively. The soil from Dzolokpuita recorded the lowest CEC among the soils.

The bulk density for the soils used was in the range of 1.03 to 1.51 Mg m^{-3}. The soil from Dzolokpuita and Bekwai series had a high bulk density of 1.51 and 1.50 Mg m^{-3} respectively

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followed by Nzima, Adenta series and soil from Dzologbogame which recorded 1.40, 1.33 and 1.27 Mg m^{-3}, respectively. The soil from Akorviefe had a low bulk density of 1.03 M gm^{-3}.

The particle size density distribution analysis showed that soils from Dzolokpuita, Dzologbogame and Akorviefe contained high sand fraction while Adenta, Bekwai and Nzima contained the higher clay fraction. From the textural analysis in Appendix 3, Adenta, Bekwai and Nzima series were sandy clay loam, soils from Akorviefe and Dzologbogame were sandy loam respectively and the soil from Dzolokpuita was loamy sand.

The population of the indigenous rhizobia specific to African yam bean was estimated using the plant infection technique (Vincent, 1970) in all the six soils. All the soils exhibited the presence of African yam rhizobia except the Bekwai series. The highest African yam bean rhizobial population was recorded in soil from Akorviefe (2.9×10^2 cell g^{-1}) and lowest in the Bekwai series (0 cell g^{-1}).

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Table 4.1: Some chemical, physical and biological characteristics of the soils used.

Parameter	Soil					
	Adenta	Bekwai	Nzima	Dz-1	Dz-2	Akorviefe
Chemical Analysis						
pH_w	6.0	5.4	5.6	5.7	5.6	6.3
pH_s (0.01M $CaCl_2$)	5.5	4.4	4.7	5.0	4.6	5.8
OC (g kg^{-1})	14.0	30.9	32.0	6.0	11.2	28.0
Available P (mg kg^{-1})	10.02	3.57	4.00	21.90	15.12	24.38
Available N (mg kg^{-1})	187.20	313.20	288.00	156.60	147.00	118.44
CEC ($cmol_c$ kg^{-1})	6.10	11.50	12.80	4.44	8.00	15.60
Physical Analysis						
Bulk Density (M gm^{-3})	1.33	1.50	1.40	1.51	1.27	1.03
Particles Size Distribution						
Sand (%)	66	44	39	80	73	74
Silt (%)	10	17	20	**7**	15	12
Clay (%)	24	39	41	13	12	14
Texture	SCL	SCL	SCL	LS	SL	SL
Biological Analysis (Rhizobial Population cells g^{-1} soil)						
African yam bean	72	0	52	140	52	290

SCL = Sandy clay loam LS = Loamy sand SL = Sandy loam

Dz-1 = Dzolokpuita Dz-2 –Dzologbogame OC = Organic carbon

CEC = Cation exchange capacity

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4.2 Symbiotic effectiveness of African yam bean (*Sphenostylis stenocarpa)* isolates in fixing nitrogen.

Effectiveness of the isolates in fixing nitrogen varied in relation to the shoot dry weights produced. Estimated values for effectiveness varied from ineffective to moderately effective with no highly effective isolate observed on the host plant based on the effectiveness index (Table 4.2). Most of the isolates from the Akuse, Adenta, Haatso and Toje series were found to be ineffective on their respective host plant (AYB) with ineffectiveness being 87%. Only 13% of the isolates were found to be moderately effective and none were highly effective (Fig. 4.1). Isolates from the Dzolokpuita, Dzologbogame and Akorviefe soils were also found to be ineffective on their respective host plant with ineffectiveness being 97% and only 3% being moderately effective and none being highly effective (Fig. 4.2).

On the whole, 65 isolates from all the soils used, 92% were ineffective on their respective host plant, 8% were moderately effective and none were highly effective (Fig. 4.3).

Rhizobia strain 29 from the Toje series was moderately effective with an effectiveness index of 64.38, strains 10, 20 and 21 exhibited moderately effectiveness values of 56.85, 57.53 and 52.74 respectively (Table 4.3a). Rhizobia isolate 55 from the Dzolokpuita soil was also moderately effective with an effectiveness index of 56.50 (Table 4.3b).

Table 4.2: Criteria for classifying rhizobia on their basis of symbiotic effectiveness

Group	Criterion
Ineffective	Isolates with effectiveness index ≤ 50%
Moderately effective	Isolates with effectiveness index between 50 – 75%
Highly effective	Isolates with effectiveness index ≥ 75%

Source: Drew *et al*. (2012).

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Table 4.3a: The influence of AYB rhizobia isolated from Akuse, Adenta, Haatso and Toje series on plant growth, nodulation and symbiotic effectiveness index.

Isolate number	SDW (g plant^{-1})	Nodule number	NDW (mg plant^{-1})	Effectiveness index (%) by SDW
Akuse series				
1	0.32	1	1.25	38.36
2	0.33	0	0.00	40.41
3	0.30	1	2.50	32.88
4	0.25	1	1.25	17.81
5	0.31	11	10.00	35.62
6	0.31	3	6.25	36.30
7	0.29	0	0.00	30.82
8	0.29	0	0.00	29.45
9	0.29	1	2.50	30.14
Mean values	**0.30**	**2**	**2.64**	**32.42**
Adenta series				
10	0.39	1	1.25	56.85
11	0.28	1	2.50	26.71
12	0.30	1	1.25	32.88
13	0.28	1	1.25	27.40
14	0.35	2	8.75	47.26
15	0.28	1	1.25	26.03
16	0.19	2	2.50	2.74
Mean values	**0.30**	**1**	**2.32**	**31.41**

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Table 4.3a continued

Isolate number	SDW (g plant^{-1})	Nodule number	NDW (mg plant^{-1})	Effectiveness index (%) by SDW
Haatso series				
17	0.21	0	0.00	8.22
18	0.27	1	1.25	23.29
19	0.26	1	2.50	22.60
20	0.39	1	2.50	57.53
21	0.37	1	62.50	52.74
22	0.21	1	2.50	6.85
23	0.28	1	2.50	27.40
24	0.34	1	2.50	43.15
Mean values	**0.29**	**1**	**9.25**	**30.22**
Toje series				
25	0.22	4	3.75	11.64
26	0.31	2	2.50	35.62
27	0.32	13	5.00	39.04
28	0.27	0	0.00	24.66
29	0.42	25	22.50	64.38
30	0.20	11	13.75	6.16
Mean values	**0.29**	**9**	**7.92**	**35.29**
No N fert.	0.18	0	0.00	0
+N	0.55	0	0.00	100

SDW = Shoot Dry Weight NDW = Nodule dry weight

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Table 4.3b: The influence of AYB rhizobia isolated from Akorviefe, Dzolokpuita and Dzologbogame soil on plant growth, nodulation and symbiotic effectiveness index.

Isolate number	SDW (g plant^{-1})	Nodule number	NDW (mg plant^{-1})	Effectiveness index (%) by SDW
Soil from Akorviefe				
31	0.28	0	0.00	23.16
32	0.20	1	6.67	42.37
33	0.14	1	6.67	23.16
34	0.13	6	8.33	19.77
35	0.10	22	8.33	10.17
36	0.32	8	6.67	28.81
37	0.38	4	10.00	40.11
38	0.32	0	0.00	29.38
39	0.23	15	5.00	13.56
40	0.34	0	0.00	32.77
41	0.30	74	15.00	25.42
42	0.19	0	0.00	6.78
43	0.25	0	3.33	18.08
44	0.37	55	13.33	37.85
45	0.29	0	0.00	24.29
46	0.32	18	5.00	28.81
Mean values	**0.27**	**13**	**5.52**	**25.29**
Dz-1				
47	0.35	0	0.00	34.46
48	0.22	1	1.67	12.99
49	0.22	0	0.00	11.86

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Table 4.3b continued

Isolate number	SDW (g plant^{-1})	Nodule number	NDW (mg plant^{-1})	Effectiveness index (%) by SDW
50	0.35	41	31.67	33.90
51	0.42	1	1.67	46.33
52	0.33	0	0.00	30.51
53	0.41	18	6.67	44.07
54	0.34	0	0.00	32.77
55	0.48	11	6.67	56.50
Mean values	**0.35**	**8**	**5.37**	**33.71**
Dz-2				
56	0.21	0	0.00	27.12
57	0.22	0	0.00	12.99
58	0.31	0	0.00	27.12
59	0.22	0	0.00	11.86
60	0.35	0	0.00	34.46
61	0.21	4	5.00	11.30
62	0.34	0	0.00	32.20
63	0.34	1	1.67	33.33
64	0.35	16	11.67	34.46
65	0.30	35	13.33	26.55
Mean values	**0.29**	**6**	**3.17**	**25.14**
No N fert.	0.15	0	0.00	0.00
+N	0.74	0	0.00	100.00

SDW = Shoot Dry Weight NDW = Nodule dry weight

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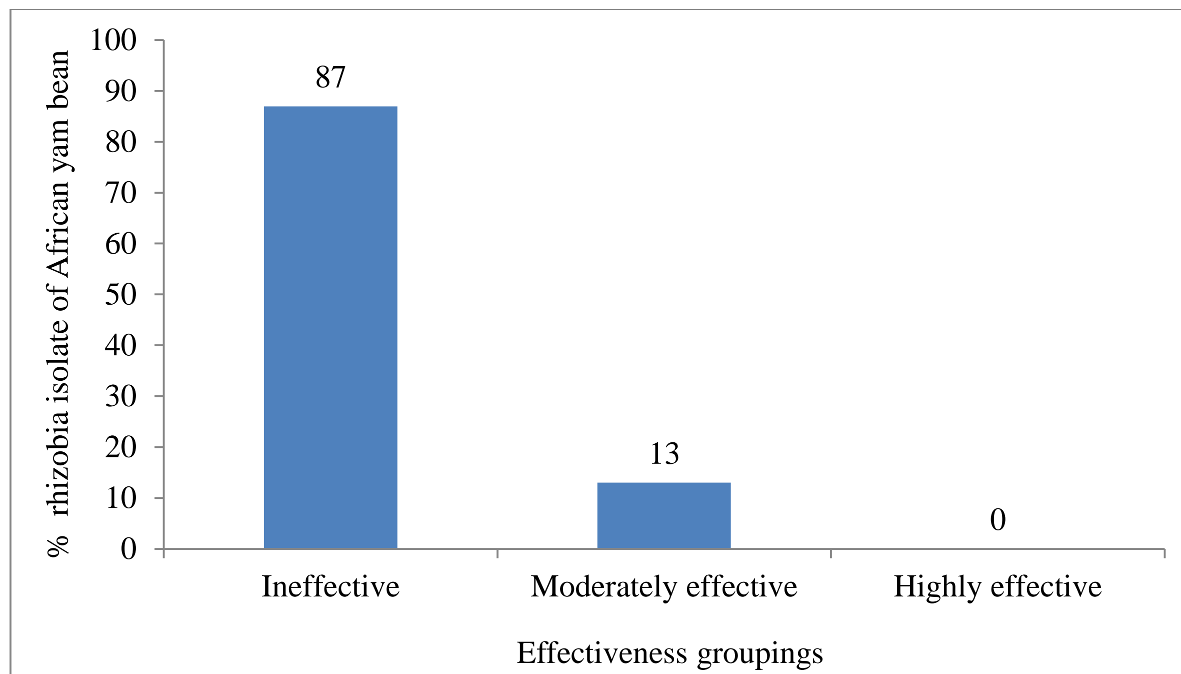

Fig 4.1: Classification of 30 isolates of African yam bean rhizobia obtained from Adenta, Toje, Haatso and Akuse series on the basis of their effectiveness.

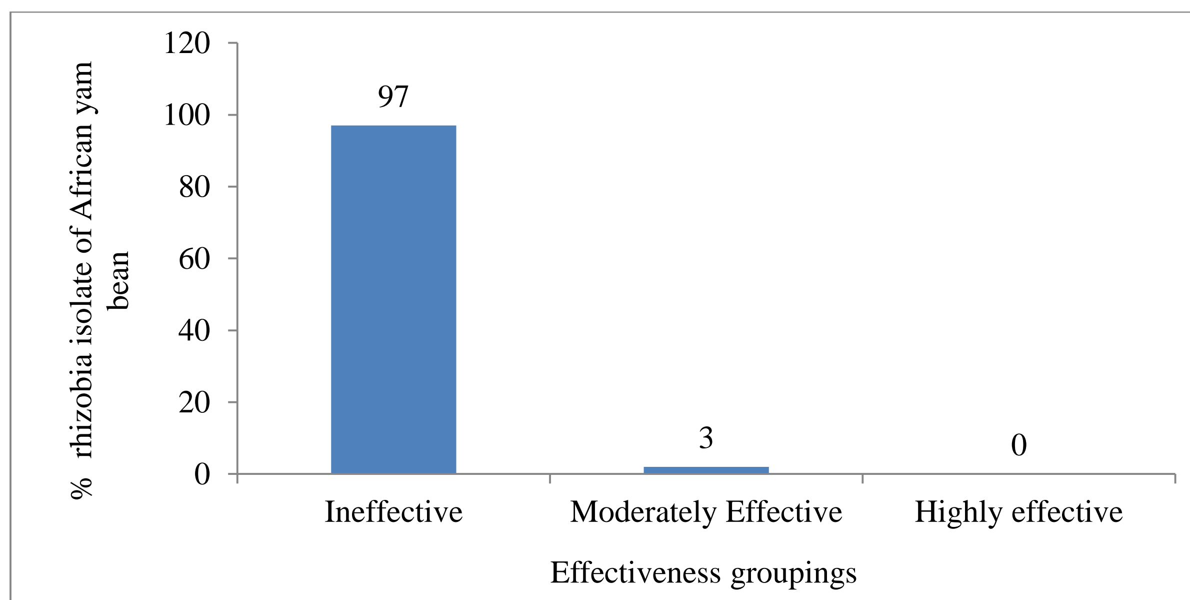

Fig**. 4.2: Classification of 35 isolates of African yam bean rhizobia obtained from soils from Dzolokpuita, Dzologbogame and Akorviefe on the basis of their effectiveness.**

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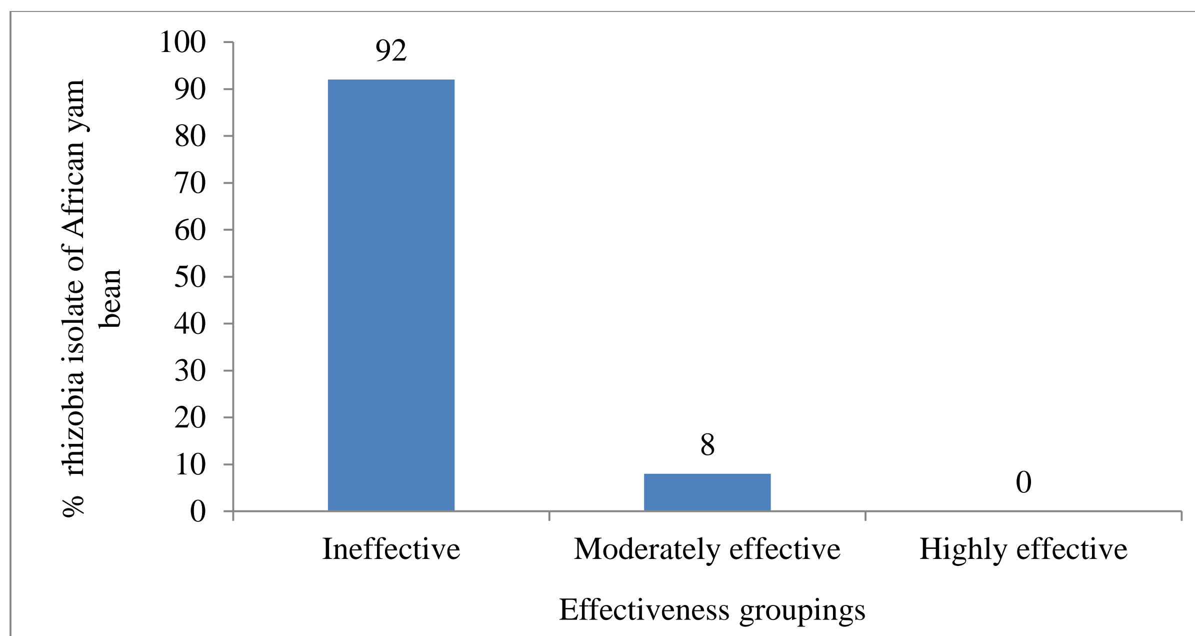

Fig 4.3: Classification of 65 isolates of African yam bean rhizobia obtained from Adenta, Toje, Haatso and Akuse series and soils from Dzolokpuita, Dzologbogame and Akorviefe on the basis of their effectiveness.

4.3 Response of African yam bean to Inoculation and Nitrogen Fertilization

4.3.1 Effect of inoculation and N application on nodule number in African yam bean.

When African yam bean (AYB) was inoculated with the inoculum I_1 (i.e. rhizobial isolate 55) and grown in the soil from Akorviefe and Adenta series, 64 and 57 nodules respectively were formed and differences in nodule number was significant ($p < 0.05$; Table 4.4). Low nodule numbers of 4 and 10 were recorded in the Nzima and the Bekwai series respectively (Table 4.4). When the inoculum I_2 (i.e. rhizobia isolate 29) was used to inoculate AYB, highest nodule number of 68 was recorded in the soil from Akorviefe followed by soil from Dzolokpuita (Dz-1) that recorded 63 nodules and the difference was significant (Table 4.4). The least nodule numbers were recorded in the Bekwai and the Nzima series with values of 3 and 10 nodules respectively when the I_2 was used to inoculate AYB in these soils. Of the two rhizobial inocula (i.e. I_1 and I_2) there was significant ($p < 0.05$) differences in their ability to cause nodulation.

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Application of 70 kg N ha^{-1} reduced nodule number in all the soils used (Table 4.4). The reduction was drastic in the Adenta series and the soils from Dzolokpuita (Dz-1) and Dzologbogame (Dz-2). Bekwai series had no nodules regardless of the level of nitrogen applied. The Nzima series recorded 1 nodule when no nitrogen was applied and no nodule when nitrogen was applied (Table 4.4). N application generally decreased nodule numbers by 41% as compared to the control. N treatment differences were significant ($p < 0.05$).

Table 4.4: Effect of inoculation and N application on nodule number per plant of African of yam bean in six Ghanaian soils.

Soil	Inoculation		Nitrogen levels	
	I_1	I_2	N_0	N_1
Adenta	57	57	43	14
Akorviefe	64	68	59	50
Bekwai	10	3	0	0
Dz-1	54	63	19	7
Dz-2	27	34	41	23
Nzima	4	10	1	0
Means of I and N	**36**	**39**	**27**	**16**
LSD (0.05): Soil:=2 I=1 Soil × I:=3 CV (%)=4.9			**LSD (0.05)=Soil: 2 N=1 Soil × N=2 CV (%)=5.9**	

N_1 = N application (70 kg N ha^{-1}) N_0 =No N application (control)
I_1 = Inoculum 1 I_2 = Inoculum 2
Dz-1= Soil from Dzolokpuita Dz-2= Soil from Dzologbogame

4.3.2 Effect of inoculation and N application on nodule dry weight in African yam bean.

When I_1 was inoculated with plants grown in Dz-1 nodule dry weight of 116.7 mg plant^{-1} was recorded followed by those grown in soil from Akorviefe which had nodule dry weight of 96.7 mg plant^{-1} (Table 4.5). There was significant ($p < 0.05$) difference in nodule dry weight between

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these two soils. The least nodule dry weights were recorded in the Nzima and the Bekwai series with values of 10 and 5 mg plant^{-1} respectively. Inoculating AYB with I_2 resulted in the highest nodule dry weight of 130.0 mg plant^{-1} in Dz-1 followed by soil from Akorviefe that recorded nodule dry weight of 128.3 mg plant^{-1}, however, there was no significant ($p > 0.05$) difference in nodule dry weights between plants grown in these two soils. Low nodule dry weights of 5.0 and 8.3 mg plant^{-1} were recorded in the Bekwai and the Nzima series with the inoculum I_2. Generally higher nodule dry weights were obtained using inoculum I_2 compared to the inoculum I_1, even though the difference was not significant ($p > 0.05$).

Higher nodule dry weights were recorded when no nitrogen was applied as compared to when nitrogen was applied at 70 kg N ha^{-1}. When no nitrogen was applied, the Adenta series had high nodule dry weight of 161.7 mg plant^{-1} followed by soil from Akorviefe which had 91.7 mg plant^{-1} (Table 4.5). Plants grown in Bekwai series did not nodulate at all nitrogen levels. The Nzima series on the other hand, recorded the lowest nodule dry weights regardless of the level of nitrogen applied. There was a significant ($p < 0.05$) difference between the nitrogen treatments.

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Table 4.5: Nodule dry weight (mg plant^{-1}) of African yam bean in six Ghanaian soils as influenced by inoculation and N application.

Soil	Inoculation		Nitrogen levels	
	I_1	I_2	N_0	N_1
Adenta	90.0	83.3	161.7	45.0
Akorviefe	96.7	128.3	91.7	48.3
Bekwai	10.0	5.0	0.0	0.0
Dz-1	116.7	130.0	43.3	8.3
Dz-2	70.0	45.0	61.7	26.7
Nzima	5.0	8.3	3.33	0.0
Means of I and N	**64.72**	**66.67**	**60.3**	**21.4**
LSD (0.05): Soil=12.7 I=NS Soil × I=18.0 CV (%)=16.2			**LSD (0.05): Soil=11.4 N=6.6 Soil × N=16.1 CV (%)=23.4**	

NS: Not significant at 5% probability level

4.3.3 Percent nitrogen derived from atmosphere (%Ndfa) of African yam bean as affected by inoculation and N application.

African yam beans grown in the soil from Akorviefe recorded high %Ndfa of 2.4 followed by soil from Dzologbogame (Dz-2) that recorded 2.0% Ndfa when inoculated with I_1(Table 4.6). Low %Ndfa of 0.4 and 0.7 were recorded for the African yam beans planted in the Nzima and the Bekwai series respectively. When the inoculum I_2 was used to inoculate AYB, Adenta series and soils from Akorviefe recorded the highest %Ndfa value of 2.5. There was no significant ($p < 0.05$) difference in %Ndfa of AYB grown in the two soils. Low %Ndfa of 0.9 and 0.5 were recorded for the Bekwai and the Nzima series respectively when I_2 was used to inoculate AYB in these soils.

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The application of nitrogen reduced %Ndfa in the six soils used. Soils from Akorviefe and Dzologbogame recorded the same %Ndfa (1.9) when nitrogen was not applied followed by Adenta soil (Table 4.6). Low values of %Ndfa were however recorded in Bekwai and Nzima series which were 0.7% and 0.4% respectively. Nitrogen effects on %Ndfa were significant ($p<0.05$).

Table 4.6: Ndfa (%) of African yam bean planted in six Ghanaian soils as influenced by inoculation and N application.

Soil	Inoculation		Nitrogen levels	
	I_1	I_2	N_0	N_1
Adenta	1.7	2.5	1.6	1.1
Akorviefe	2.4	2.5	1.9	1.8
Bekwai	0.7	0.9	0.7	0.2
Dz-1	1.5	1.8	1.5	1.1
Dz-2	2.0	2.2	1.9	1.7
Nzima	0.4	0.5	0.4	0.1
Means of I and N	**1.5**	**1.7**	**1.3**	**1.0**
LSD (0.05): Soil=0.4 I=0.3 Soil × I=NS CV (%)=23.0			**LSD (0.05): Soil=0.2 N=0.1 Soil × N=0.3 CV (%)=12.9**	

NS: Not significant at 5% probability level

4.3.4 Total N_2 fixed by African yam bean as affected by inoculation and N application.

The N difference method was used to calculate the total N fixed. The total N fixed and how it was influenced by applying N fertilizer and different types of inocula in the various soils is presented in Table 4.7.

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Inoculating with inoculum I_1, African yam bean planted in the soil from Akorviefe had the highest total N_2 fixed of 68.9 mg plant^{-1} followed by those planted in Dz-2 which recorded 37.4 mg N fixed plant^{-1}. There was significant ($p < 0.05$) difference in total N_2 fixed among the different treatments. African yam beans planted on the Nzima and the Bekwai series fixed low total N of 2.7 mg and 7.5 mg plant^{-1} respectively while with plants grown in soils from Dzolokpuita and Dzologbogame, moderate total N was fixed. Inoculating AYB with I_2, soil from Akorviefe recorded the highest total N_2 fixed of 74.4 mg plant^{-1} followed by Dz-2 with total N_2 fixed of 43.8 mg plant^{-1}. There was significant ($p < 0.05$) difference in total N_2 fixed by plants between the two soils. Low total N_2 fixed of 9.4 and 3.3 mg plant^{-1} were recorded in the Bekwai and the Nzima series with the inoculum I_2.

When nitrogen was applied, total N_2 fixed was recorded for plants grown in soil from Akorviefe (57.4 mg plant^{-1}) which was followed by Dz-2 in which was recorded 34.9 mg plant^{-1}. Low total N_2 fixed was recorded in Bekwai and Nzima (2.5 and 1.1 mg plant^{-1} respectively). Total N_2 fixed by the African yam bean was not depressed by the application of nitrogen for soil from Akorviefe, Dzolokpuita, Dzologbogame and also Adenta series but rather there was a depression of N_2 fixed per plant in Bekwai and Nzima series as compared to when nitrogen was not applied. Significant ($p < 0.05$) differences in total nitrogen fixed among the different soils were observed.

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Table 4.7: Effect of inoculation and N application on total N_2 fixed (mg plant^{-1}) by African yam bean in six Ghanaian soils.

Soil	Inoculation		Nitrogen levels	
	I_1	I_2	N_0	N_1
Adenta	14.9	24.8	13.4	14.5
Akorviefe	68.9	74.4	52.0	57.4
Bekwai	7.5	9.4	5.8	2.5
Dz-1	17.5	29.3	13.5	19.5
Dz-2	37.4	43.8	33.2	34.9
Nzima	2.7	3.3	2.3	1.1
Means of I and N	**24.8**	**30.8**	**20.0**	**21.6**
LSD (0.05): Soil=5.9 I=3.4 Soil × I=NS CV (%)=17.9			**LSD (0.05): Soil=3.1 N=NS Soil × N=4.4 CV (%)=12.5**	

NS: Not significant at 5% probability level

4.3.5 Shoot N (%) of African yam bean as affected by inoculation and N application

Plants inoculated with inoculum I_1 in the soil from Akorviefe had a % shoot N of 5.27 (Fig 4.4) followed by the Dz-1 (4.48). There was significant ($p < 0.05$) difference in % shoot N between the two soils. When AYB was inoculated with I_2, Akorviefe recorded the highest value of 5.30 followed by Dz-1 (4.71). Plants from Adenta and Nzima recorded low % shoot N values of 4.03 and 3.70 respectively, with inoculum I_2. When 70 kg N ha^{-1} was applied, higher % shoot N values were recorded as compared to where nitrogen was not applied. When nitrogen was applied, plants from Akorviefe soil had the highest % shoot N of 5.95 followed by Dz-1 (5.25). Low values were recorded for Adenta and Nzima 4.04 and 3.92 respectively. The same trend was observed in % shoot N for all the soils when nitrogen was not applied. Application of N did not depress the % shoot N but rather improved the % shoot N of the African yam bean in the various soils. The soil and nitrogen interaction was significant ($p < 0.05$) in % shoot N.

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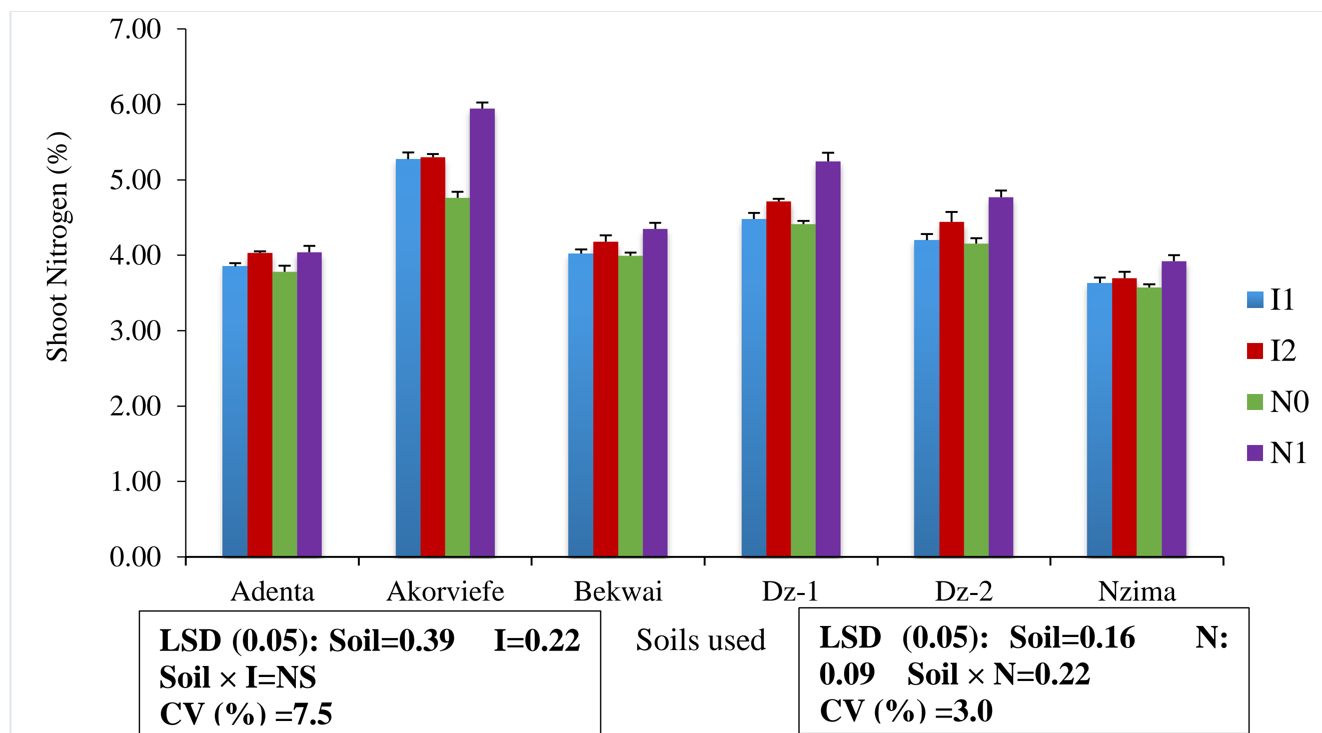

Fig 4.4: Shoot N (%) of African yam bean in six Ghanaian soils as affected by inoculation and N application.

4.3.6 Total N in shoot of African yam bean as affected by inoculation and N application

A high total N value of 149.1 mg plant^{-1} was recorded in the shoot of AYB grown in the Akorviefe soil followed by Dz-2 which recorded 79.7 mg plant^{-1} when inoculated with I_1(Table 4.8). Low total N in the shoot of AYB was recorded in the Nzima and the Adenta series with values of 23.5 and 33.5 mg plant^{-1} respectively. When AYB was inoculated with I_2, there was a high total N of 160.0 mg plant^{-1} recorded for soil from Akorviefe followed by Dz-2 soil recording 87.9 mg plant^{-1}. Low total N of 43.8 and 25.4 mg plant^{-1} were recorded in the Bekwai and the Nzima series respectively with the inoculum I_2. Inoculation application was significant ($p < 0.05$) as well the soils used.

When nitrogen was applied, high total N of 195.4 mg plant^{-1} was recorded for the AYB grown in the soil from Akorviefe followed by Dz-2 (98.4 mg plant^{-1}). Low total N was recorded in Bekwai

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and Nzima with values 46.7 and 31.9 mg plant^{-1} respectively (Table 4.8). Applying N increased total N in shoot with percent increment of 56% compared to no N application. Significant soil and N interaction was obtained ($p<0.05$).

Table 4.8: Influence of inoculation and N application on total N in shoot (mg plant^{-1}) of African yam bean in six Ghanaian soils.

Soil	Inoculation		Nitrogen levels	
	I_1	I_2	N_0	N_1
Adenta	33.9	46.0	31.3	54.0
Akorviefe	149.1	160.0	128.6	195.4
Bekwai	40.6	43.8	32.8	46.7
Dz-1	50.9	77.9	40.6	96.2
Dz-2	79.7	87.9	71.7	98.4
Nzima	23.5	25.4	22.9	31.9
Means of I and N	**62.9**	**73.5**	**54.6**	**87.1**
LSD (0.05): Soil: 4.4 I: 2.6 Soil × I: 6.3 CV (%): 5.5			**LSD (0.05): Soil: 3.3 N: 1.9 Soil × N:4.6 CV (%): 3.9**	

4.3.7 Effect of inoculation and N application on shoot dry weight in African yam bean.

When AYB was inoculated with inoculum I_1, shoot dry weight of 2.83 g plant^{-1} was recorded for AYB grown in the soil from Akorviefe (Fig 4.5) followed by Dz-2 (1.90 g plant^{-1}). AYB grown in the Adenta and Nzima series recorded low shoot dry weights of 0.88 g plant^{-1} and 0.65 g plant^{-1} respectively. Inoculating AYB with I_2, a high shoot dry weight of 3.02 g plant^{-1} was recorded for AYB grown in the soil from Akorviefe followed by Dz-2 recording 1.98 g plant^{-1}. Low shoot dry weights of 0.98 and 0.69 g plant^{-1} were recorded for the AYB planted in the Adenta and the Nzima series respectively when inoculated with inoculum I_2. Significant ($p < 0.05$) differences

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were observed in all the soils. Comparing inoculum I_1 and I_2, inoculum I_2 had 11% increase in total shoot dry weight of AYB in all the soils compared to inoculum I_1.

Applying nitrogen improved the shoot dry weight of the AYB planted in the various soils. High shoot dry weight of 3.29 g plant^{-1} was recorded for AYB planted in the soil from Akorviefe followed by Dz-2 which recorded 2.06 g plant^{-1} (Fig 4.5). Low shoot dry weights of 1.07 g plant^{-1} and 0.81 g plant^{-1} were recorded in Bekwai and Nzima series respectively. Applying N increased shoot dry weight with percent increment of 36% when compared to no N. This shows the positive effect of nitrogen application on shoot dry weight of legumes. Differences in soils treatment were significant ($p < 0.05$).

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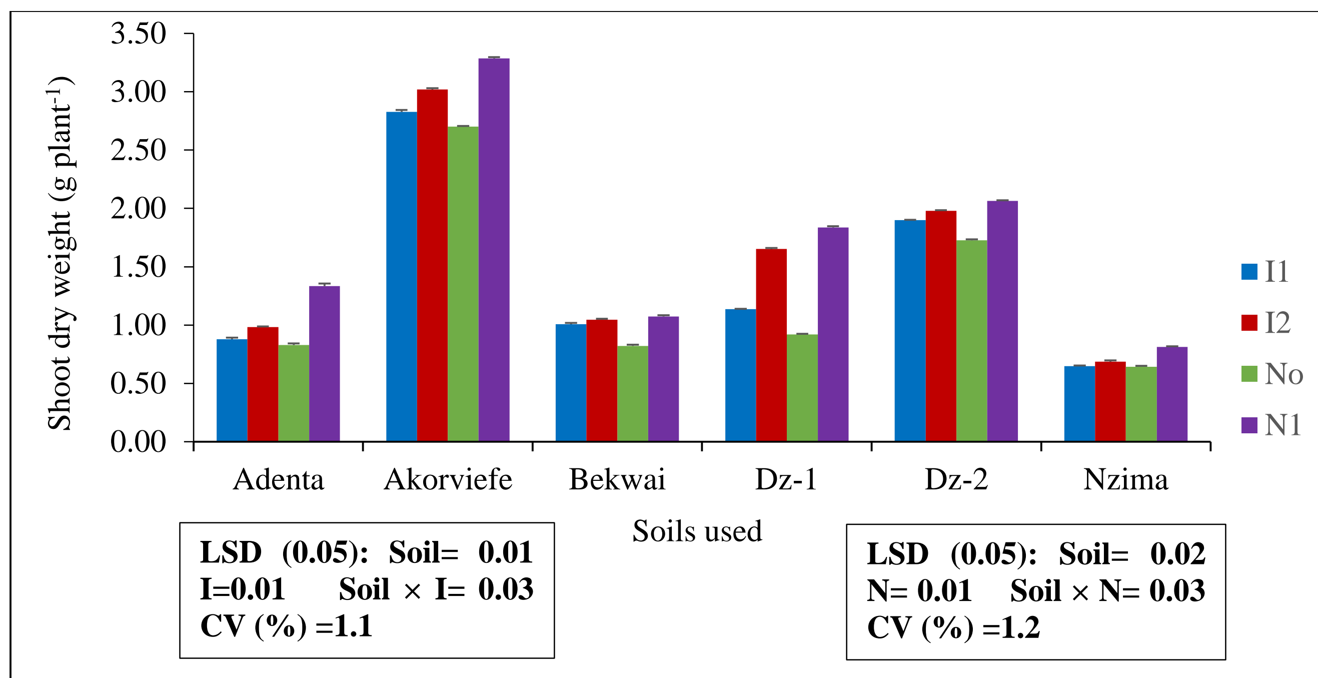

Fig 4.5: Shoot dry weight (g plant^{-1}) of African yam bean planted in six Ghanaian soils as affected by inoculation and N application.

4.4 Diversity of Indigenous African Yam Bean Rhizobia

4.4.1 Cross Inoculation Studies (Host Range Analysis)

The degree of compatibility of African yam bean *Rhizobium* with other selected legumes was examined to determine how promiscuous or specific the *Rhizobium* organism was. The cross inoculation results showed differences in nodulation among the host legumes inoculated with the different African yam bean isolates (Table 4.9). Most of the legumes used showed the presence of nodules when inoculated with the African yam bean isolates. Cowpea (*Vigna unguiculata*) showed much compatibility (56%) while pigeon pea (*Cajanus cajan*) and soybean (*Glycine max*) showed lesser compatibility (33 and 11% respectively) with African yam rhizobia isolates (Fig 4.6). No nodules were observed on the uninoculated control plants.

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Table 4.9: Test of ability of nine African yam bean *Rhizobium* isolates to cross inoculate three host legumes.

	Nodulation Score			
Isolate	AYB	Cowpea	Pigeon pea	Soybean
AYB 1	+	+	+	+
AYB 2	+	+	-	-
AYB 3	+	+	+	-
AYB 4	+	-	-	-
AYB 5	+	-	-	-
AYB 6	+	-	-	-
AYB 7	+	+	-	-
AYB 8	+	-	-	-
AYB 9	+	+	+	-

AYB = African yam bean + = presence of nodules - = absence of nodules

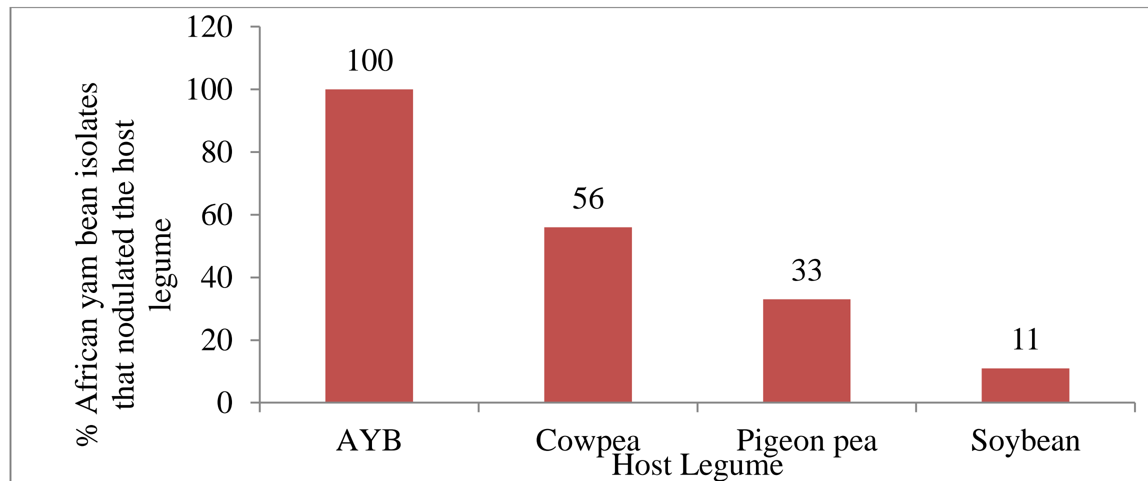

Fig 4.6: Ability of nine African yam bean *Rhizobium* isolates to form nodules on three host legumes.

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In the reverse experiment, rhizobial isolates from cowpea, pigeon pea and soybean were inoculated on African yam bean and the difference in nodulation varied among the isolates used (Table 4.10). It was observed that African yam bean was compatible with 80% of the isolates from soybean and 40% from cowpea but none of the rhizobia isolates obtained from pigeon pea were able to form nodules on African yam bean (Fig. 4.7). No nodules were observed in uninoculated control plants.

Table 4.10: Test of ability of cowpea, soybean and pigeon pea rhizobia isolates to form nodules on African yam bean.

Isolate	Host Legume (AYB)	Isolate	Host Legume (AYB)	Isolate	Host Legume (AYB)	Isolate	Host Legume (AYB)
AYB 1	+	CP 1	+	PP 1	-	SB 1	-
AYB 2	+	CP 2	-	PP 2	-	SB 1	+
AYB 3	+	CP 3	-	PP 3	-	SB 1	+
AYB 4	+	CP 4	-	PP 4	-	SB 1	+
AYB 5	+	CP 5	+	PP 5	-	SB 1	+

CP = Cowpea SB = Soybean PP = Pigeon pea AYB = Africa yam bean

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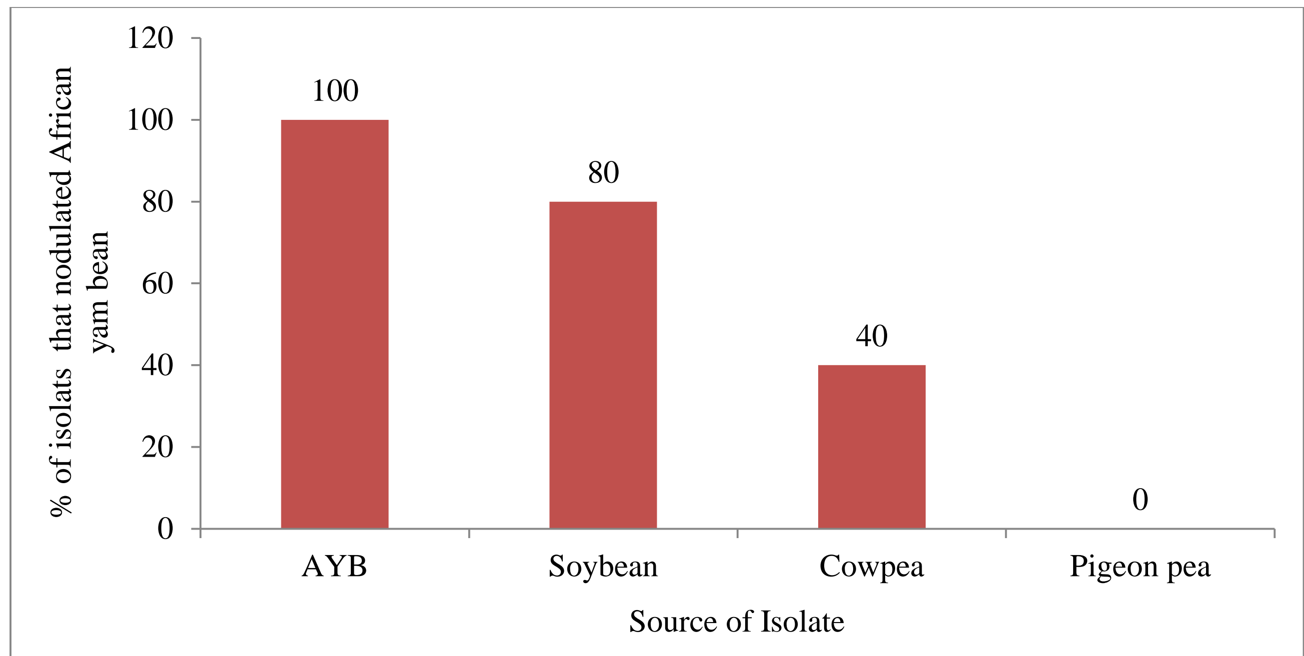

Fig.4.7: Ability of rhizobia isolates obtained from three different legumes to form nodules on African yam bean

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CHAPTER FIVE

5.0 DISCUSSION

5.1 Soil Characterisation

The pH determined in water for all the six soils recorded higher values than those recorded in salt. This describes the soils having negatively charged colloidal surface which signifies the potential for attracting positive charged compounds. It also suggests that the soils are highly weathered as documented by Abekoe and Sahrawat (2001).

The high organic carbon accumulation in the Bekwai, Nzima series and soil from Akorviefe could be due to high litter deposition from canopies as well as the low organic matter turnover due to less cultivation in Bekwai and Nzima series and the associated low temperatures in these forest regions (Jordan, 1985). The appreciable amount of clay content in Nzima and Bekwai series describes the level of organic matter in them. Jones *et al*. (2006) documented that clay fraction has been found to accommodate more organic carbon and other soil nutrients than sand and silt. Soils from Dzolokpuita and Dzologbogame recorded less organic carbon content and this could be due to less litter falls and much tillage on these soils. The high sand and low clay contents of the soils from Dzolokpuita and Dzologbogame could be attributed to less organic matter due to less litter falls. The low organic carbon content in the Adenta series reflects the characteristic of soils in the coastal savanna agro-ecological zones where there is high rate of mineralization due to high temperatures which reduced the accumulation of organic carbon (Dowuona *et al.,* 2012). The Adenta series recorded high available nitrogen contents. Therefore, the low C/N ratio of the Adenta series confirmed the high rate of decomposition of organic

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matter. The available N values of 156.6 mg kg^{-1} and 147 mg kg^{-1} for soils from Dzolokpuita and Dzologbogame may be due to the application of nitrogen by farmers at that site.

The available phosphorus contents in the Bekwai and Nzima series were low and could be due to P-fixation through reactions with Fe and Al hydroxide considering their soil pH of 5.4 and 5.6 respectively (Owusu-Bennoah *et al.,* 2000). Available phosphorus was high for soils from Akorviefe, Dzologbogame and Dzolokpuita. These soils might had received phosphorus amendment recently as they were sampled from a cultivated field.

The high CEC recorded for soil from Akorviefe could be due to the high amount of organic carbon recorded, since soils with high amount of organic matter tend to have high CEC (soilquality.org.au, 2011). However, the low CEC recorded in the Adenta series could be attributed to the presence of low activity clays like kaolinite which confirms high weathering activity in that soil as documented by Dowuona (1985) while that in soil from Dzolokpuita could be due the low organic matter and the high sand content recorded.

The medium bulk density of Adenta series reflects the sandy clay loam characteristic of soils in the coastal savanna agro-ecological zone while the high bulk density in Bekwai series could be attributed to high activity clays. The use of traction on the soil from Dzolokpuita could have caused compaction of the soil and contributed to the high bulk density. Medium bulk densities for Nzima and soil from Dzologbogame could also be attributed to the high activity clay in both soils as well as the use of some level of traction in soil from Dzologbogame as explained by Brosnan *et al.* (2009). The low bulk density in soil from Akorviefe could be due to less traction on the soil.

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Cells of *Rhizobium* strains capable of nodulating African yam bean were found to be present in all the soils used except the Bekwai series. Soils from Akorviefe recorded high number of *Rhizobium* cells. African yam bean had been cultivated in that area and it is likely that there is a buildup of rhizobia cells in the soil with cultivation of the bean as explained by Sajjad *et al*. (2008). In a similar way, soils from Dzologbogame and Dzolokpuita also recorded high *Rhizobium* populations which could be attributed to rhizobia released from decayed nodules of previous crop since these soils have a history of AYB cultivation (Kawaka *et al.,* 2014; Thies *et al.,* 1995,). The high numbers of *Rhizobium* strains in the soil from Akorviefe could also be attributed to the sandy clay texture of the soil since light-textured soils were found to be beneficial for the proliferation and survival of root-nodule bacteria (Martynuik and Oron, 2008). The absence of *Rhizobium* strains in the Bekwai series could be due to the acidic nature of the soil (pH of 5.4) since rhizobia are influenced by the pH of the soil (Niste *et al.,* 2013 and Mapfumo *et al.,* 2000, Vessey and Luit, 1999; Peoples *et al.,* 1995). The absence of nodules in Bekwai series may also be due to the absence of native rhizobia compactible with African yam bean in that soil.

5.2 Symbiotic effectiveness of African yam bean (*Sphenostylis stenocarpa)* isolates in fixing nitrogen.

To select rhizobial strains for inoculum production, symbiotic effectiveness of the native rhizobia population is a necessary tool that helps to influence legume response to inoculation (Thies *et al.,* 1991). However, in the development of legume inoculum, one needs to determine highly effective rhizobia in fixing nitrogen (Kawaka *et al.,* 2014). In the present study, it was observed that none of the isolates was highly effective, few were moderately effective (13% and 3%) and the majority were ineffective (87% and 97%). Ferreira and Marques (1992) observed

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differences in effectiveness among 170 strains isolated from native clover. In the same way differences in effectiveness were observed for the 65 rhizobial strains used for the study.

The higher % ineffective rhizobia compared to the moderately effective isolates could be attributed to the highly competitive and abundant ineffective native rhizobia in the soils giving them an advantage over the few highly effective ones if present in the soil in occupying nodules. Mathu *et al* (2012) also observed the presence of ineffective indigenous rhizobia strains in Kenyan soils when cowpea and green gram were grown to assess the ability of the indigenous rhizobia in nodulating these legumes. However, Kawaka *et al.* (2014) reported of cowpea isolates being effective and attributed that to the presence of more competitive effective strains of the native indigenous rhizobia in the sampled farms.

Symbiotic effectiveness was higher in strains from the coastal savanna agro-ecological zones (Adenta, Toje, Haatso and Akuse soils) where African yam bean was not cultivated resulting in higher moderate effective isolates than soils from semi deciduous agro-ecological zone (soils from Dzolokpuita, Dzologbogame and Akorviefe) where African yam bean was cultivated. One would have expected that soils from the semi deciduous agro-ecological zone would have a higher percentage of the effective isolates as a result of the frequent cultivation of the African yam bean than in the coastal savanna agro-ecological zone where African yam bean was not cultivated. However, this was not observed which could be due to the abundance and competitiveness of the ineffective indigenous and native rhizobia of the African yam bean in the cultivated soils compared to the uncultivated as documented by Mathu *et al.* (2012) on cowpea and green gram rhizobia.

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5.3 Response of African yam bean to Inoculation and Nitrogen Fertilization

5.3.1 Response of African yam bean to Nitrogen application.

The response of African yam bean to nitrogen application was evaluated in all the soils and nitrogen had an inhibitory effect on the nodulation of the crop. The results showed that nodule numbers decreased significantly from 59 nodules plant^{-1} in the control to 50 nodules plant^{-1} when N fertilizer was applied in the soil from Akorviefe. Significant decreases were also recorded in the other soils except Bekwai series which did not record any nodule number. Similar trend was also observed with the nodule dry weight of the various soils when N was applied.

The inhibitory effects of nitrogen on nodulation and nitrogen fixation of legumes have been reported by many researchers. For instance, Kamanu *et al*. (2012) reported that the application of inorganic fertilizer depressed nodule numbers and nodule dry weight in snap bean. There was reduction of the number of nodules in garden pea with the application of nitrogen (Voisin *et al*., 2002). Streeter (1988) observed the inhibition of symbiotic N_2 fixation by nitrate which resulted in a high root biomass than nodule biomass leading to reduced nodules formed. Voisin *et al.* (2003) later reported that the relationship between nodule growth and nitrogen application had not been well established. However, reports by other researchers have suggested that the application of N deprived nodules of carbohydrates and the product of nitrate metabolism such as glutamine and asparagine inhibited nodule formation (Vessy and Waterer, 1992, Neo and Layzall, 1997, Bacanamwo and Harper, 1997). Gordon *et al*. (2002) also reported that decreased diffusion of O_2 into the nodules restricted respiration of bacteriods when N was applied resulting in reduced N formed. This inhibitory effect of N made Gentili *et al. (*2006) to report that applying high levels of nitrogen in *Alnus incana* resulted in the inhibition of early cell divisions in the cortex of the nodules of the *Alnus sp.* Ani *et al*. (2007) also observed that there was down

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regulation of *nin* gene which performed a key function in nodule ontogenesis in the presence of high levels of N levels.

Dry matter is the measure of growth of plant (Noggle and Fritz, 1983; Joel *et al.,* 1997) and from the present study, the application of N resulted in an increase in shoot biomass accumulation in all the soils. Studies by many researchers have reported the increment of dry matter yield due to N application. The addition of nitrate to mungbean resulted in increase in shoot and root dry weight (Elahi *et al.,* 2004). The growth of faba bean and pea was increased in the presence of 5.0 and 7.5 mM nitrate (Herdina and Salisbury, 1989). Shoot dry matter was improved by the application of fertilizer to lima bean, green gram and common bean (Otieno *et al.,* 2009). The stimulated growth of legumes by the application of N was due to the high amounts of nitrogen absorbed by plants that increased the N content and weight of leaves, which led to the enhancement of photosynthetic ability and advancement of carbohydrate storage in leaf hence the higher dry matter in the fertilized plant than the control (Mae, 1997). A similar observation was made by Makino *et al.* (1992) who noted that N application increased photosynthetic leaves through increased amounts of stromal and thylakoid proteins in leaves causing an increase in dry matter and N accumulation. The higher dry matter yield in the N applied plants (or N fertilized plants) compared to the control indicated that N_2 fixation was not supplying the plant with enough nitrogen for growth or the occurrence of indigenous rhizobia strains in the soils were ineffective in fixing nitrogen. This confirms conclusions by Mathu *et al.* (2012) who attributed the poor growth of cowpea and green gram to the presence of ineffective indigenous rhizobia strains in Kenyan soils. Also Chemining'wa *et al.* (2004) noted that low shoot dry weight was recorded for pea cultivated in Kenya that was nodulated by the abundant and competitive indigenous rhizobia strains of pea implying that these indigenous rhizobia were ineffective.

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Among the soils used, the soil from Akorviefe promoted higher dry matter yield for AYB than the other soils. This could be due to the high available phosphate of 24.38 mg kg^{-1} that could have contributed substantially to the growth of a nitrogen fixing crop like African yam bean since Tang *et al.* (2001) noted that P deficiency impaired nodulation and N_2 fixed. Also the high CEC value of the soil (15.60 $cmol_c$ kg^{-1}) could have helped the plants obtain more nutrients for growth (Hazleton and Murphy, 2007).

The significant decrease in N_2 fixation by African yam bean with the application of N is in line with the results by Ngau (1998) who observed decrease in biological N_2 fixed in legumes when N was applied. Root nodulating legume prefer available N to fixing N because symbiotic system requires high energy for development, maintenance and function compared to N uptake (George and Singleton, 1992). Also, the poor performance of AYB to nitrogen fixation when chemical N fertilizer was applied could be due to the sensitivity of the crop to mineral nitrogen and the addition of no or low N can improve the N_2 fixing capacity of the crop (Walch, 1995; Buttery, 1990; Naisbitt and Sprent, 1993). Atkins (1986) also documented that nodulating plants preferred available N to fixing atmospheric nitrogen due to the high energy cost involved.

High total N accumulation, dry matter yield, N fixed were observed for the AYB cultivated on the African yam bean soils (i.e. soils from Akorviefe, Dz-1 and Dz-2) than soils (Adenta, Bekwai and Nzima series) not previously cultivated with the Africa Yam Bean. The African yam bean cultivated soils had a history of farmers cultivating crops on these soils and applying fertilizers that might have built up with time as was shown with some of the chemical parameters in table 4.1. This might have contributed to the higher available nutrients so that African yam bean grown in the AYB soils obtained more nutrients and performed better.

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The number of nodules and nodule dry weight were not drastically reduced when inorganic N fertilizer was applied to the African yam bean grown in the African yam bean cultivated soils as compared to the other soils where no AYB had been planted. Thus, low inhibitory effect on nodulation in the African yam bean cultivated soils was observed as compared to soils that had not been previously cultivated with the bean. This effect might be due to the high available P in the African yam bean cultivated soils than soils not previously cultivated with the AYB that counteracted the inhibitory effect of nitrogen fertilizer that depressed the nodule number and the nodule dry weight as explained by Gentili and Huss-Danell (2003).

5.3 2 Response of African yam bean to inoculation.

Rhizobial inoculation of the African yam bean promoted nodule development in all the soils and hence higher nodulation. The stimulatory effect of inoculation on nodulation was reported by Otieno *et al.* (2009) who discovered that the application of rhizobia significantly increased nodule numbers and nodule dry weights of lima bean, Lablab, green gram and common bean compared to nitrogen and manure application. However, the low dry matter of AYB produced by applying inoculum compared to nitrogen application indicates that the strains were not fixing much N_2 to meet the nitrogen requirement of the plant and hence were not highly effective in fixing N_2. This confirms the findings of Herridge (2002) who documented that introducing highly effective rhizobia at the time of sowing of legumes will usually result in sufficient N being fixed by the crop to fulfill its requirement of growth.

From the results, rhizobia inoculum 2 (i.e. strain 29 of effectiveness over 64%) performed better than rhizobia inoculum 1 (strain 55 of effectiveness 56%) in terms of all the parameters tested (nodule number and nodule weight, %Ndfa, total N fixed, shoot total N and shoot dry weight). This agrees with findings made by Gicharu *et al.* (2013) who stated that when climbing bean

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cultivars were inoculated with USDA 2676 inoculum, the plants generated higher nodules compared to the other rhizobia inocula. Solomon *et al.* (2012) found that the inoculum TAL 379 gave a significantly higher nodule numbers than inoculum TAL 378 compared to the uninoculated control soybean varieties. The high measurement made for inoculum 2 compared to inoculum 1 with respect to the parameters shows how effective the rhizobia in inoculum 2 were. Thus inoculum 2 was more effective than inoculant 1.

The poor response of AYB to inoculation and N_2 fixation in the African yam bean cultivated soils could be attributed to the ineffectiveness of the indigenous rhizobia whiles the introduced strains in the inoculant were more effective. Singleton and Tavares (1986) noted that inoculation may be successful when the native rhizobia populations in soil were ineffective. The positive response of AYB to inoculation in the Bekwai and Nzima series might be due to those soils containing low population of native rhizobia; a similar observation was made by Hafeez *et al.* (2000) with garden pea.

5.4 Diversity of Indigenous African Yam Bean Rhizobia.

5.4.1 Cross Inoculation Studies (Host Range Analysis)

The Cross inoculation group concept has been an effective tool in demonstrating the ability of rhizobia isolates in nodulating a group of legume host species which has helped in classifying *Rhizobium* as specific or promiscuous (Fred *et al.*, 1932). The concept was therefore applied to this study to identify the promiscuity or specificity of the native African yam bean *Rhizobium* obtained from the screening experiment. The results showed that African yam bean *Rhizobium* was compatible with cowpea and soybean rhizobia but not compatible with pigeon pea rhizobia. This could mean that AYB could be grouped with either soybean or cowpea in the same cross inoculation group. Experiments conducted by other researchers showed that the African yam

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bean *Rhizobium* was promiscuous. Oagile (2005) observed that African yam bean is a promiscuous legume as it was able to form nitrogen fixing nodules with three strains tested and classified as both *Rhizobium* and *Bradyrhizobium* species. Oagile (2012) also confirmed that African yam bean also formed nodules with a wide range of rhizobial strains indigenous to Ghanaian soils which also confirmed its promiscuity (Oagile, 2012). *Rhizobium* spp. CP 279 and ORS 302 both being broad host range were inoculated on African yam bean landraces and formed nitrogen fixing nodules within 28 days (Oagile, 2005). Assefa and Kleiner (1997) also reported that *Bradyrhizobium* spp. AUEB20 isolated from Ethiopian tree *Erythrina brucei* was promiscuous in nodulating a number of African yam bean plants and the nodules were found to be fixing nitrogen. Promiscuity of the African yam bean was also confirmed by Obiagwu (1995) who stated that African yam bean has the ability to match cowpea in terms of nodulation because Singleton *et al.* (1992) documented that cowpea rhizobia indigenous to African soils were promiscuous and nodulated a wide range of legumes.

Isolates from pigeon pea from the results were not able to nodulate African yam bean while that of African yam bean were able to nodulate pigeon pea which agrees with work done by Boakye (2013) on tree legumes which had similar nodulating patterns. This however, shows the specificity of pigeon pea rhizobia and confirmed the promiscuity of African yam bean isolates. The results also indicated the nature of diversity of the rhizobia that nodulate these legumes and confirmed the complexity or confusion associated with the cross inoculation concept.

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CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The main focus of this study was to enumerate the population of African yam bean rhizobia in some Ghanaian soils (soils cultivated and uncultivated with AYB), screen for highly effective rhizobia of AYB capable of being used as future inoculant and assess the effect of N and inoculating highly effective rhizobia on African yam bean.

From the MPN results, except for Bekwai series which contained no compactible indigenous African yam bean rhizobia cells (0 cell g^{-1} soil), the rest of the soils contained high population of African yam bean nodulating rhizobia. Also soils from Akorviefe recorded the highest African yam bean nodulating rhizobia (290 cell g^{-1} soil), followed by Dz-1, Adenta series, Dz-2 and Nzima series of 140, 72, 52 and 52 cell g^{-1} soil respectively.

Screening for highly effective rhizobia in these soils recorded no highly effective *Rhizobium* strains but there were moderate and ineffective strains. Interestingly soils uncultivated with African yam bean recorded 87% ineffective and 13% moderately effective rhizobia while soils cultivated with African yam bean recorded 97% ineffective and 3% moderate effective rhizobia.

The application of N fertilizer at the rate of 70 kg Nha^{-1} and rhizobia inoculation had an effect on the various parameters measured such as nodule number, nodule dry weight, %Ndfa, total N_2 fixed, % N in shoot, total N in shoot and shoot dry weight. Application of N decreased nodulation and N_2 fixed. However, it increased total N accumulation and shoot biomass in all the soils. Rhizobia inoculation improved nodulation, N_2 fixed, N accumulation and shoot biomass in

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the soils used. N application and rhizobia inoculation both improved N accumulation and growth of African yam bean. Inoculum 2 was more effective than inoculum 1.

Cross inoculation study showed African yam bean rhizobia as being compatible with pigeon pea, soybean and cowpea hence promiscuity of AYB rhizobia. Also soybean and cowpea rhizobia nodulated African yam bean but not rhizobia from Pigeon pea, showing AYB as being broad host range.

6.2. Recommendations

The following recommendations are made from the results obtained from the study:

1. The study could be repeated in various soil types to test the authenticity of these results whether or not African yam bean nodulating rhizobia exist in different Ghanaian soils.
2. More screening experiment should be conducted to identify highly effective rhizobia in different Ghanaian soils.
3. To observe the effect of rhizobial inoculation and N application on yield of African yam bean, the crop should be allowed to mature.
4. Other research should be conducted on the response of AYB to phosphorus and other soil amendments such as biochar.
5. Classification of AYB rhizobia isolates should be conducted through morphological and biochemical techniques.
6. Genetic diversity should be carried to confirm the traditional diversity (CIG).

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APPENDICE

Appendix 1: Composition and Preparation of Yeast Extract Mannitol (YEM) Agar

Reagent	Quantity (g L^{-1})
Mannitol	10.0
Dipotassium Phosphate (K_2HPO_4)	0.8
Potassium dihydrogen phosphate (KH_2PO_4)	0.2
Magnesium Sulphate ($MgSO_4 . 7H_2O$)	0.2
Sodium Chloride (NaCl)	0.1
Calcium Chloride ($CaCl_2$)	0.1
Yeast Extract	0.5
Agar	15.0
Distilled water	1 L

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Appendix 2: Composition and Preparation of N-free Nutrient Solution

Stock solution	Form	Quantity (g L^{-1})
1	$CaCl_2.2H_2O$	249.1
2	KH_2PO_4	135.1
3	Fe-citrate	6.70
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.2H_2O$	0.048

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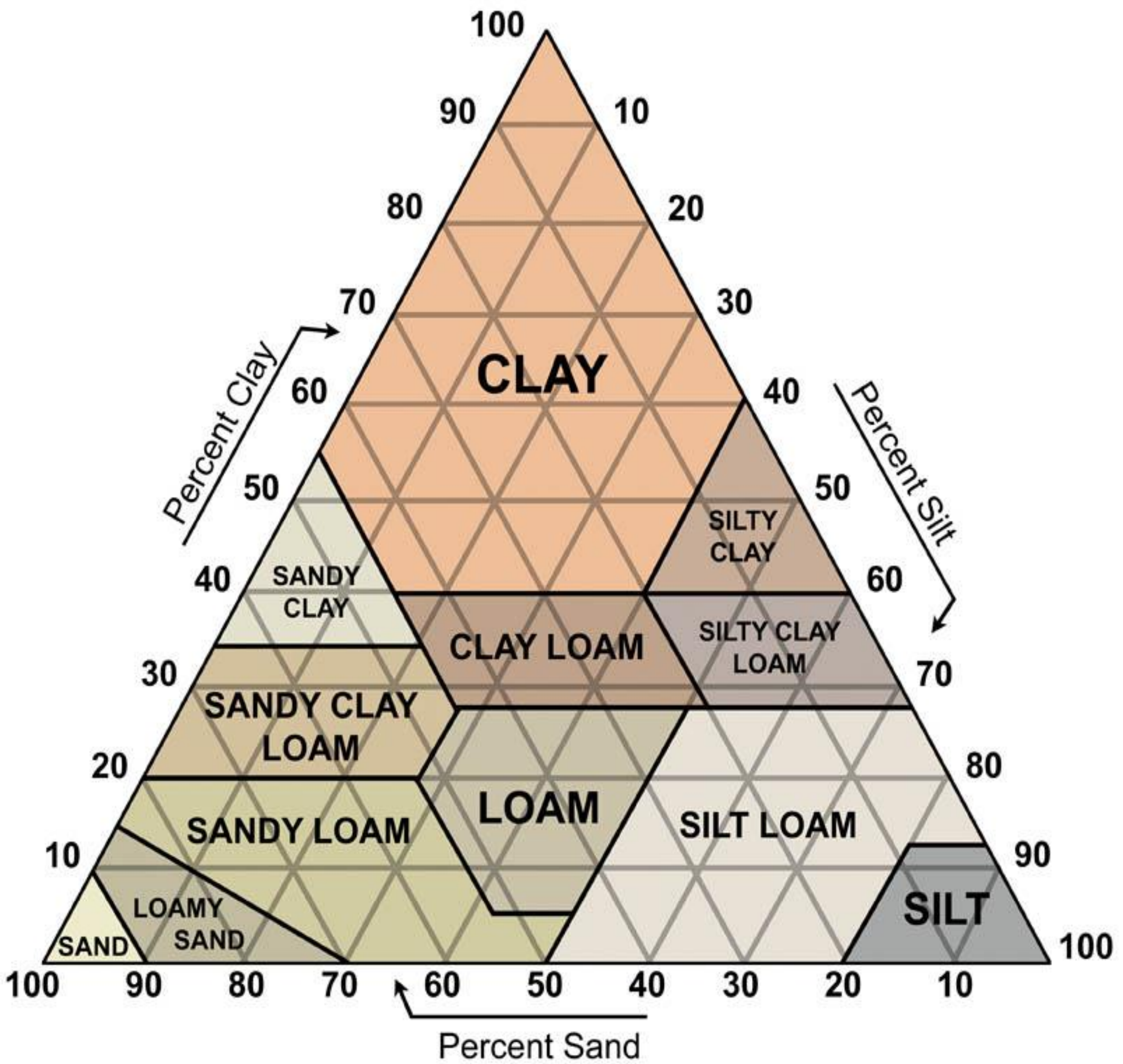
Appendix 3: Soil texture triangle



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Appendix 4: Anova table for nitrogen effect on the growth parameters of African yam bean in six Ghanaian soils.

Variate: Nodule number plant^{-1}

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	13283.979	2656.796	1627.99	<.001
Treatment	1	1139.062	1139.062	697.98	<.001
Soils × Treatment	5	906.479	181.296	111.09	<.001
Residual	24	39.167	1.632		
Total	35	15368.687			

Variate: Nodule Dry Weight (mg plant^{-1})

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	49166.67	9833.33	108.09	<.001
Treatment	1	13611.11	13611.11	149.62	<.001
Soils × Treatment	5	13313.89	2662.78	29.27	<.001
Residual	24	2183.33	90.97		
Total	35	78275.00			

Variate: Ndfa (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	13.36842	2.67368	119.31	<.001
Treatment	1	1.05404	1.05404	47.04	<.001
Soils × Treatment	5	0.17631	0.03526	1.57	0.205
Residual	24	0.53782	0.02241		
Total	35	15.13660			

Variate: Total N_2 fixed (mg plant^{-1})

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	12169.008	2433.802	357.69	<.001
Treatment	1	23.234	23.234	3.41	0.077
Soils × Treatment	5	98.480	19.696	2.89	0.035
Residual	24	163.300	6.804		
Total	35	12454.023			

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Variate: Shoot N (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	10.87637	2.17527	122.86	<.001
Treatment	1	3.22801	3.22801	182.32	<.001
Soils × Treatment	5	0.95371	0.19074	10.77	<.001
Residual	24	0.42493	0.01771		
Total	35	15.48302			

Variate: Total N in shoot (mg plant^{-1})

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	73023.829	14604.766	1945.93	<.001
Treatment	1	9481.462	9481.462	1263.30	<.001
Soils × Treatment	5	4116.886	823.377	109.71	<.001
Residual	24	180.127	7.505		
Total	35	86802.304			

Variate: Shoot Dry Weight (g plant^{-1})

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	20.8897722	4.1779544	12154.05	<.001
Treatment	1	1.9182250	1.9182250	5580.29	<.001
Soils × Treatment	5	0.5510833	0.1102167	320.63	<.001
Residual	24	0.0082500	0.0003437		
Total	35	23.3673306			

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Appendix 5: Anova table for inoculation effect on the growth parameters of African yam bean in six Ghanaian soils

Variate: Nodule number plant^{-1}

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	21308.951	4261.790	1239.79	<.001
Treatment	1	98.340	98.340	28.61	<.001
Soils × Treatment	5	270.951	54.190	15.76	<.001
Residual	24	82.500	3.438		
Total	35	21760.743			

Variate: Nodule Dry Weight (mg plant^{-1})

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	77345.1	15469.0	135.83	<0.001
Treatment	1	34	34	0.3	0.590
Soils × Treatment	5	2795.1	559	4.91	0.003
Residual	24	2733.3	113.9		
Total	35	82907.6			

Variate: Ndfa (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	19.0921	3.8184	28.41	<.001
Treatment	1	0.6074	0.6074	4.52	0.044
Soils × Treatment	5	0.6430	0.1286	0.96	0.463
Residual	24	3.2254	0.1344		
Total	35	23.5678			

Variate: Total N_2 fixed (mg plant^{-1})

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	18955.93	3791.19	153.08	<0.001
Treatment	1	328.19	328.19	13.25	0.001
Soils × Treatment	5	143.31	28.66	1.16	0.358
Residual	24	594.37	24.77		
Total	35	20021.80			

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Variate: Shoot N (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	8.8583	1.7717	16.63	<0.001
Treatment	1	0.6074	0.6074	5.70	0.025
Soils × Treatment	5	0.6430	0.1286	1.21	0.336
Residual	24	2.5574	0.1066		
Total	35	12.6660			

Variate: Total N in shoot (mg plant^{-1})

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	66651	13330.26	958.59	<0.001
Treatment	1	1004.02	1004.02	72.2	<0.001
Soils × Treatment	5	617.25	123.45	8.88	<0.001
Residual	24	333.75	13.91		
Total	35	68606.33			

Variate: Shoot Dry Weight (g plant^{-1})

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Soils	5	20.8028639	4.1605728	15642.88	<.001
Treatment	1	0.2352250	0.2352250	884.40	<.001
Soils × Treatment	5	0.2524750	0.0504950	189.85	<.001
Residual	24	0.0063833	0.0002660		
Total	35	21.2969472			