NODULATION, NITROGEN FIXATION AND DIVERSITY OF THE AFRICAN YAM BEAN RHIZOBIA IN FOUR GHANAIAN SOILS

BY

ABIGAIL TETTEY

(10230237)

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

Department of Soil Science,
School of Agriculture,
College of Basic and Applied Sciences,
University of Ghana, Legon,
Accra, Ghana.

JULY, 2014

DECLARATION

I hereby declare that, except for references to other peoples work, which have been duly cited and acknowledged, this thesis is the result of my own original research and has not been presented elsewhere either in part or in whole for another degree.

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Abigail Tettey (Student) Date

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Prof. S. K. A. Danso (Principal Supervisor) Date

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Dr. (Mrs.) S. Asuming–Brempong (Co–supervisor) Date

DEDICATION

I dedicate this thesis to the blessed memory of Prof. Seth Kofi Akyea Danso, who suddenly passed away at the final stages of this project. May his soul rest in eternal Peace.

I also dedicate this thesis to Prof. Emmanuel Owusu–Bennoah, for the special interest he took to help me come this far. God richly bless you.

ACKNOWLEDGEMENTS

Glory be to the Almighty God for His love, abundant grace and mercy upon my life without which this thesis would not have been completed successfully.

I express my sincere appreciation and gratitude to my principal supervisor, the late Professor S. K. A. Danso for his invaluable guidance, suggestions, encouragement and financial support which have helped me complete this work successfully. I am particularly appreciative of his strictness and concern. May the Almighty God richly bless him.

A very special thank you to my co–supervisor; Dr. (Mrs) S. Asuming–Brempong for her support, guidance, understanding and encouragement towards the successful completion of this work. God bless her abundantly.

I am extremely grateful to Prof. E. Owusu–Bennoah for obtaining a Council for Scientific and Industrial Research (CSIR)–West African Agricultural Productivity Programme (WAAPP), CSIR–WAAPP Fellowship for me, without which this work could not have been finished successfully. I am also very grateful to Dr. (Mrs) R. Mamaa Entsua–Mensah, the Deputy Director–General, CSIR and all the Coordinators of WAAPP for providing the financial package for this work.

I am grateful to Dr. I. Y. D. Lawson for reading through this thesis and making the necessary inputs when Prof. Danso suddenly passed away.

I am also thankful to Dr. T. A. Adjadeh (Head of Department), all Lecturers and technicians of the Department of Soil Science for their encouragements, suggestions and support in the course of my study.

A big thank you to all the technicians of Ecological Laboratory of University of Ghana, Legon, for granting me the permission and also assisting me to carry out my research in their laboratory.

My appreciation also goes to Dr. S. Amiteye, Mr Ofori Ayerh, Mr Robert Appiah, and all staff of Biotechnology and Nuclear Agriculture Research Institute (BNARI)–Ghana Atomic Energy Commission (GAEC) for granting me the permission and helping me to undertake part of my research in their laboratory.

A special thank you to Mr Benjamin Otu and Mr K. Acquah all of Biotech laboratory, University of Ghana for the assistance they gave me in the course of my project.

I thank my parents, Mr J. T. Kpabitey and Madam Paulina Ayerkie, and my siblings (Abraham, Felix and Naomi) for their support and encouragement throughout my course of study.

There are people without whose help this research could not have been a success. In no particular order, but with equal gratitude for their expertise and support, I wish to thank Mr J. A. Narternor, Mr M. S. Elegba, Mr D. K. Tsatsu, Mr W. A. Asante, Mr E. N. A. Bonaparte, Mr A. N. Odonkor, Dr J. Bawole, Mr C. Fasemkye, Mr E. T. Nanor, Mr N. Agyekum and all staff of University of Ghana Experimental Farms, Legon.

A special thank you goes to all my colleagues especially Daniel Fianko Ansah for the support he gave me during the period of this research. God bless you.

Finally, to all those who helped me directly and indirectly, I say thank you very much.

ABSTRACT

Indigenous and traditional crops are very essential in the survival strategies of local farmers, since they have multiple uses such as a variety of plant parts that can be consumed as well as for medicinal purposes. Traditional crops play an important role in achieving food security as well as conserving biodiversity. Some of these indigenous crops (e.g. legumes) have the capacity to establish and grow relatively well on poor fertility soils where crops such as maize (a major staple cereal) may fail to give good yields. These under–exploited legumes include the African yam bean *(Sphenostylis stenocarpa*). Efforts to exploit them in agriculture have been made in some countries, but little has been reported on the cultivation and performance of similar crops in Ghanaian soils.

This study was conducted to assess the nodulation, nitrogen fixing potential, the abundance, and diversity of the rhizobia that nodulate the African yam bean in four Ghanaian soils as well as assess the response of African yam bean to nitrogen and phosphorus fertilization.

The result of the Most Probable Number (MPN) estimates showed that the four soils contained varied numbers of indigenous rhizobia capable (from 9.3×10 cells g^{-1} soil in Toje series to 2.0×10^2 cells g^{-1} soil in the Akuse series) of nodulating African yam bean. That of cowpea (used for comparison) ranged from 1.2×10 cells g^{-1} soil in the Toje series to 6.8×10^2 cells g^{-1} soil in the Adenta series, with about 75% of the soils tested, containing more than 10^2 rhizobia cells per gram of soil.

For the Toje series, African yam bean formed the lowest number of nodules (mean 14 nodules $plant^{-1}$), with plants in Haatso forming the highest (mean 39 nodules $plant^{-1}$). Nodulation in the Adenta series like that in Haatso series was significantly higher than nodulation in the Akuse series.

Nitrogen fertilization had a significant adverse effect on nodulation, nodule dry weight and N derived from N_2 fixation, whiles there was an increase in dry matter yield. In contrast, P fertilization had a positive effect on all the parameters mentioned above.

The cross–inoculation studies revealed that the African yam bean rhizobia were able to form nodules on cowpea, yardlong bean and Pueraria, but not on bambara, lima bean and crotalaria, even though rhizobia isolated from bambara, lima bean and yardlong bean formed nodules on the African yam bean.

Random amplified polymorphic DNA (RAPD) analysis with RPO4 showed that the 30 isolates examined consisted of two major genetic groups, with a mean similarity of 60%. A similar phylogenetic clustering of these isolates was observed with the RPO1 primer with mean similarity of 54%. However, the RPO1 primer revealed a higher diversity among the African yam bean isolates in Haatso and Adenta series than did the RPO4 primer, based on the Shannon–Weiner diversity index. The amplification of the 16S–23S rDNA (ITS) gene of almost all the isolates gave band of sizes from 350 bp to 850 bp. Combined restriction analysis of digested ITS region with *Hha*I and *Hind*III endonucleases, also distinguished the isolates into two major phylogenetic groups at 56% similarity level. Characterization of the test rhizobia isolates based on PCR amplification of the 16S rDNA gene gave almost a single band of 1500 bp. Restriction of the 16S gene with *Hae*III and *Hha*I enzymes also clustered the isolates into two major groups at 53% similarity level.

From the results, it can be concluded that, African yam bean rhizobia may be widespread and diverse in Ghanaian soils. Also, the African yam bean appears to be a relatively permissive host. This character however may not guarantee effectiveness in N_2 fixation and may sometimes lead to the formation of less effective symbioses with less specific and ineffective strains. It is therefore recommended that the study be conducted in various areas with different soils types to

establish the validity of the conclusion, that the African yam bean nodulating rhizobia are common in most if not all Ghanaian soils. Further taxonomic study of indigenous African yam bean *Rhizobium* isolates in different soils should be carried out by taking as many morphological, biochemical and genetic characters as possible to have a more complete picture of the taxonomy and the evolutionary relationship between the African yam rhizobia that nodulate other commonly given legumes, such as cowpea, lima bean and bambara groundnuts.

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CHAPTER ONE

INTRODUCTION

Even though nodulated legumes have been used by indigenous people in Africa for centuries, it is sometimes doubtful if their full potential has been realized (Sprent *et al.,* 2010). Legumes are recognized as a source of dietary protein and energy in the countries where cost of animal protein is high (Amarowicz & Pegg, 2008). Protein contents in legume grains range from 17% to 40%, contrasting with the 7–13% of cereals, and being equal to the 18–25% protein contents of meat (Genovese & Lajolo, 2001). The leaves are sometimes eaten, either raw or cooked, the grain is eaten fresh or dried, and the haulms are used as forage.

A hallmark trait of legumes is their ability to nodulate and fix atmospheric nitrogen in symbiosis with compatible soil bacteria (rhizobia). Most studies on legume–*Rhizobium* symbiosis focus on biomass productivity, nodulation, nitrogen fixation and its residual effect of domesticated food legumes (Mapfumo *et al.,* 1999; Mafongoya *et al.,* 2002), with little attention to indigenous legumes. Vast collections of indigenous legumes are present in Africa, ranging from large rainforest trees to small annual herbs (Lock, 1989). Most indigenous crops have multiple uses and a variety of plant parts that can be consumed. They produce both edible seeds and tubers and thus have the potential of expanding the food basket of Africans, including Ghanaians. Thus, traditional crops play an important role in achieving food security and in conserving biological diversity (Warren, 1991). However, there is very little evidence of their domestication and cultivation. Most indigenous crops (e.g. legumes) have the capacity to establish and grow relatively well on poor fertility soils where crops such as maize (a major staple cereal) have failed to give good yields (Mapfumo *et al.,* 2005).

Poor and declining soil fertility, increasing cost of mineral fertilizers and the associated environmental effects, low availability and poor quality of organic amendments remain major challenges to food security in Africa (Smaling *et al.*, 1997; Giller *et al.*, 1997). High demand for land for non–agricultural purposes has also significantly reduced the fallowing durations, as a traditional soil fertility regeneration practice (Mafongoya *et al.*, 2006). Thus traditional crops are very vital in the survival strategies of most African farmers, since most indigenous crops are able to grow on marginal, leached or eroded land.

Grain legumes constitute the main source of protein in the diets of the average Ghanaian home (Klu *et al.*, 2001). The widely used ones include cowpea *(Vigna unguiculata)*, groundnut *(Arachis hypogaea)*, lima bean *(Phaseolus lunatus)* among others (Klu *et al.*, 2001). However, there are other pulses (grain legumes) that could help meet human dietary needs but are cultivated only in localized areas and used less. These under–exploited legumes include African yam bean *(Sphenostylis stenocarpa*), bambara groundnut *(Vigna subterranea)* and pigeon pea *(Cajanus cajan)* (Klu *et al.*, 2001).

In Ghana however, except for the highly popular legumes such as cowpea, soybean, groundnut, bambara, few reliable data are available on the abilities of other legumes to nodulate and fix nitrogen with naturally occurring strains of rhizobia in various soils. However, there are several others which have not been well studied with regards to their ability to fix nitrogen into the soil and improve soil fertility, and even serve as food sources. Efforts to exploit them in productive agriculture have been made in some countries, but little has been reported on the cultivation and performance of similar species in Ghanaian soils.

Food security and sustainability is a serious global concern in recent times, and many indigenous food crops of Africa which promise to ameliorate food and nutritional insecurities are presently neglected and under–utilized (Adewale & Odoh, 2012). African yam bean is one of such crop with great nutritional potentials. Thus the subsistence nature of production of the crop may have been occasioned by its poor acceptability as a valuable crop among middle–aged farmers in Africa (Adewale & Odoh, 2012). The poor awareness about the taxonomy, nodulation, and nitrogen fixing ability, variety and nutritional value of edible parts of the African yam bean may be due to limited research on it.

Further research and development of these and other species is essential for human livelihood, to retain agricultural production and biodiversity in a changing world that is being increasingly dominated by a few crops. Thus, screening rhizosphere soils of legumes for the presence and abundance of highly effective native *Rhizobium* strains in soils is therefore very crucial and highly effective in efforts to promote greater exploitation of biological nitrogen fixation as a substitute or supplement to nitrogen fertilization (Dogbe *et al.,* 2000). This information is somehow not adequate or is lacking. Besides, since many soil types that occur in Ghana are found in the West African sub–region, it is likely that such information could be useful to farmers in other countries of the sub–region.

Therefore to contribute towards bridging of this knowledge gap, this study was carried out to investigate the nodulation and N_2 fixation of the African yam bean in some Ghanaian soils, as well as to evaluate the homologous rhizobia of African yam bean found in four Ghanaian soils.

Specific study objectives are:

1) Enumerate the populations of indigenous rhizobia capable of nodulating the African yam bean in four soils in Ghana.

2) Assess how nitrogen and phosphorus fertilization influence nodulation, N fixation and growth of the African yam bean.

3) To assess the diversity of the obtained rhizobia isolates.

Research Questions

1) Does the indigenous rhizobia have the potential to fix N_2 in African yam bean?

2) How compactible are the rhizobia that nodulate the African yam bean with tropical food and forage legumes?

3) How genetically diverse is the African yam bean nodulating *Rhizobium*?

CHAPTER TWO

LITERATURE REVIEW

2.1 Nitrogen in the Biosphere

Nitrogen is an important component of many organic molecules such as DNA, RNA and proteins which are the building blocks of life. Nitrogen constitutes 78% of the air we breathe in terms of volume. Even though the majority of the air we breathe is N_2, most of this is unavailable for plants use. This is because of the strong triple bond between the N atoms in the N_2 molecule, making it somewhat inert. Thus, in order for plants and animals to use nitrogen, it must be converted to either ammonium (NH_4^+), nitrate (NO_3^-) or organic nitrogen. Nitrogen is a versatile element that exists in both organic and inorganic forms as well as in many different oxidation states (Harrison, 2003).

The movement of nitrogen between the atmosphere, biosphere, and geosphere is summarised in the nitrogen cycle in Figure 2.1 (http://www.ianlee.co.uk/oldweb/nitrogen.htm).

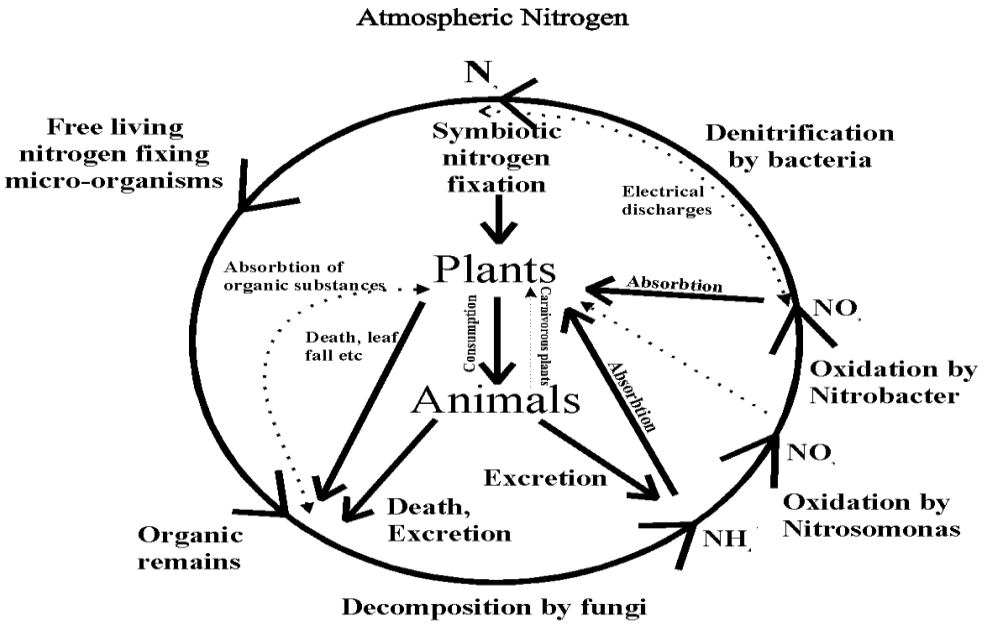


Figure 2.1: The Nitrogen Cycle.

Nitrogen (N), one of the macronutrients, is most often found limiting for plant growth. This is attributed to the continual loss of nitrogen from the reserve of fixed nitrogen or combined, present in soil and available for use by plants. Available soil nitrogen is constantly depleted by processes such as microbial denitrification, soil erosion, leaching, chemical volatilization, removal of nitrogen–containing crop residues from the land surface. Thus, the nitrogen reserve in agricultural soils must be replenished intermittently, in order to maintain an adequate level of nitrogen for crop production. Soil nitrogen is normally replenished through the addition of inorganic nitrogenous fertilizers or by the activity of biological nitrogen fixation systems.

2.2 The Concept of Nitrogen Fixation

Plant growth is often limited by the availability of nitrogen in the soil even though 78–80% of the atmosphere is composed of nitrogen gas (N_2). Therefore atmospheric nitrogen can only be used by plants, when it is 'fixed'; that is, split and combined with hydrogen or oxygen. This process of nitrogen fixation requires a large input of energy (Wagner, 2012) and can occur either biologically, within the cells of various bacteria, chemically (in fertilizer industries) or during lightning.

Nitrogen fixation occurs both biologically and non–biologically. Non–biological nitrogen fixation occurs through the effects of lightning, and in industry primarily through the Haber–Bosch process. Industrial nitrogen fixation in the form of nitrogen fertilizer has increased from 3.5 million tons in 1950 to 80 million tons in 1989 (Hardy, 1993) and 1.4×10^{14} Mg N $year^{-1}$ (Canfield *et al.*, 2010) in response to the needs of high–yielding crops. Lightning worldwide is also reported to contribute approximately 1×10^{10} kg NH_3 per year (Cheng, 2008). Cheng (2008) reported that approximately 2 tonnes of industrially–fixed nitrogen are needed as fertilizer for

crop production to equal the effects of 1 tonne of nitrogen biologically–fixed by legume. Biological nitrogen fixation is estimated at 1.1×10^{14} Mg N $year^{-1}$ (Canfield *et al.*, 2010).

2.2.1 Industrial Nitrogen Fixation (Haber–Bosch process)

The split of the stable triple bond of molecular nitrogen (N≡N) is very energy demanding and is accomplished at high temperatures (600–800°C) and pressures (as much as 5.06×10^{7} Pa) (Cheng, 2008). At a temperature of about 600°C, atmospheric nitrogen and hydrogen (usually derived from natural gas or petroleum) are combined chemically in a vessel to form ammonia (NH_3). Catalyst (usually iron with oxides of aluminium and potassium) are added to the vessel to speed up the chemical reaction. The Haber process is a widely used process for the commercial production of ammonia. The process is summarised in the following equation (http://www.reference.com/browse/haber+process).

$$N_{2(g)} + 3H_{2(g)} \xleftrightarrow{heat,\ pressure\ \&\ metal\ catalyst} 2NH_{3(g)} \quad (\Delta H = 92.4\ kJ\ mol^{-1})$$

2.2.2 Biological Nitrogen Fixation (The Legume–*Rhizobium* Symbiosis)

Biological nitrogen fixation involves the fixing of atmospheric nitrogen through symbiotic and asymbiotic means (Raven *et al.*, 1992). Biological nitrogen fixation is an important and environmentally friendly natural process that supports life on this planet (Salisbury & Ross, 1992), since plants and animals obtain nitrogen from either nitrogen–fixing organisms or from nitrogen fertilizers. Many kinds of microbes live in close association with specific host plants, and hence benefit from these associations by obtaining carbon and other nutrients from their hosts. A variety of bacteria, fungi, nematodes and protozoa colonize the rhizosphere of plants, existing either as free–living organisms in the soil or attached to surface of plant roots (Bowen & Rovira, 1999), since carbon and minerals sources are very much in abundance in the zone

(Walker *et al.*, 2003). Marschner (1993) also attributed this is due to the fact that plants exude high levels of nutrients from their roots, including low–molecular–weight root exudates (such as amino acids, organic acids, sugars, aromatics and other secondary metabolite) and high–molecular–weight root exudates (such as polysaccharides and proteins).

Soil bacteria belonging to various genera of the order *Rhizobiales* (collectively called *rhizobia*) are able to invade legume roots in nitrogen–limiting environments, thus leading to the formation of root nodules (Soto *et al.*, 2006). In the root nodules through the induction of the nitrogenase complex, the *Rhizobium* is able to convert atmospheric nitrogen into ammonia, which is used by the plant, while the plant supplies carbon source to the bacteria for the reduction of nitrogen. Furthermore the process involves the release of flavonoids from the root of the legume host, induce transcription of nodulation genes in compatible *Rhizobium*, leading to the formation of lipochito–oligosaccharide molecules that, in turn, signal the host plant to begin nodule formation (Long, 1996).

2.2.2.1 Nitrogen Fixation by Free–Living Heterotrophs (Asymbiotic fixation)

Many heterotrophic bacteria live in the soil and fix significant levels of nitrogen without the direct interaction with other organisms (Wagner, 2012). Examples of free–living nitrogen–fixing bacteria include species of *Azotobacter*, *Bacillus*, *Clostridium*, and *Klebsiella*. These bacteria must find their own source of energy, typically by oxidizing organic molecules released by other organisms or from decomposition (Wagner, 2012). However there are some free–living organisms that have chemolithotrophic abilities and can thereby are able to utilize inorganic compounds as sources of energy to fix atmospheric nitrogen (Wagner, 2012). Because the activity of nitrogenase can be inhibited by oxygen, free–living organisms behave as anaerobes or microaerophiles during the process of nitrogen fixation. However, because of the scarceness of

suitable carbon and energy sources for the free–living organisms, their contribution to global nitrogen fixation rates is generally considered minor (Wagner, 2012). However, Vadakattu and Paterson (2006) in an intensive wheat rotation farming system study in Australia demonstrated that free–living microorganisms contributed 20 kg N ha^{-1} yr^{-1} to the cropping system (i.e. about 30–50% of the total nitrogen needs). Nonetheless, maintaining wheat stubble and reduced tillage in this system, was reported by Vadakattu and Paterson (2006) to provide the necessary high–carbon, low–nitrogen environment to optimize the activity of the free–living organisms.

Associative Nitrogen Fixation

Species of *Azospirillum* are able to form close associations with several members of the grass family, *Poaceae*, comprising agronomically important cereal crops, such as rice, wheat, corn, oats, and barley (Tarrand *et al.*, 1978). Appreciable amounts of nitrogen fixed by these bacteria have been reported within the rhizosphere of the host plants. Vlassak and Reynders (1997) commented that the level of associative nitrogen fixation is influenced by several factors, including soil temperature, the ability of the host plant to provide a rhizosphere environment low in oxygen, the availability of host photosynthate for the bacteria, the competitiveness of the bacteria, and the efficiency of nitrogenase.

2.2.2.2 Symbiotic Nitrogen Fixation

Symbiotic nitrogen fixation occurs when nitrogen fixing bacteria such as *Rhizobium* form symbiotic relationships with legume host plants. In this relationship, the nitrogen fixing bacteria inhabit the legume root nodules and receive carbohydrates as well as a favourable environment from their host plant in exchange for the atmosphere nitrogen they fix (Harrison, 2003). The energy requirements of BNF are met by renewable sources such as plant–synthesized carbohydrates rather than from non–renewable fossil fuels such as natural gas. The plant

provides the rhizobia with sugars from photosynthesis (for energy), micronutrients and ultimately provides shelter from desiccation and predation (Sprent & Raven, 1992). The fixed nitrogen is exported from the nodule in several different forms (White *et al.,* 2007) and are taken up by the host plant for protein synthesis and its growth and development (Sessitsch *et al.*, 2002). This mutualistic relationship between the *Leguminosae* and the *Rhizobiceae* forms the basis for the ecological importance of legumes in natural and agricultural ecosystems. Thus, symbiotic legumes contribute largely to the N economy of the ecosystems, with asymbiotic and symbiotic biological systems fixing an estimated 100–175 million metric tons of nitrogen annually (Burns & Hardy, 1975). The process of biological nitrogen fixation has been studied extensively (Leigh, 2002), however, the nitrogen reduction process is a very complex mechanism which has not yet been fully elucidated (Franche *et al.,* 2009). The result of net reduction of molecular nitrogen to ammonia is generally accounted for by the following equation (Vance, 2008):

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

The above chemical equation essentially requires the molybdenum (Mo) and Fe proteins of the nitrogenase enzyme, an electron source and a supply of ATP (Howard & Rees, 1996). The process has to overcome the presence of oxygen (O_2) released when ATP is generated in the bacteroids (rhizobia transformed in compartmentalised cells in which they are capable of N_2 fixation) (Schauser *et al.*, 2008), as oxygen inactivates nitrogenase synthesis (Hill *et al.*, 1981). This problem is overcome by plant–derived leghaemoglobin which binds with O_2 and facilitates O_2 diffusion through the bacteroids at a low and buffered concentration (Burris & Haas, 1994).

An example is the water fern *Azolla*'s symbiosis with a cyanobacterium *Anabaena azollae*. *Anabaena* colonizes the cavities formed at the base of *Azolla* fronds. The cyanobacteria fixes nitrogen in specialized cells called heterocysts. Rice paddies are typically covered with *Azolla*

blooms that fix up to 600 kg N ha^{-1} during the growing season (Fattah, 2005). The second example is the symbiosis between actinorhizal trees with the actinomycete, *Frankia*. Actinorhizal plants are found in many ecosystems including alpine, xeric, forest, glacial till, riparian, coastal dune, and arctic tundra environments (Benson & Sylvester, 1993). Even though the symbiotic partners described above play an important role in the worldwide ecology of nitrogen fixation, by far the most important nitrogen–fixing symbiotic associations are the relationships between legumes and *Rhizobium* bacteria.

2.3 The Legume Component

Leguminous plants are relevant economic crops, because of their diversity, manifested in their ability to adapt to a wide range of ecological conditions, the variety of their edible parts, high protein content of the grains, their use as pastures, etc. (Morel *et al.,* 2012). A hallmark trait of legumes is their ability to nodulate and fix atmospheric nitrogen in symbiosis with compatible *Rhizobium* strains. This ability of many legumes to nodulate is an issue of ecological and economic importance (Zahran, 2009).

Legumes are flowering plants that produce seedpods while some produce root tubers (Morel *et al.*, 2012). They have colonized several ecosystems (Schrire *et al.*, 2005), and have been found in most of the archaeological annals of plants (Morel *et al.*, 2012).

*Leguminosae (*alternatively *Fabaceae)* is the third most populous family of flowering plants (behind *Asteraceae* and *Orchidaceae)* with 670 to 750 genera and 18,000 to 19,000 species (Rivas *et al.,* 2009). Traditionally, three main subfamilies are distinguished: *Caesalpinoidae, Mimosoidae* and *Papilionidae.* The *Caesalpinoidae* has very few nodulating members, whereas most of the important agricultural crops are members of the *Papilionidae* (Giller, 2001). The *Mimosoidae* has recently received attention, since, in many cases, bacteria recovered from their

nodules belong to the beta subclass of Proteobacteria, while *Papilionidae* symbionts belong to the alpha subclass (Chen *et al.*, 2003). However, not all species of the *Leguminosae* can form symbioses with *Rhizobium* (Sprent, 2001). It is estimated that only 5% of the genera within the *Caesalpinioideae* can be nodulated, while within the *Mimosoideae* and *Papilionoideae*, this proportion increases to approximately 88% and 97%, respectively (Sprent, 2008).

Parasponia spp is the only non–legume woody plant that has been reported to form symbiosis with *Rhizobium* and utilize nitrogen fixed by the bacteria (Becking, 1992). The *Parasponia–Rhizobium* association can be highly effective with levels of nitrogen fixation comparable with those observed in legume–*Rhizobium* symbioses (Trinick, 1980). Both fast and slow–growing rhizobia (*Rhizobium* and *Bradyrhizobium*) are capable of nodulating *Parasponia species* (Trinick & Hadobas, 1988).

Leguminous plants are very diverse in morphology, habitat, and ecology, ranging from annual herbs to giant forest trees (Singh *et al.*, 2007). Some 25% of the world's major crop production is derived from legumes, and more than one–third of humanity's nutritional nitrogen requirement comes from legumes (Rivas *et al.*, 2009). Grain legumes are widely recognized as essential sources of food and feed proteins (Howieson *et al.*, 2000). In many regions of the world, legume seeds are the unique supply of protein in the diet and they often represent a necessary supplement to other protein sources (Rivas *et al.*, 2009). Legumes also contain many health–promoting components, such as fibre, resistant starch, minerals, and numerous phytochemicals endowed with useful biological activities (Amarowicz & Pegg, 2008). Hydrophilic phytochemicals, such as ascorbic acid, phenolic acids and polyphenols, have been associated with an enhancement of the immune system functionality and reduced cancer risk, whereas lipophilic phytonutrients,

such as carotenoids and tocopherols, may prevent the risk of cardiovascular and some eye diseases (Dillard & German, 2000).

Besides, legumes are very important both agriculturally and ecologically since they are responsible for a substantial part of the global flux of nitrogen from atmospheric N_2 to fixed forms such as ammonia, nitrate, and organic nitrogen. They are harvested as food for human and as feed for animal consumption as well as used for fuel–woods, timber, oil production, and medicinal purposes. They are also cultivated as ornamentals, used as living fences and firebreaks, etc (Lewis *et al.,* 2005). They are also utilized as cover crops, intercropped with cereals and other staple foods, used as agro–forestry trees due to their nitrogen fixing abilities. In rotation with cereals, legumes provide a source of slow–release nitrogen that contributes to sustainable cropping systems (Popelka *et al.,* 2004).

Legumes, by virtue of their capacity to fix nitrogen, are widely distributed in all tropical soils displaying a wide range of tolerance to acidity, alkalinity, water logging, mineral deficiency and drought (Rengel, 2002). They can colonize marginal lands and impoverished soils, thus offering some advantages in rehabilitating degraded and marginalized soils (Assefa, 1993). Legumes are environmentally friendly plants since all the nitrogen they fix in association with the rhizobia is assimilated by the plants with no leaching and ground water pollution (Diriba, 2007). Legumes play a critical role in natural ecosystems, agriculture and agroforestry where their ability to fix nitrogen in symbiosis with rhizobia makes them excellent colonizers of low–nitrogen environments (Graham & Vance, 2003).

Grain legumes (beans, peas, lentils, etc) provide about 33% of the dietary protein requirements of humans (Vance *et al.,* 2000). Soybean and peanut, provide more than 35% of the worlds processed vegetable oils. Grain legumes, fodder–pasture legumes, N_2–fixing shrub and tree

legumes are important for cropping systems in Africa (Dakora & Keya, 1997). They are able to grow in diverse ecological zones in the continent, including low fertility soils, and nodulate with a wide diversity of rhizobia and bradyrhizobia (Dakora & Keya, 1997).

Cowpea (*Vigna unguiculata),* bambara groundnut (*Vigna subterranean),* Kersting's bean (*Macrotyloma geocarpum)* are among the major indigenous legumes cultivated throughout sub–Saharan Africa (Dakora & Keya, 1997). Nonetheless, imported species such as common bean, groundnut, pigeon pea and soybean have also become important components of the traditional cropping systems in sub–Saharan Africa (Dakora & Keya, 1997).

Research attention has started to focus on the undomesticated/neglected/underutilized food legumes in Africa (Dakora & Keya, 1997). The most significant of these species, with regards to food use and value, include, marama bean *(Tylosema esculentum*), *Sphenostylis stenocarpa* (African yam bean), *Vigna Zobatifolia* and *Vigna vexillata,* which produce tubers with protein content ranging from 8 to 15%; levels higher than any conventional tuber crop (Dakora & Keya, 1997). The seeds are also rich in protein and essential amino acids such as methionine and lysine. The scientifically unimproved marama bean rivals soybean and groundnut in both protein and oil contents (Dakora & Keya, 1997). These undomesticated food legumes are similar in their ability to grow in highly leached and low fertility soils, and thrive in harsher conditions. The capacity of these legumes to grow in poor nitrogen soils, and yet store great quantities of protein in seeds and tubers, suggests a dependence on symbiotic fixation for N nutrition.

2.3.1 The African yam bean (*Sphenostylis stenocarpa)*

African yam bean (*Sphenostylis stenocarpa* [Hochst. Ex. A. Rich.] Harm) like other tuberous legumes (e.g. *Pachyrhizus* sp.) belongs to the *Fabaceae* family and the order *Fabales* and by its name is indigenous to tropical Africa (Potter, 1992). Harms (1899) evolved the generic name

Sphenostylis as the botanical genus to describe a group of distinctive leguminous taxon formerly grouped within the *Dolichos* and *Vigna* genera (Adewale & Odoh, 2012).

The African yam bean *(Sphenostylis stenocarpa)* should not be confused with the *Pachyrhizus spp* which in many places in the literature are referred to as "yam bean", Mexican yam bean, Mexican turnip Jicama, ahipa, etc (Adewale & Dumet, 2011). *Pachyrhizus spp*. is a more popular tuberous legume that is common in the tropics of Asia and Southern America (Adewale & Dumet, 2011). There are seven species within the genus, *Sphenostylis*, however, *Sphenostylis stenocarpa* is economically, the most important species (Potter & Doyle, 1994). The taxonomic profile of African yam bean is presented in Table 2.1.

Table 2.1: Taxonomic profile of African yam bean.

Rank	Scientific /Common Name
Kingdom	*Plantae*
Subkingdom	*Tracheobionta* (vascular plants)
Super division (phylum)	*Spermatophyta* (seed plants)
Division (sub phylum)	*Magnoliophyta* (flowering plants)
Class	*Magnoliopsida* (Dicotyledons)
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabaceae* (Pea family)
Sub family	*Papilionoideae*
Tribe	*Phaseoleae*
Sub tribe	*Phaseolinae*
Genus	*Sphenostylis* E. Meyer (*Sphenostylis)*
Species	*Sphenostylis stenocarpa* (Hochst. Ex. A. Rich.) Harms. (AYB)

Source: http//plants.usda.gov/core/profile? Symbol=spst8.

African yam bean is the most important, most cultivated and most valuable species in the genus *Sphenostylis* (Potter & Doyle, 1992). This tuberous legume is important in most indigenous African food cultures and in peasant agriculture (Adewale & Dumet, 2011). There is no record of the origin of the crop in any other continent except Africa (Potter & Doyle, 1994). This confirms the claim that the crop is a tropical African legume. The African yam bean is common in Central and West Africa, particularly in Cameroon, Cote d'Ivoire, Ghana, Nigeria, Togo (Porter, 1992), Gabon, Democratic Republic of Congo, Ethiopia, parts of East Africa, Malawi and Zimbabwe (Uttech, 2007). Adansi (1975) reported that it was introduced into Ghana from Togo. It is usually cultivated as a secondary crop with yam and cassava in Ghana and Nigeria (Adewale, 2010). In Ghana it is found in localized areas in the Volta Region, where it is grown by peasant farmers as a security crop (Klu *et al.*, 2001). Farmers who still hold some seed stocks, especially the white with black–eye pattern, plant it at the base of yam mounds in June or July (Amoatey *et al.*, 2000). The African yam bean flourishes and takes over the stakes from senescing yam.

African yam bean is a vigorously climbing herbaceous vine whose height can reach 1.5–3 m or more (Adewale & Dumet, 2011). The main vine/stem produces many branches which also twine strongly on available stakes (Adewale & Dumet, 2011). African yam bean flowers profusely in 100 to 150 days, producing brightly–coloured flowers, which may be pink, purple or greenish white, which mature into slightly woody pods containing about 20 to 30 seeds (Klu *et al.*, 2001). The flowers seem to exhibit self–pollination; up to six pods/peduncle results after fertilization with usually linear and long pods which turn brown when mature (Dukes, 1981). The pods which are sometimes flat or raised in a ridge–like form on both margins are usually prone to shattering; they dehisce along the dorsal and ventral sutures when dry. The seeds are sometimes round, oval, oblong, or rhomboid with different colours (Oshodi *et al.*, 1995) and size (Adewale *et al.*, 2010).

Mono–coloured seeds are white, grey, cream, light or dark brown, purple, or black. The stem of the plant produces small underground tubers of various sizes and shapes (Adewale & Dumet, 2011). The seeds are hard and diverse in colour and shape, usually spherical, ellipsoid with dark–brown, creamy–white or brownish yellow colour (Edem *et al.,* 1990). The tubers are white, watery and very similar to sweet potatoes (Kay, 1987). The seeds and tubers are the two major organs of economic importance as food; however, there are cultural and regional preferences (Adewale & Dumet, 2011). In West Africa for instance, the seeds are preferred to the tubers, while the tubers are appreciated in East and Central Africa (Potter, 1992).

The African yam bean is found in forests, open and wooded grasslands, rocky fields as well as marshy grounds, occurring both as a cultivated crop and as weed (Potter, 1992). The African yam bean grows on a wide range of soils including acid and highly leached sandy soils at altitudes, ranging from sea level to 1,950 m (Duke *et al.,* 1977). The suitability of the African yam bean for different ecologies, varied soil and climatic conditions (Schippers, 2000) suggests that it can potentially serve as an important crop for food security. This ability of the crop to survive in diverse agro ecological conditions of Africa must have aided its continual existence over times (Klu *et al.,* 2001). In Ghana and Nigeria, only a small sector of farmers are involved in its cultivation, hence, they are the holders of the crop's genetic resources (Adewale *et al.,* 2012).

African yam bean seed has higher amino acid content than that found in pigeon pea, cowpea, and bambara groundnut (Uguru & Madukaife, 2001). The African yam bean seeds were found to be rich in potassium and phosphorus (Oshodi *et al.,* 1995), calcium, iron, magnesium and zinc but low in sodium and copper (Edem *et al.,* 1990). Achinewhu and Ackah (2003) analysed raw samples of African yam bean and cowpea processed into "moimoi" (a local dish) and porridges

for chemical, functional and sensory properties analysis. Sensory evaluations showed that African yam beans do not differ significantly from cowpea. The African yam bean is also reported to have medicinal importance (Potter, 1992). African yam bean has low susceptibility to most field and storage leguminous pests (Omitogun *et al.*, 1999). African yam bean seeds contain lectins, a group of carbohydrate binding proteins, found to have strong insecticidal effect against *Callosobruchus maculatus (*cowpea weevil) (Machuka *et al.*, 2000).

Like other legumes, the African yam bean also nodulate profusely and fix atmospheric nitrogen, thereby helping to replenish soil nitrogen (Klu *et al.*, 2001). Assefa and Kleiner (1997) remarked that African yam bean has very high nitrogen–fixing ability. Although the vast genetic, economic and nutritional potentials of African yam bean have begun to be recognized, especially in reducing malnutrition among Africans, the crop has not received adequate research attention compared to major legumes such as cowpea, groundnut and soybean (Saka *et al.*, 2004). It is thus a minor legume classified as neglected, underutilized or underexploited species (Saka *et al.*, 2004). Its usefulness and attractiveness to consumers could be enhanced if it receives adequate attention and breeding efforts are geared towards its improvement. African yam bean needs to be improved genetically for farmer and consumer preferred traits like disease resistance, drought tolerance and early flowering to reduce the length of the crop cycle, reduced seed coat hardness to make for easier cooking and increased nutritional value and/or reduced anti–nutritional factors which are reported to cause discomfort and flatulence when consumed (Onyeike & Omubo–Dede, 2002). Figure 2.2 shows some pictures of the different varieties of African yam bean grains as well as the root tuber of the crop.

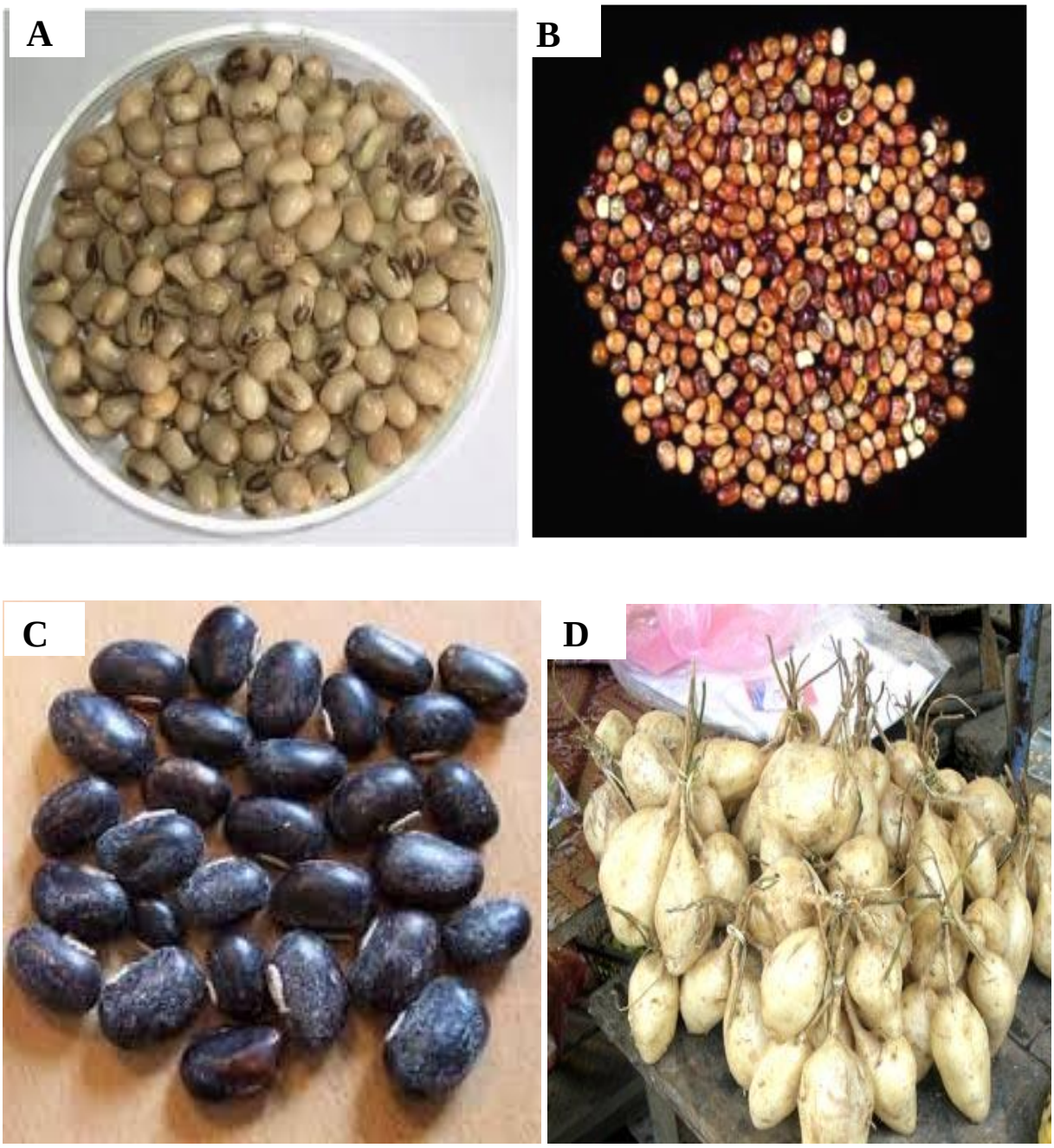


Figure 2.2: A, B, and C are samples of the different varieties of African yam bean grains, and D is a sample of the edible root tuber of the African yam bean plant.

2.4 The *Rhizobium* (Nitrogen Fixing Bacterium)

Discovery of the legume–*Rhizobium* symbiosis has been attributed to the German scientists Hellriegel and Wilfarth, who in 1886 reported that legumes bearing root nodules could use molecular (gaseous) nitrogen. In 1888, Beijerinck, a Dutch microbiologist, succeeded in isolating a bacterial strain (*Rhizobium leguminosarum*) from root nodules, and pure cultures were shown to induce nodules when inoculated to the same host plants (Franche *et al.,* 2009). These bacteria were named *Bacillus radicicola* by Beijerinck. The generic name, *Rhizobium* was formally adopted in 1962 by Buchaman (Giller, 2001). The current taxonomy of rhizobia consists of several genera in the subclass Alpha– and Beta– Proteobacteria: *Rhizobium*, *Mesorhizobium*, *Ensifer* (previously *Sinorhizobium*), *Azorhizobium*, *Methylobacterium*, *Bradyrhizobium*, *Phyllobacterium*, *Devosia* and *Ochrobactrum* are genera that belong to rhizobial Alpha–Proteobacteria (Morel *et al.*, 2012).

Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria (Somasegaran & Hoben, 1994) which elicit nodule formation on legumes (Denarie *et al.*, 1996). Rhizobia are a ubiquitous part of the soil micro–flora in a free–living state in the rhizosphere of legumes (Allen & Allen, 1981; Somasegaran & Hoben, 1994). The ability to form symbiotic relationships with members of the plant family *Fabaceae*, is a unique feature associated with bacteria belonging to the family *Rhizobiaceae* (Pepper & Upchurch, 1991). Despite the widespread distribution of leguminous crops, many soils remain void of rhizobial strains (Brockwell *et al.,* 1995).

Rhizobium is gram negative, motile, rod–shaped (approximately 0.5–0.9 µm in width and 1.2–3.0 µm in length) and heterotrophic (Somasegaran & Hoben, 1994). Root nodule bacteria generally grow under 25–30°C (optimum) and in the pH range of 6–7 (Somasegaran & Hoben,

1994). *Rhizobium* growth normally occurs under aerobic conditions. However, when fixing nitrogen, low levels of oxygen are required, to protect the enzyme *nitrogenase* (Rending & Taylor, 1989) and hence, *Rhizobium* is able to grow in microaerophilic conditions (Somasegaran & Hoben, 1994). *Rhizobium* differs from most other soil microorganisms by taking dual forms, i.e., a free–living in soils and symbiotic form inside of host legumes (Fujihara, 2009).

2.4.1 Rhizobia Diversity in Soils

Rhizobia are diverse and their classification has undergone great changes due to new phylogenetic data leading to the description of new taxa (Zakhia & de Lajudie, 2001). It is an expanding field because more and more rhizobia are being isolated and characterised, especially from the Mediterranean and tropical zones, where diversity is still poorly documented (Zakhia & Lajudie, 2001). Population diversity among rhizobia compatible with a particular legume is likely to be greatest in the center of origin of that host (Lie *et al.*, 1987). However, varied rhizobial populations can arise in symbiosis with species that are not indigenous to a particular region (Sadowsky & Graham, 1998).

Rhizobia are arguably the best known soil bacteria, and are also the most widely studied plant symbionts, because of their ability to fix atmospheric nitrogen in the root nodules of compatible leguminous plants (Hirsch, 1996). There has been considerable work over the past half–century on the size and dynamics of natural populations in different agricultural soils, and the behaviour and survival of inoculant strains (Hirsch, 1996). The advent of modern molecular techniques has resulted in proposals of differing credibility to improve upon the study of symbiotic N_2 fixation.

Rhizobial numbers vary depending on the soil type, environment and type of cultivation of the soil. Hirsch (1996) mentioned that since populations in bulk soil rarely exceed 10^6 cells g^{-1} soil, compared with an estimated 10–10^9 viable bacterial cells g^{-1} soil, they invariably comprise a

minority group, although they can reach much higher numbers in the rhizosphere. In agricultural soils, the population of rhizobia is enhanced by the presence of the cultivated legume it can nodulate (Thies *et al.*, 1992; Sadowsky & Graham, 1998). In the past, *Rhizobium* species and biovars were generally defined according to the host plants which they nodulate. However, recent advances in DNA sequence–based taxonomy have facilitated a move from this functional classification (Hirsch, 1996).

When soil samples are diluted and used to inoculate seedlings, the Most Probable Number (MPN) of infective (nodulating) cells in the original sample can be calculated from the number of plants with root nodules (Vincent, 1970). It appears that a single rhizobial cell normally initiates and colonizes each nodule, multiplying by many orders of magnitude in the nutrient-rich environment provided by the host plant, although nodules containing more than one strain have been reported (Johnston & Beringer, 1975). However, increases in rhizobia populations, are often seen under a recently planted legume crop, months before any boost in numbers precipitated by nodule senescence, indicating that the rhizobia-host interaction involves more than nodulation (Hirsch, 1996).

2.5 Methods of Studying *Rhizobium* Diversity in Soils

2.5.1 Cross Inoculation Group Concept

Based on early investigations of legume–*Rhizobium* symbiosis that largely focused on agriculturally important crops, the concept of cross–inoculation groups was introduced (Fred *et al.*, 1932). Of the cross–inoculation groups that were originally described, six were generally considered to be sufficiently unique to have species epithets assigned to them. Cultural, morphological, biochemical and serological methods have been applied to identify, characterize

and classify isolates of *Rhizobium* over the years. The legume–*Rhizobium* association is specific (each *Rhizobium* strain establishes a symbiosis with only a limited set of host plants and vice versa). Plants which are mutually compatible with the same species of rhizobia were listed in the cross–inoculation groups and were used in rhizobial taxonomy. Table 2.2 presents the various cross inoculation groups.

Table 2. 2: 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 lupini	lupinus	Lupine
Bradyrhizobium japonicum	*Glycine max*	Soybean
Cowpea Miscellany	*Vigna sp., Macroptilium spp., Lablab spp.*, Lima bean *Stylosanthes spp*., etc	

Source: Alexander (1978).

The occurrence of a wide diversity of the root nodulating bacteria in a particular soil increases the opportunity for a legume host to find compatible rhizobia. This principle is commonly used for the isolation of efficient and well adapted rhizobial strains (Florentino *et al.,* 2010).

2.5.2 Cultural and Metabolic Methods

Many previous studies or researchers have used a large range of biochemical and metabolic tests to differentiate, characterise and classify rhizobial species. 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, tolerance to pesticides, effects of temperature, growth rate in peptone broth, nitrate reduction, production of catalase, production of acid or alkali, etc. (Graham *et al.,* 1991; Somasegaran & Hoben, 1994; Mpepereki *et al.,* 1997; Odee *et al.,* 1997). Rhizobia species differ significantly in carbohydrate metabolism and substrate utilization (Somasegaran & Hoben, 1994). The utilization of carbohydrates such as glucose, sucrose, lactose, fructose, arabinose and succinate, serve as a diagnostic test in the differentiation of *Rhizobium* species (Graham *et al.,* 1991; Somasegaran & Hoben, 1994). Fast–and slow–growing rhizobia variably utilized sugars and other carbohydrates (Mpepereki *et al.,* 1997). Rhizobia nitrogen requirements can be satisfied by inorganic nitrogenous salts (e.g., nitrate and ammonium salts) and by many amino acids and short chain peptides (Zhang *et al.*, 1991).

2.5.3 The Molecular Method

Biologist have employed a variety of molecular techniques to address questions about phylogeny, evolution, and population diversity during the last decade (Boyer *et al.,* 2001). Random amplified polymorphic DNA (RAPD) developed by Williams *et al.* (1990) is still among the most widely used because the technique is simple and relatively inexpensive (Harris, 1999).

Initial investigations of Rhizobium genomic profiles concentrated on estimating diversity at conserved regions of genes like 16S rDNA genes using a technique called Amplified Ribosomal

DNA restriction analysis (ARDRA) (Pandey *et al.*, 2004). Also PCR amplification with primers specific to the repetitive gene elements; REP (Repetitive Extragenic Pallindromic), ERIC (Enterobacterial Repetitive Intergenic Consensus) and BOX (composed of the box A, B and C subunits) techniques have been used for genomic fingerprinting of bacteria (Louws *et al.*, 1994). These repetitive elements, located in the intergenic regions of many bacterial genomes, are considered to be highly conserved (Martin *et al.*, 1992) which make them useful for elucidating relationships within and between bacterial species (Chen *et al.*, 2000).

Restriction Fragment Length Polymorphism (RFLP) analysis is another technique that has been used to discriminate differences in restriction enzyme profiles of amplified DNA (Laguerre *et al.*, 1996; Odee *et al.*, 2002). Multi–locus sequence typing analysis where sequence variation is examined for multiple loci have also been used for population studies (van Berkum *et al.*, 2003).

2.5.3.1 Importance of Molecular Biology in *Rhizobium* Studies

Prior to the advent of molecular techniques, the study of rhizobia diversity involved phenotypic characterisation as well as classification (Schwinghamer & Dubman, 1980). These phenotypic methods provided important information for formalising taxonomic organisation and structure, rhizobial population structure and species diversity (Thies *et al.*, 2001). However, these phenotypic techniques were reportedly limited in many ways (Thies *et al.*, 2001). These phenotypic techniques were reported particularly to be highly labour intensive and with low discriminatory power compared to the molecular techniques (Streeter, 1994; Thies *et al.*, 2001).

Thies *et al.* (2001) reported that since the late 1980s, molecular–based methods for differentiation and characterising bacterial isolates have been available. These methods that have revolutionised the study of *Rhizobium* ecology are based upon the polymerase chain reaction

analysis (Graham, 2008). These molecular methods generally have higher discriminatory power and are faster than most phenotypic methods (Handley *et al.,* 1998). However, some of them especially the PCR–based techniques, are reported to have low reproducibility and greatly dependent on colony age, source and concentration of reagents and also on DNA purity and extraction protocol (Sato *et al.,* 1999).

The assessment of rhizobial genetic diversity is contributing both to the worldwide knowledge of diversity of soil microorganisms, and to the usefulness of rhizobial collections (Ogutcu *et al.,* 2008), and in developing long–term strategies to increase contributions of legume–*Rhizobium* nitrogen fixation to agricultural productivity.

The use of genotypic approaches, applied to isolates obtained from a large number of legume species and from different regions, have resulted in reclassification of known rhizobia species as well as in an increased number of new species (Prevost & Antoun, 2006). Thus genetic methods have usually provided additional information and made classification of rhizobia much more definitive. Recent development in PCR techniques have facilitated genetic analysis of a larger portion of the rhizobia genome and have aided classification greatly. The use of PCR and sequencing techniques have resulted in the creation of new species *(*e.g. *R. ciceri*) (Nour *et al.,* 1994), and the confirmation of the identity of some species such as *R. tropici* and *R. etli, R. Fredii* (Javis *et al.,* 1992) in the genus *Rhizobium.*

Molecular techniques have helped to develop easy and quick methods to microbial characterization including distinguishing genera, species and even strains (Schneider & de Brujin, 1996; Giongo *et al.,* 2008). Polymerase Chain Reaction (PCR) techniques and the use of primers corresponding to consensus repetitive sequences scattered in the bacteria genome,

thought as ERIC 1 & 2, BOX, etc can create highly characteristic patterns when distinguished in agarose gels, providing well separation on strain level (Adiguzel, 2006). ERIC sequences are highly protected among rhizobia genomes and they were used to select and classify different rhizobia strains in population works (Giongo *et al.*, 2008).

2.6 Assessment of Biological Nitrogen Fixation

To ensure full recognition of the benefits of the Legume–*Rhizobium* symbiosis, it is necessary to be able to assess the extent of effective nodulation and if possible nitrogen fixed. Several methods such as nodule number, nodule mass, nodule colour, total N uptake, shoot dry matter yield, percent of nitrogen derived from atmosphere using the N difference and A–value methods (Beck e*t al.*, 1993; Hardarson & Danso, 1993) have been used.

2.6.1 Nodulation and plant total N content

Evaluation of the capacity of a crop in fixing nitrogen can be assessed by assessing nodulation parameters such as nodule number, nodule mass, nodule colour, distribution and longevity of the nodule population. Nodule number and nodule mass or nodule weight per unit dry weight of the whole plant or root system are often used in trial comparisons; however, similar information can be obtained by visually scoring nodulation on a 0–5 basis taking into account nodule number, size, pigmentation and distribution (Peoples *et al.*, 1989). Effectiveness of nodules can generally be estimated by the degree of pink/red coloration of nitrogen fixing bacteroids tissue inside the nodule. The rankings as reviewed by Peoples *et al.* (1989) should be regarded as a guide only. Ideally, visual ratings should be consistently done by one person throughout an experiment, but if more than one person is involved, division of labour should be on the basis of replicates and not treatments (Peoples *et al.*, 1989). The procedure involves carefully digging–up 20 plants at

random across a crop (ensuring the root system and nodules are recovered) and scoring each plant using pre–determined classification criteria.

Regardless of the method used to measure nitrogen fixation, it is necessary to determine amounts of total plant nitrogen (Peoples *et al.,* 1989). Commonly, the wet Kjedahl digestion method is used. In this method, organic and mineral N is reduced to NH_3 in hot, concentrated sulphuric acid in the presence of a catalyst. The NH_3 is recovered by distillation and estimated by titration or calorimetrically (Bergersen, 1980).

2.7 Measurement of Biological Nitrogen Fixation

Several methods for estimating nitrogen fixation in plants are available, although all have their advantages and disadvantages. Most of these methods have been reviewed by various researchers (e.g. Rennie & Rennie, 1983; Danso, 1985). The commonly applied methods to measure the rates of nitrogen fixation in grain legumes include the acetylene reduction (AR) assay, ^{15}N assimilation technique, xylem solute technique and total nitrogen difference method. Most of these techniques have some advantages and disadvantages (Hardarson & Danso, 1993; Witty & Day, 1978). To optimize the amount of nitrogen fixed in any system it is necessary to be able to accurately estimate the magnitude of fixation under different conditions with the appropriate techniques.

2.7.1 The Total N Difference Method (TND)

The total nitrogen difference method is the simplest of all the methods (Danso *et al.,* 1992). It involves quantification of total nitrogen in both the fixing plant and the non–fixing plant (Hardarson & Danso, 1993). The technique operates upon the assumption that two genotypically similar plants that differ only in the ability to nodulate and fix nitrogen should absorb the same amount of soil N whether the nitrogen fixing species is actively or not fixing under uniform

environmental conditions. Therefore by analysing the total N in the two plants, the amount of nitrogen fixed from the atmosphere could be estimated as the difference between the two (Danso & Herridge, 1987). One problem with the total nitrogen difference method is the difficulty in getting an appropriate non–fixing plant for the reference. Also another limitation is that there is the probability that the fixing legume and non-fixing reference plant may be taking up different amounts of soil N, which could be a major source of error (Danso, 1985). Danso *et al.* (1986) recommended that soils with marginal N content should be used when applying the total nitrogen difference method in order to minimize errors. This means that errors occur when this method is employed under conditions where the available N supply is high. For example, vermiculite was used as an N–free growth medium for the study of associative N_2 fixation (Rennie & Larson, 1979), but later research showed that significant quantities of mineral N can be released from vermiculite when it is incubated under warm moist conditions (Giller *et al.*, 1986). Despite the above limitations of the TND approach, the method has the advantage of giving a measure of the total amount of N fixed over the length of the experiment and is indispensable for many laboratory based studies (Giller & Wilson, 1991).

2.7.2 Acetylene Reduction Assay (ARA)

The Acetylene Reduction assay relies on the preferential reduction of acetylene (C_2H_2) to ethylene (C_2H_4) by nitrogenase, instead of reducing nitrogen to ammonia (Montoya *et al.*, 1996). However, the AR assay has not escaped criticism during the past four decades of its use, predominantly due to potential indirect effects of C_2H_2 on microbial metabolism and the reliability of the factor used to extrapolate rates of C_2H_4 production to rates of nitrogen fixation (Giller, 1987).

The acetylene reduction assay is a useful diagnostic tool for the detection of nitrogenase activity and has been widely used in all areas of nitrogen fixation research because of its high sensitivity and simplicity. However, its reliability is now questioned for even comparative studies since acetylene reduction provides only an instantaneous measure of nitrogenase activity under the prevailing assay conditions, therefore its accuracy has always been restricted by the requirement for many repeated determinations to adjust for marked diurnal and seasonal changes in nitrogen fixing activity (Peoples *et al.,* 1989). Further errors in the field can arise due to the use of an inappropriate calibration factor to relate ethylene production to nitrogen fixation, incomplete recovery of a plant's total nodule population, nodule detachment or damage prior to assay, plant disturbance, or an acetylene–induced decline in nitrogenase activity during assay (Witty & Minchin, 1988). Although in situ procedures with flow–through gas systems have been devised to overcome some of these technical problems, the procedure has only limited application for the measurement of nitrogen fixation in the field (Peoples *et al.,* 1989).

2.7.3 ^{15}N Isotope Dilution Method

Most of the above mentioned techniques are based on indirect criteria, and cannot distinguish various sources of nitrogen in a fixing plant. The ^{15}N assimilation technique follows the net incorporation of the ^{15}N tracer into cellular biomass after a predetermined incubation period. However, its use is limited because of the high cost of the ^{15}N isotopic tracer and the expensive equipment needed for the determination of the ^{15}N/^{14}N isotopic ratios (especially in developing countries).

There are two stable isotopes of nitrogen, ^{14}N and ^{15}N. The heavy isotope, ^{15}N, occurs in atmospheric nitrogen at a constant abundance of 0.3663 atoms percentage (total variation ranges from 0.36628 to 0.36632) (Mariotti *et al.,* 1983). If the ^{15}N abundance in plant is lower than this,

an estimate of the proportions of legume N derived from each source can be made. In many cases the very small differences in natural abundance of ^{15}N between soil N and N_2 can be used, provided a suitably precise mass spectrometer is available. More usually, the difference between soil nitrogen and atmospheric nitrogen is extended by incorporation into the soil of ^{15}N enriched nitrogenous compounds (e.g. of between 5 and 95 atoms % ^{15}N).

2.8 Factors Affecting Biological Nitrogen Fixation

Grain legumes fix about 15–210 kg N ha^{-1} seasonally in Africa, and hence feature prominently in the cropping systems of traditional farmers (Dakora & Keya, 1997). However, increased exploitation of this biological N is constrained by various environmental and nutritional factors. Thus the ability of deriving full benefits from biological nitrogen fixation depends substantially on the edaphic, climatic and biotic characteristics of the soil and the environment. The amount of atmospheric N_2 fixed by legumes varies widely, depending on the management of the abiotic and biotic soil environmental components (Dakora & Keya, 1997). Several factors have been noted to affect biological nitrogen fixation in legumes. There have already been many extensive reports on the factors that affect biological nitrogen fixation in legumes (Sprent, 1976; Vincent, 1980).

2.8.1 Edaphic Factors

Edaphic factors which relate to the soil include: soil moisture, drought, soil pH, soil phosphorus levels, soil mineral nitrogen concentration, and availability of micronutrients: Iron (Fe), Molybdenum (Mo), Cobalt (Co) and Boron (B). Dakora and Keya (1997) reported that soil constraints play a major role in limiting crop yields in Africa. The intrinsically low fertility of African soils and their highly heterogeneous nature, tends to limit land use and crop choice as wel1 as nutrient–supplying capacity (Munns & Franco, 1981). Thus nutrient deficiencies involving P, K, S, Mo and Zn are quite common.

2.8.1.1 Effect of Soil Nitrogen on Nodulation and Nitrogen Fixation

The nitrogen fertility of soils is crucial for increased production in the African savanna (Dakora & Keya, 1997). About 90% of mineral N in the continent is found in living plants, with only a small fraction left in soils while the reverse ratio is more typical in temperate environments (Borlaug, 1991).

The heterogeneous nature of African soils affects legume nodulation and N_2 fixation through differences in soil N status (Peoples & Herridge, 1990). High amounts of soil N are documented to adversely affect N_2 fixation in many nodulating legumes (Peoples & Herridge, 1990). This appears to be a mechanism presumably to avoid 'wasting' photosynthate on supporting a nitrogen–fixing symbiosis when it is not necessary, owing to abundant soil available N (Hirsch, 1996). Numerous studies (Eaglesham, 1982; Ssali & Keya, 1986), etc. have shown that N_2 fixation in cowpea, soybean, common bean and groundnut is reduced in the presence of combined N. In contrast, some indigenous African legumes such as bambara groundnut and Kersting's bean exhibit nitrate tolerance (Dakora *et al.,* 1992). Thus, nodulation might be inhibited at root intracellular nitrate levels where nitrogen fixation theoretically could occur (Hirsch, 1996). Due to this inhibition phenomenon, estimations on the distribution of rhizobia based on host plant nodulation in the field may be false if soil nitrate is high, in contrast with plant infection tests with dilutions of field soil carried out *in vitro* (Hirsch, 1996).

2.8.1.2 Effect of Soil Phosphorus on Nodulation and Nitrogen Fixation

Phosphorus (P) is an essential macronutrient for plant growth and function. The P requirements of host plants for optimal growth and symbiotic nitrogen fixation processes have been assessed by determination of nodule development and functioning (Sa & Israel, 1991). The influence of P on symbiotic nitrogen fixation in leguminous plants has received considerable attention. Robson

and O'Hara (1981) concluded that P nutrition increased symbiotic nitrogen fixation in subterranean clover (*Trifolium subterraneum* L.) by stimulating host plant growth rather than by exerting specific effects on rhizobial growth or on nodule formation and function. Decreased specific–nitrogenase activity in nodules of P–deficient soybean plants was associated with decreased energy status of host plant cells of nodules. During the process of photosynthesis, ATP is generated when light energy is transformed and stored in the form of ATP for later use by the plant. Thus phosphorus has a specific involvement in symbiotic nitrogen fixation as an energy source when 16 molecules of adenosine triphosphate (ATP) are converted to adenosine diphosphate (ADP) as each molecule of N_2 is reduced to NH_3 (Anonymous, 1999; Cheng, 2008). Inadequate P restricts root growth, the process of photosynthesis, translocation of sugars, and other such functions which directly or indirectly influence N_2 fixation by legume plants (Anonymous, 1999).

In legumes, phosphorus deficiency specially affects symbiotic nitrogen fixation by limiting the growth and survival of rhizobia (O'Hara *et al.,* 1988), nodule formation (Drevon & Hartwig, 1997), nodule functioning (Tang *et al.,* 2001a) and host plant growth (Tsvetkova & Georgiev, 2003). Furthermore, phosphorus deficiency has been reported to decrease nodule mass more than host growth in soybean (Drevon & Hartwig, 1997). Nonetheless, reports on P effect on nodule formation and functioning are controversial: phosphorus deficiency may increase, decrease or not affect the nodule number per unit of shoot mass (Vadez *et al.,* 1996), as well as the specific nitrogenase activity and the amount of N fixed per nodule mass (Tang *et al.,* 2001b). Differences in the response of nodule formation and functioning to P deficiency appear to be related to legume species, soil and environmental conditions as well as the duration and severity of the stress.

2.8.1.3 Soil pH

Rhizobial survival in soil is adversely affected by extremes of pH and high salt content, although a few leguminous hosts will grow under saline or alkaline conditions (Hirsch, 1996). Very acid soils are not conducive to survival of rhizobia or their host plants, but in general there is a correlation between acid–tolerance of the host plant species and the rhizobial symbionts (Danso, 1977). This is illustrated in a survey of 60 different soil types stated by Catroux and Amarger (1992). The symbiont of acid–intolerant *lucerne, S. meliloti*, was present at 10^3 nodulating cells g^{-1} soil in alkaline soils' pH 7–8, whereas if it was not present at all in soils more acid than pH 6.0, where fewer than 10 nodulating cells g^{-1} were detected. Conversely, the symbiont of acid–tolerant lupins, *Bradyrhizobium sp*. (*Lupinus*) was present at around 10^2 nodulating cells g^{-1} soil at pH 4.5–5.5, but could not be detected in soils above pH 6 (Hirsch, 1996). In soils with pH 5.5–7.5, the biovars of *R. leguminosarum* were present at 10^4–10^5 nodulating cells g^{-1} but below or above this pH range their populations were lower than those of *Bradyrhizobium sp. (Lupinus)* or *S. meliloti*, respectively (Hirsch, 1996). Lowendorf (1980) compared the results from six different researchers and found that there is a general consensus that the most acid soil in which *B. japonicum* can survive is pH 4, for *R. leguminosarum biovars trifolii* and *viciae* the limit is pH 4.7, and for *S. meliloti*, pH 5. *Rhizobium leguminosarum bv. trifolii* in laboratory cultures grew more slowly in media at pH 4.5 than in those at pH 5.5 (Wood & Cooper, 1988).

2.8.1.4 Availability of Heavy Metals

Consequence of low soil pH is the increased solubility and hence the bioavailability of some metal ions. Heavy metals have been shown to be detrimental to rhizobial survival (Brady *et al.*, 1990). In plots at Woburn which had received sewage sludge over a long period, resulting in heavy–metal contamination, the population of *R. leguminosarum bv. trifolii* was reduced

dramatically, in addition, only one strain able to nodulate clovers was found, and it was ineffective on white clover (Hirsch *et al.,* 1993). This strain was shown to be more resistant to Cd, Cu, Ni and Zn than were diverse isolates from adjacent control plots (Chaudri *et al.*, 1992). However, *S. meliloti* inoculant introduced into metal–contaminated soil survived better than *R. leguminosarum bv. trifolii* and *R. loti* (Giller *et al.,* 1993), indicating that rhizobial species might vary in their sensitivity to heavy–metal contamination. Similarly, Chaudri *et al.* (1993) found increasing levels of Zn, Cu, Ni and Cd, in combination with different soil pH and organic carbon levels, reduced *R. leguminosarum bv. trifolii* numbers in German soils, and concluded that Zn was the metal most toxic to rhizobia.

2.8.1.5 Soil moisture.

There are many reports on the effects of desiccation on rhizobial survival; in some cases rhizobia survive for many years following desiccation; in others, especially where it is combined with elevated temperatures, numbers drop. However, long–term studies on survival of *B. japonicum* in Italian soils indicate that moisture content of soil is the most obvious factor affecting population level, waterlogging leading to a dramatic decrease (Beringer & Kay, 1993). Similarly, flooding on *R. leguminosarum* populations, although there are contradictory reports in the literature that waterlogging or flooding has no effect on rhizobial numbers. It seems likely that there are complex interactions between soil type and climatic conditions, rhizobial species studied and method of assessment.

2.8.1.6 Drought

Water stress reduces N_2 fixation, nodule respiration, and cytosolic protein content of the nodule (Bordeleau & Prevost, 1994). Soil moisture deficiency reduces the rhizobial population density in the soil (Tate, 1995), rhizobial migration, nodule number and size (Williams & De Mallorea,

1984) and has a pronounced effect on N_2 fixation since nodule initiation, growth and activity are more sensitive to water stress than are generally root and shoot metabolism (Albrecht *et al.,* 1994). In dry soil, infection of root hairs is restricted because they become short, stubby and inadequate for rhizobial infection (Lie, 1981). Eaglesham and Ayanaba (1984) stated that excess water is detrimental to N_2 fixation because it lowers oxygen diffusion for nodule functions and may lead to a build–up of CO_2 (which inhibits nodule formation) and ethylene (which restricts nodulation at lower concentration).

A reduction in root nodule flux has been associated with the inhibition of nitrogen fixation under drought (Sanginga *et al.*, 2000a). The legume–*Rhizobium* symbiosis is strongly related to the physiological state of the host plant (Cochard *et al.,* 2002). Thus a limiting factor like drought might impose a limitation on the vigour of the host legume (Cavender–Bares & Bazzaz, 2000). Consequently, nodulation fails to occur through loss of infection sites due to changes in the morphology of effectible root hairs (Eaglesham & Ayanaba, 1984). Schulze *et al*. (1991) found that in the desert and savannah areas of Namibia, symbiotic acacias spend more water per unit carbon assimilated than non–legumes. The waters spent on carbon assimilation probably represents the cost of supplying extra carbohydrate for nitrogen fixation (Danso *et al.*, 1992) thus suggesting that in the dry savannah and Sahelian zones of Africa water cost of nitrogen fixation can itself constrain the symbiotic process and reduce biological N yield.

2.8.1.7 Salinity

Salinity has been identified as one of the factors that affect the growth and activity of nitrogen fixing legumes in arid and semi–arid climates (Zahran, 2001). Tate (1995) reported that increased salt concentration may have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress. Effects of salt stress include inhibition

of initial step of rhizobia–legume symbiosis such as curling of root hairs, reduction in nodule respiration (Delgado *et al.,* 1994; Walsh, 1995), reduction in production of leghaemoglobin and reduction in photosynthetic activities and growth of the plant (Delgado *et al.,* 1994). In contrast to their host legumes, some rhizobia can survive in the presence of high level of salt both in the culture and soil (Bordeleau & Prevost, 1994). Many species of bacteria adapt to saline conditions by intracellular accumulation of low molecular weight organic substances (Csonke & Hanson, 1991).

2.8.2 Environmental (Climatic) Factors

Two important climatic determinants affecting biological nitrogen fixation are temperature and light.

2.8.2.1 Temperature.

Various stages of nodulation and nitrogen fixation of herbaceous legumes are affected by soil temperature (Sprent, 1979). Soil temperature affect the survival and thus the population of *Rhizobium* or *Bradyrhizobium* in soil, with lower temperatures being more favourable than high temperatures (Danso, 1977). High soil temperature can prevent nodulation, and even if nodulation does occur, these temperatures could inhibit the process of nitrogen fixation in legumes (Day *et al.,* 1978). The much lower optimum temperature for symbiotic plants (25°C) than that reported for nitrogenase activity of detached nodules (40°C) (Bond & Macintosh, 1975), suggests that the temperature effect on plant growth was a major determinant in the symbiotic system. A study conducted on *Casuarina species* by Reddell *et al.* (1985) suggested that such temperature requirements for significant nodulation and nitrogen fixation affects the natural distribution of *Casuarina species. Casuarina spp*. occurs in soils with low N and seems to be dependent almost entirely on BNF. Rhizobia cannot survive under extreme soil

temperatures. Their response to heat appears to be strain–dependent and is affected by soil type, moisture content etc.

2.8.3 Biotic Factors

Among the biotic factors affecting biological nitrogen fixation, the major ones include; absence/presence of the required *Rhizobium* species, plant physiological defects (e.g. excessive defoliation of host plant), crop competition (cropping system), insects and pest attack, etc.

2.8.3.1 Presence of Compatible *Rhizobium*

An important pre–requisite for effective nodulation and nitrogen fixation is the presence of an effective and competitive strain in the soil (Danso & Owiredu, 1988). The survival and persistence of an adequate number of effective *Rhizobium* in soils independent of a hosts` stimulant is essential to ensure nodulation of young seedlings (Dudeja & Khurana, 1989).

One factor causing reduction in rhizobial populations could be increased predation by protozoa (Habte & Alexander, 1977). Protozoa will be more mobile as moisture content increases (Vargas & Hattori, 1986) and rhizobia more available for predation (Postman, *et al.*, 1989).

Soils with high clay content appear to be more conducive to rhizobial survival than are low clay soils (Danso, 1977). This might be caused by the higher protection offered by clay against adverse conditions such as drought and high temperatures (Danso, 1977). This would reduce the size of the neck of the pores, effectively protecting the bacteria which inhabit them from protozoan predation (Heijnen & van Veen, 1991).

Other biotic factors that could reduce rhizobial numbers include bacteriophages, bacterial parasites such as *Bdellovibrio,* antibiotics produced by other soil microbes and bacteriocins produced by other rhizobia (Hirsch, 1996). Although *Bdellovibrio* are present in many soils, and

they can be shown to parasitize rhizobia in dense liquid culture, they did not significantly reduce rhizobial numbers, and appropriate conditions for *Bdellovibrio* infection are unlikely to occur in field soils (Keya & Alexander, 1975). Studies of bacteriocin production of more than 100 *R. leguminosarum* isolates showed that mutual antagonism was frequent (Hirsch, 1979).

2.8.3.2 Crop Effects

There are numerous reports of rhizobial population fluctuations in response to different crops, and over time (Hirsch, 1996). Observations differ widely, depending upon plants, rhizobial species, soil type, climate and agronomic practices (Hirsch, 1996). There are studies of the response of indigenous populations to host plants, other crops and different soil treatments; and some information on natural colonization of soils by rhizobia, where inoculation is not involved. Counts of rhizobia are usually higher in the host rhizosphere, and following soil cultivation procedures which disperse the bacteria released from senescent nodules of a well–nodulated crop. This has been observed in *B. japonicum;* populations increased in the presence of soya beans (Weaver *et al.,* 1972) and again in the following winter, the latter increase was attributed to release from nodules (Kuykendall *et al.,* 1982). Soils with no record of ever having grown a particular legume can still contain significant numbers of the compatible rhizobia (Hirsch, 1996). These might reflect ancient vegetation patterns, or alternatively rhizobia may have invaded from nearby sites.

2.8.3.3 Cropping System

Nitrogen fixation in legumes is also influenced by the cropping system used. An evaluation of traditional cropping systems in Africa shows that crop rotation involving legume and cereal monocultures is by far more sustainable than intercropping, the most dominant cultural practice in the continent (Dakora & Keya, 1997). Eaglesham *et al.* (1981) in monitoring root nodule

formation in cropping systems involving maize and four grain legumes (cowpea, Bambara groundnut, Kersting's bean and groundnut) discovered changes in nodule abundance dynamics in response to the cropping pattern. Cowpea and groundnut showed decreased nodule numbers with intercropping. However, Bambara groundnut and Kersting's bean formed more nodules when intercropped than when sole cropped. The fact that nitrogen fixation in cowpea was unaffected by intercropping with maize in Nigeria (Eaglesham *et al.,* 1981) might suggest cultivar differences in symbiotic response to cropping systems. It is clear that introduced species like soybean show reduced nitrogen fixation under mixed cropping (Nambiar *et al.,* 1982). With some species, symbiotic activity in intercropped legume can be stimulated if the associated cereal in the mixture exerts intense competition for soil N. In that case the legume is forced to rely more on symbiosis for its N nutrition (Danso *et al.,* 1987). Danso *et al.* (1987) showed that by increasing the population of the cereal in the mixture, this increased the proportion of the nitrogen derived from the symbiotic fixation. Tree legumes can also fix about 43–581 kg N ha yr^{-1} making their leaf prunings an important component of sustainability in agroforestry and alley cropping systems.

Rotational cropping involving legumes and cereals have been found to be a more sustainable system of increasing food production (Dakora & Keya, 1997). The ability of African yam bean to form effective nodules with a wide range of rhizobial strains makes it a suitable for sustainable development purposes in most cropping systems. The use of African yam bean and other legumes as cover crops, can increase the efficiency of fertilizer utilization and the amount of organic matter for maintenance of high soil productivity (Dakora & Keya, 1997). Although legumes of all categories contribute to nitrogen fertility in various cropping systems, the extent

of their nodulation could be constrained by a range of factors amongst which is the environment relating to both the host plant and its microsymbiont (Dakora & Keya, 1997).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Soil Characterization and Description

Four soil types namely, Toje, Adenta, Haatso and Akuse series' (Local names), located in the coastal savanna zone of Ghana were used for the experiment. Toje, Adenta and Haatso series' form part of the soil series along a catena down the Legon hill.

The soils had ustic moisture and isohyperthermic temperature regimes (Eze *et al.*, 2010). Total annual rainfall is about 900 mm (Dowuona *et al.*, 2012) and is bimodally distributed. The rainfall regime consists of a main wet season which falls between March to June/July, and a minor rainy season around September to October. This area is further characterized by a distinct dry season between December and February. Relative humidity ranges between 59% in the afternoon and 93% at night with a mean annual temperature of about 28°C (Dowuona *et al.*, 2012). The vegetation is grassland with substantial amounts of clumps and thickets. Quartzite and quartzitic sandstones are the underlying geological formations with evidence of fractures and groundwater reserves typical of the hydrogeology of the Togo Structural Units of Ghana (Dapaah–Siakwan & Gyau–Boakye, 2000). The soils were sampled to a depth of 0–20 cm from an uncultivated field at the University of Ghana Experimental farm at Legon.

Adenta series has been classified as Savannah Ochrosol (Brammer, 1962), Typic Kandiustalf (Eze *et al.*, 2010) according to USDA classification system and as Haplic Luvisol by Soil Research Institute (1999) according to FAO–UNESCO classification system (1990). The Adenta soil is a well–drained soil series which occurs extensively on gentle middle slope with site gradient of 1–2% on a catena along the Legon hill. The Adenta series has a profile which

consists of light yellowish brown sandy clay loam topsoil with friable granular structure and has an altitude of 82 meters above sea level (Eze *et al.*, 2010).

Toje series is classified as Rhodic Kandiustalf and Haatso as Kandic Paleustalf according to USDA classification system (Eze *et al.*, 2010). These soil series' have an altitude of 108 metres and 66 meters respectively above sea level (Eze *et al.*, 2010). Toje series is located at the lower upper slope with slope less than 1% whiles Haatso series is located at the lower slope positions. Haatso series at the foot of the catena is formed on colluvial and alluvial deposits underlain by iron-stained quartzite or sandstone at 0.6–0.9 meters (Brammer, 1967). The topsoil of Toje series is dark reddish brown grading to red with depth. The structure is granular at the surface, with friable consistency; this changes to weak, fine sub–angular blocky below the surface.

The Akuse soil series is classified as a Calcic Vertisol (Eze, 2008), according to USDA classification system. The parent material is garnetiferous hornblende gneiss and it occurs on upper-middle slope with an altitude of 18 m above sea level (Yangyuoru *et al.*, 2006). The relative humidity at night time to the early hours of the day ranges from 70 to 100% throughout the year (Yangyuoru *et al.*, 2006). The afternoon relative humidity falls to a range of 20–65% during the year. The texture of the soils is mainly sandy to silty clay.

3.2 Soil Sampling and Analysis

Three representative surface soil samples from the plough layer (0–20 cm) of each of four soil series (Adenta, Akuse, Haatso and Toje) were sampled in bulk. Prior to potting and crop establishment, soil samples were crushed and sieved through a 2 mm sieve to obtain the fine earth fraction.

3.2.1 Chemical Analysis

3.2.1.1 Soil pH

Soil pH was determined in both distilled water and 0.01M calcium chloride using the electrometric method by Peech (1965). Twenty grammes (20 g) of 2 mm sieved soil sample were weighed into a 50 mL beaker and 20 mL of distilled water was added (1:1, soil to water ratio). The soil–water suspension was stirred vigorously for 30 minutes and allowed to stand for about 20 minutes, most of the particles to settle. The pH meter (Hanna H19017 microprocessor pH meter) was standardized with solutions at pH of 4 and 7. The pH meter electrode was partly immersed gently into the supernatant and the reading on the pH meter was recorded as pH in water (pH_W). The pH meter electrode was rinsed in–between pH readings with distilled water. The procedure was repeated with 20 g of soil and 40 mL of 0.01M $CaCl_2$ solution (1:2, soil to salt solution ratio). The pH reading was recorded as the soil pH in salt (pH_S)

3.2.1.2 Available Phosphorus Determination

Available phosphorus was determined using Bray 1 method (Bray & Kurtz, 1945). Five grammes (5 g) of air–dried and sieved soil were weighed into extraction bottle and 50 mL of Bray1 **(**mixture of 0.03M NH_4F and 0.025M HCl**)** solution was added and shaken on a mechanical shaker for three minutes. The suspension was centrifuged and filtered through No. 42 Whatman filter paper into a 100 mL volumetric flask and made up to the volume. Five millilitres (5 mL) aliquot of the filtrate was taken into a 50 mL volumetric flask in duplicate. The pH was adjusted using P–nitrophenol indicator and neutralized with a few drops of 4M NH_4OH until the solution turned yellow. Eight millilitres (8 mL) of reagent B; prepared by dissolving 1.056 g of ascorbic acid in 200 mL of reagent A [12 g of ammonium molybdate + 0.2998 g of antimony potassium tartrate dissolved in 250 mL of distilled water]. The dissolved reagents were added to

1000 mL of 2.5M H_2SO_4, mixed thoroughly and made to the 2000 mL mark (Watanabe & Olsen, 1965). The solutions were mixed thoroughly by shaking and allowed to stand for 15 minutes for the colour to stabilize (the colour changed to blue of different intensity depending on the concentration of phosphorus available in the soil). A blank was prepared with distilled water and 8 mL of reagent B. The spectrophotometer was calibrated using 25 mg L^{-1} standard P solution prepared in the same manner as above. The intensity of the blue colour was measured using the Philips PU 8620 spectrophotometer at a wavelength of 712 nm. The available P concentration in the soil sample was read and calculated using the spectrophotometer reading as follows:

$$\text{P}\ (\%) = \frac{\text{R} \times \text{Vol. of extract}}{\text{Vol. of aliquot} \times \text{Weight of soil} \times 10^6} \times 100 \qquad [3.1]$$

Where R = Spectrophotometer reading (mg L^{-1}).

3.2.1.3 Total Nitrogen Determination

Half of a gram (0.5 g) of soil was weighed into a 250 mL Kjedahl flask and a tablet of digestion accelerator, selenium catalyst, was added followed by 5 mL concentrated sulphuric acid. The mixture was digested until the digest became clear. The flask was then cooled and its content transferred into a 100 mL volumetric flask with distilled water and quantitatively made up to volume. A 5 mL aliquot of the digest was taken into a Markhan distillation apparatus. Five millilitres (5 mL) of 40% NaOH solution was added to the aliquot and the mixture distilled. The distillate was collected in 5 mL of 2% boric acid solution. Three drops of a mixed indicator containing methyl red and methylene blue were added to the distillate in a 50 mL Erlenmeyer flask and then titrated against 0.01M HCl acid solution (Bremner, 1965). The % nitrogen was calculated as:

$$\text{N}(\%) = \frac{\text{Titre} \times \text{Molarity of HCl} \times \text{Vol. of extract} \times 0.014}{\text{Vol. of aliquot} \times \text{Weight of soil}} \times 100 \qquad [3.2]$$

Where 0.014 = milliequivalent weight of nitrogen

3.2.1.4 Soil Organic Carbon Determination

Organic carbon was determined by the wet combustion method of Walkley and Black (1934). A half gram (0.5 g) of air–dried and sieved soil sample was weighed into a conical flask. Ten millilitres (10 mL) of potassium dichromate and 20 mL of concentrated sulphuric acid (H_2SO_4) solutions were added. The flask was swirled to ensure that all the soil particles were in contact with the solution to allow for digestion. The content of the flask was allowed to settle for 30 minutes to ensure an efficient combustion after which 200 mL of distilled water was added. Ten millilitres (10 mL) of orthophosphoric acid and barium phenylalanine sulphate (an indicator), were added to the solution. The solution was then titrated against ferrous ammonium sulphate solution until colour changed. The titre value was used to calculate the percent carbon (%C) as:

$$\%\mathrm{SOC} = \frac{[10 - (\mathrm{XN})] \times 0.003 \times 1.33}{\mathrm{W}} \times 100 \qquad [3.3]$$

Where X = titre value of the ferrous ammonium sulphate.

N = molarity of the ferrous ammonium sulphate.

W = weight of the soil sample, **1.33** = correction factor (*f*) assuming more than 75% recovery of organic carbon by wet combustion procedure, **0.003** = Milliequivalent weight of carbon (g).

3.2.1.5 Cation Exchange Capacity (CEC)

Ten grammes (10 g) of soil was weighed into an extraction bottle and 100 mL of 1N NH_4OAc solution at pH 7 was added and agitated for 30 minutes. The suspension was filtered through a Number 42 Whatman filter paper. The non–adsorbed ammonium ions were then washed off with methanol and the ammonium saturated soil was leached with acidified 1M KCl solution. A 10

mL aliquot of the decanted leachate (filtrate) was pipetted into a Kjeldahl flask and the NH_3 was liberated into 5 mL of 2% boric acid solution as described in Section 3.2.1.3 for total nitrogen determination. The ammonium ion concentration in the KCl filtrate was determined and the CEC of the soil in $cmol_c\ kg^{-1}$ soil estimated.

3.2.2 Physical Analysis

3.2.2.1 Soil Bulk Density

The bulk density was determined using the core method by Blake (1965). Soil samples were taken at each site using cylindrical metal core samplers (5 cm diameter x 5 cm height) after clearing the vegetation cover. The core sampler was driven into the soil far enough to fill the volume of the core. The filled core sampler was carefully removed and both ends trimmed. Three core samples of each soil were carefully transferred into moisture cans, of known weight (W_1) and dried at 105^0 C for two days (48 hours). The weight of dried soil plus moisture can was determined and recorded as W_2. The bulk density of soil was determined as the ratio of mass of dried soil to the volume of soil. It is assumed that the volume of the soil sample was equal to the volume of the core sampler (volume of a cylinder); which is calculated as: $V\ (cm^{-3}) = \pi r^2 h$, **r** is the radius of the core sampler and **h** is the vertical height of the core sampler. Thus the bulk density (ρb) of the soil is calculated as:

$$\text{Bulk Density, } \rho b\ (g cm^{-3}) = \frac{W_2 - W_1}{V} \qquad [3.4]$$

3.2.2.2 Particle Size Analysis

The particle size analysis was determined using the Bouyoucos Hydrometer method modified by Day (1965). Forty grammes (40 g) of air–dried and 2 mm sieved soil sample were weighed into a dispersing bottle and 100 mL of 5% Calgon (Sodium Hexametaphosphate) solution was added

and shaken for two hours. The suspension was thereafter transferred into a 1 L graduated sedimentation cylinder and was brought to the mark by adding distilled water. A plunger was used to stir the suspension vigorously by moving it in and out of the suspension several times. The first and second hydrometer readings were taken at 5 minutes and 5 hours after the plunger was removed, a hydrometer was gently lowered into the content and the scale at the top of the meniscus was noted and recorded. These readings represented the silt plus clay fraction and the clay fraction, respectively. The suspension was poured from the sedimentation cylinder into a 47–micron (µm) sieve and effluent discarded. Tap water was run through the sediment on the sieve to wash off most of the fine material. The sand particles left in the sieve were transferred into a moisture can, oven dried for 24 hours, cooled in a desiccator and the dry weight determined. The percentage of sand, silt and clay fractions were calculated based on the oven dry weight of the soil sample taken as follows:

$$\%(\text{Clay} + \text{Silt}) = \frac{\text{Hydrometer reading at 5mins}}{\text{weight of soil (g)}} \times 100 \qquad [3.5]$$

$$\text{Clay}\ (\%) = \frac{\text{Hydrometer reading at 5 hours}}{\text{weight of soil (g)}} \times 100 \qquad [3.6]$$

$$\text{Silt}\ (\%) = \%(\text{Clay} + \text{Silt}) - \text{Clay}\ (\%) \qquad [3.7]$$

$$\text{Sand}\ (\%) = \frac{\text{Weight of oven dry sand retained on the 47µm seive}}{\text{weight of soil (g)}} \times 100 \qquad [3.8]$$

The textural classes were determined using the texture triangle.

3.3 Estimation of the populations of rhizobia in the soils using the Most Probable Number plant infection technique.

The estimated population of rhizobia in the soils capable of nodulating the test legumes was enumerated by the Most Probable Number (MPN) plant infection assay (Vincent, 1970) using a modified Leonard jar assembly. The assembly was composed of a plastic cup tapered to a similar cup at the bottom. The cup containing the rooting medium (acid–wash sand) was inserted into a similar plastic cup containing the nutrient solution. Hundred millilitres (100 mL) of N–free nutrient solution (appendix B) was added to the lower container of each Leonard jar assembly. The rooting medium was irrigated with a cotton wick connecting the upper and the lower units, after which the whole assembly was autoclaved to get rid of microorganisms. The legume seeds were surface sterilized in 70% alcohol for 3 minutes and rinsed thoroughly in several changes of sterilized distilled water (Somasegaran & Hoben, 1994). The seeds were pre–germinated on moist filter paper in petri dishes until the radicles were about 2 cm long. A pair of sterilized forceps was used to prepare holes in the rooting medium in each assembly. Seeds with radicle length of about 1–2 cm were picked up with the sterile pair of forceps and placed one per hole, with the radicle facing downwards. The holes were deep enough to accommodate pre–germinated seeds 0.5 cm below the surface. Two seedlings were transplanted into each sterilized plastic cup containing autoclaved acid–washed sand. The assembly were randomly arranged in a greenhouse. One millilitre (1 mL) of ten–fold dilutions (i.e. ten grams of the soil sample was diluted in 90 mL of sterile distilled water) of soil suspension was used to inoculate each jar (Somasegaran & Hoben, 1994) with four replicates of each dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) step. The plants were supplied with N–free nutrient solution when necessary. The plants were assessed for the presence of nodules 6 weeks after planting and the most probable number of

rhizobia cells per gram of soil per legume calculated (Vincent, 1970). At harvest, the total number of nodulated units was obtained by summing up the nodulated units at each dilution level. Uninoculated controls were used to check for sterile conditions. The MPN was calculated using the formula:

$$MPN = \frac{m \times d}{v} \qquad [3.9]$$

Where: **m** is the most likely number from MPN table,

d is the lowest dilution in the series and

v is the aliquot used for inoculation (Somasegaran & Hoben, 1994).

3.4 The Greenhouse Experiment

3.4.1 Nodulation Potential of African Yam Bean

The ability of African yam bean to nodulate in the various soils was evaluated in plastic pots with top internal diameter of 16.3 cm and bottom interior diameter of 11.2 cm containing 2 kg of each soil type. The pots were perforated at the bottom to allow for drainage of excess water. They were placed in plastic plates to collect any excess drained water. Four surface sterilised and pre–germinated seeds were planted in each pot, which were later thinned to two plants. There were three replicate per each soil. The pots were randomly arranged, kept at the greenhouse and watered regularly. The plants were harvested six weeks after planting and assessed for nodulation.

3.4.2 Nitrogen and Phosphorus Response Experiment

The response of African yam bean to nitrogen and phosphorus fertilizers was evaluated. The experiment was conducted in plastic pots (14.5 cm high, 16.3 cm wide at the top and 11.2 cm

wide at the base). The pots were filled with 2 kg each of the various soils that have been sieved through 2 mm sieve. Four seeds were planted in each pot and later thinned to two after germination. Isotope dilution was done with ^{15}N labelled $(NH_4)_2SO_4$ and ordinary $(NH_4)_2SO_4$ fertilizer applied at 0, 50, 100, and 200 kg N ha^{-1}. Phosphorus was applied in the form of triple superphosphate (TSP) at 0, 40, 80 and 120 kg P ha^{-1}. The test crops used were African yam bean (*Sphenostylis stenocarpa*, cream colour) and maize (*Zea maiz* Cultivar Obaatampa used as a reference plant). The nitrogen fertilizer was applied in two splits; 5 days after germination and three weeks after germination. The experiment was arranged as randomised complete design with four replicates. The plants were kept in the greenhouse and watered daily. The plants were harvested at seven weeks after planting by cutting the stem at the soil surface level. The roots were carefully washed in slow running water and observed nodules were collected and counted. The vegetative parts were oven–dried at 70^oC until a constant dry weight was obtained. The shoot dry weight was obtained and the plant samples were milled for chemical analysis.

3.5 Chemical Analysis of Plant Material

3.5.1 Digestion of Plant Material

One–tenth of a gram (0.1 g) of the milled plant sample was weighed into a 25 mL flask and 5 mL of concentrated sulphuric acid (H_2SO_4) was added. The flasks were swirled intermittently to facilitate contact between the sample and the sulphuric acid. The flask was allowed to stand overnight for the sulphuric acid to dissolve the plant sample entirely. Thereafter, each solution was heated for some time after which Hydrogen Peroxide (H_2O_2) was added until the solution became clear. Distilled water was added and the solutions were allowed to stand overnight to cool and settle after which they were decanted into a 100 mL flask. Aliquots of the digest or extract were taken for nitrogen and phosphorus determination.

3.5.2 Determination of Plant Total Nitrogen

A 5 mL aliquot of the digest (described in Section 3.5.1 above) was taken into a Markhan distillation apparatus. Five millilitres (5 mL) of 40% NaOH solution was added to the aliquot and the mixture distilled. The distillate was collected in 5 mL of 2% boric acid. Three drops of a mixed indicator containing methyl red and methylene blue were added to the distillate in a 50 mL Erlenmeyer flask and then titrated against 0.01M HCl acid solution (Bremner, 1965). The percentage nitrogen was calculated as:

$$\mathrm{N}(\%) = \frac{\text{Titre} \times \text{Molarity of HCl} \times \text{Vol. of extract} \times 0.014}{\text{Vol. of aliquot} \times \text{Weight of plant sample}} \times 100 \qquad [3.10]$$

Where 0.014 = milliequivalent weight of nitrogen

The shoot total N (mg/plant) was derived from the shoot biomass (shoot dry matter yield: **SDW**) and the plant N content (% N) as:

$$\text{Shoot Total N } (mg/plant) = SDW\ (g\ /plant) \times \frac{\text{shoot \% N}}{100} \times 1000 \qquad [3.11]$$

The percentage of ^{15}N atom excess in the milled plant samples (African yam bean and maize) were analysed in Belgium by KULEUVEN Research and Development Laboratory. The percentage of N derived from the atmosphere (%Ndfa) by the legume was calculated using the formula by IAEA (2001):

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom \% } {}^{15}N \text{ excess in Nitrogen fixing plant}}{\text{atom \% } {}^{15}N \text{ excess in reference plant}}\right) \times 100 \qquad [3.12]$$

$$\text{Total N fixed } (mg/plant) = \frac{\text{\% Ndfa}}{100} \times Shoot\ total\ N\ \ (mg/plant) \qquad [3.13]$$

3.5.3 Determination of Plant Total Phosphorus

Five millilitres (5 mL) aliquot of the digest (described in section 3.5.1 above) was taken into a 50 mL volumetric flask in duplicates. The pH was adjusted using P–nitrophenol indicator and neutralized with a few drops of 4M NH_4OH until the solution turned yellow. Eight millilitres (8 mL) of reagent B; prepared by dissolving 1.056 g of ascorbic acid in 200 mL of reagent A [12 g of ammonium molybdate + 0.2998 g of antimony potassium tartrate dissolved in 250 mL of distilled water]. The dissolved reagents were added to 1000 mL of 2.5M H_2SO_4 (148 mL of conc. H_2SO_4), mixed thoroughly and made to the 2000 mL mark (Watanabe & Olsen, 1965). The solutions were mixed thoroughly by shaking and allowing to stand for 15 minutes for the colour to stabilize. The colour changed to blue of different shades depending on the concentration of phosphorus available in the soil. A blank was prepared with distilled water and 8 mL of reagent B. The spectrophotometer was calibrated using 25 mg L^{-1} standard P solution prepared in the same manner as above. The intensity of the blue colour was measured using the Philips PU 8620 spectrophotometer at a wavelength of 712 nm. The P concentration in the plant tissue was calculated as:

$$\text{P}\ (\%) = \frac{\text{R} \times \text{Vol. of extract}}{\text{Vol. of aliquot} \times \text{Weight of soil} \times 10^6} \times 100 \qquad [3.14]$$

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

The shoot total P (P uptake) was derived from the shoot biomass (dry matter yield) and the shoot P content (% P) as:

$$\text{Shoot Total P}\ (mg/plant) = Shoot\ dry\ weight\ (g) \times \frac{\text{shoot \% P}}{100} \times 1000 \qquad [3.15\ ]$$

The phosphorus utilization efficiency (PUE) was also calculated for the different rates of P using the formula of Elliott and White (1994):

$$\text{PUE } (g\ SDMY/\ mg\ P\ taken\ up) = \frac{SDMY\ (g)}{P\ Uptake\ \ (mg/plant)} \qquad [3.16\ ]$$

SDMY = shoot dry matter yield

3.6 Isolation of Rhizobia

Representative nodules were sampled from the initial screening study and *Rhizobium spp* were isolated for further studies. The nodules were first surface sterilized with 70% alcohol for 3 minutes and then with 0.1% mercuric chloride (HgCl) for another 3 minutes and rinsed with several washes of sterile distilled water (Somasegaran & Hoben, 1994). The nodules were each crushed in a drop of sterile distilled water in a petri dish with a sterile rod. A loopful of the suspension was then streaked on yeast extract mannitol (YEM) agar (Appendix A) plates and incubated at 28^{o}C. A total of 30 *Rhizobium* isolates were obtained.

3.7 Cross Inoculation Studies (Host Range Analysis)

The ability of each isolate to nodulate different leguminous plant species was assessed using the cross inoculation concept. The legumes used as host plants included both grain and forage legumes. They included cowpea (*Vigna unguiculata*), yardlong bean (*Vigna sesquipedalis*), groundnut (*Arachis hypogea),* lima bean (*Phaseolus lunatus*), African yam bean (*Sphenostylis stenocarpa),* bambara groundnut *(Voandzeia subterranean),* crotalaria (*Crotalaria spp*) and Pueraria. Seeds of the selected host legumes were surface–sterilized and germinated in sterile Petri dishes containing moist sterile filter paper. The germinated seedlings were transplanted at two seedlings per growth pouch. Growth pouches containing sterile nitrogen free nutrient

solution were inoculated with 1 mL of 4 days old YEM broth culture (containing about 10^9 cells mL^{-1}) of the corresponding rhizobia isolate (Somasegaran & Hoben, 1994). Uninoculated pouches served as control treatment. The pouches were randomly arranged and kept in the greenhouse. Seedlings were watered with sterile N–free nutrient solution whenever necessary. Nodule formation was assessed five weeks after inoculation.

3.8 Molecular Characterisation

Thirty (30) rhizobial isolates were randomly isolated from nodules of African yam bean (*Sphenostylis stenocarpa*) grown in four Ghanaian soils. The nodules were selected mostly based on the presence of the pink/red colouration in the nodule and the size of the nodule. Total genomic DNA isolated from African yam bean rhizobia was analysed by Polymerase Chain Reaction (PCR) technique using an arbitrary primer (RPO4: 5′–GGAAGTCGCC–3′), a 20–base oligonucleotide primer corresponding to a conserved *nif* gene promoter region (RPO1: 5′–TGCGGCTGGATCACCTCCTT–3′) (Richardson *et al.*, 1995). The Amplified Ribosomal DNA Restriction Analysis (ARDRA) of the 16S rDNA gene was done. Also, the Internal Transcribed Spacer (ITS) was amplified and the size fractionated.

3.8.1 Genomic DNA Extraction

Genomic DNA was extracted by using a Qiagen DNeasy kit. The rhizobial isolates were grown in YEM broth in an incubator shaker at 150 rpm and 28°C for 72 hours. About 1–2 mL of rhizobial cultures were centrifuged at 8500×g for 10 minutes and the supernatant discarded. The cell pellets were harvested and resuspended in 400 µL of preheated AP1 buffer (lysis buffer), vortexed and incubated at 65^{o}C for 15 minutes in a water bath. The tubes were inverted every 5 minutes during the incubation period. The cell pellets were added with 130 µL of AP2 buffer (precipitation buffer), vortexed and incubated on ice for 5 minutes. The lysate was transferred

into a DNA Mini spin column placed in a 2 mL collection tube and centrifuged for 5 minutes at 20,000×g (14,000 rpm). The flow–through fraction was transferred into a new tube and 1.5 volumes of AP3/E was added and mixed by pipetting. An aliquot of 650 µL of the mixture was transferred into a DNeasy Mini spin column in a 2 mL collection tube and centrifuged for 1 min at 6000×g, after which the flow–through was discarded. Thereafter, 500 µL of AW buffer was added and centrifuged for 2 minutes at 20,000×g. The spin column was carefully transferred into a new 2 mL micro centrifuge tube, and 100 µL of AE buffer was added for elution. The mixture was incubated at room temperature for 5 minutes and then centrifuged at 6000×g for 1 min. The column was discarded the DNA was stored appropriately (4°C for short term, −20°C for long term storage).

3.8.2 PCR Amplification of Genomic DNA using RPO1 and RPO4 Primers

Total genomic DNA of the rhizobial isolates were amplified using RPO1 and RPO4 primers. Amplification reaction was carried out in a 12.5 µL mixture solution containing 1 µL of 12.5 mM $MgCl_2$, 1.25 µL of ×10 buffer, 0.25 µL of dNTPs, 0.25 µL of *Taq polymerase*, 1 µL of primer, 1.5 µL of template DNA and 7.25 µL of water. Polymerase chain reaction (PCR) amplifications were carried out in a BOI–RAD iCycler system with an initial denaturation of temperature of 92°C for 30 s, followed by 35 cycles of denaturation (30 seconds at 94°C), annealing (2 min at 40°C), and extension (90 seconds at 78°C); followed by a final extension at 72^{o}C for 3 min. The concentration and size of the PCR products were examined on a 2% agarose gel pre–stained with ethidium bromide in 1×Tris–acetate EDTA (TAE) buffer. The gels were run for 120 minutes at 90v and photographed under UV illumination in a GeneFlash (Syngene BIO Imaging) unit.

3.8.3 PCR Amplification of 16S–23S Intergenic Spacer (ITS) and 16S rDNA Gene

Polymerase chain reaction (PCR) of the 16S–23S rDNA (ITS) and the 16S rDNA gene was performed with the primer sets; ITS 149072F (5′–TGCGGCTGGATCCCCTCCTT–3′), ITS 13238R (5′–CCGGGTTTCCCCATTCGG–3′), 16SF (5′–AGAGTTTGATCCTGGCTCAG–3′) and 16SR (5′–AAGGAGGTGATCCAGCCGCA–3′). Amplification reactions were performed in a 25 µL volume, containing 2 µL $MgCl_2$ (25 mmol L^{-1}), 0.5 µL of dNTPs, 2.5 µL of 10× buffer, 0.5 µL *Taq polymerase*, 12.8 µL of H_2O, 2.0 µL of each primer and 2 µL of template DNA. The thermal profile used was an initial denaturation step at 95 °C for 1 minute, 35 cycles consisting; of 1 min denaturation at 94 °C, 1 minute of primer annealing at 50 °C and 2 min of extension at 72 °C, plus a 3 min final extension at 72 °C. The concentration and size of the PCR products were examined on a 2% agarose gel pre–stained with ethidium bromide in 1×Tris–acetate EDTA (TAE) buffer. The gels were run for 120 minutes at 90v and photographed under UV illumination in a GeneFlash (Syngene BIO Imaging) unit.

3.8.4 Restriction Fragment Length Polymorphism (RFLP) Analysis

Polymerase chain reaction (PCR) products were digested with the restriction endonucleases *Hind*III and *Hha*I for ITS analysis and *Hha*I and *Hae*III for 16S rDNA gene analysis. The restriction enzymes were used to digest 5 µL aliquots of amplified DNA and incubated at 37°C for at least one hour. The digested DNA fragments were separated by horizontal gel electrophoresis on a 2.5% agarose gel pre–stained with ethidium bromide in 1×TAE buffer. The gels were run for 90 minutes at 100v and photographed under UV illumination in a GeneFlash (Syngene BIO Imaging) unit.

3.9 Data Analysis

3.9.1 Statistical Analysis

The data collected from the various experiments were subjected to general Analysis of Variance and the means obtained were compared by the Least Significant Difference (LSD) at 5 % level of significance using GENSTAT software version 9. Microsoft Excel was used to effectively order and summarize the mean values into graphs, as well as run correlation analysis where necessary.

3.9.2 Cluster Analysis

Polymerase chain reaction (PCR) products were scored as presence (1) and absence (0) of band at specific band sizes for each of the primers used. A data matrix with fragments in columns and rhizobia isolates in rows was assembled from these scores. Cluster analysis was performed to reveal the genetic relationship among the isolates. Genetic associations among varieties were evaluated using simple matching similarity coefficients for pair–wise comparisons based on the proportion of shared bands produced by the primers. Similarity matrices were generated using the SIMQUAL sub–program of NTSYS–PC software (Rohlf, 1998). Similarity coefficients were used for cluster analysis of varieties using the SAHN sub–program of NTSYSPC software and dendrograms were obtained using the un–weighted pair–group method with arithmetic average (UPGMA) sub–program of NTSYS–PC V.2.1.

3.9.3 Diversity, Richness and Evenness Indices

The diversity among rhizobia isolates from the various soils was estimated using the Shannon–Weaver Index (Shannon & Weaver, 1949). This Index of diversity ***(H')*** was estimated based on the number of isolates belonging to each group of profiles in RPO1 and RPO4 PCR amplifications, considering an 80% similarity in the cluster analysis. The idea behind this index

is that the diversity of a community is similar to the amount of information in a code or message. The diversity is calculated using the following equation:

$$H' = -\sum_{i-1}^{n} P_i ln\, P_i \qquad [3.17]$$

Where P_i is the proportion of individuals found in species **i.** For a well–sampled community, this proportion as $P_i = n_i/N$, where n_i is the number of individuals in species **i** and N is the total number of individuals in the community. Since by definition the p is between zero and one, the natural log makes all of the terms of the summation negative, which is why the inverse of the sum is taken.

The Margalef index was used to calculate the quantity of richness ***(R)*** among the isolates (Margalef, 1958):

$$R = S - {}^{1}/_{ln\, N} \qquad [3.18]$$

Where S in this case is the number of groups or clusters and N is the total number of isolates in all clusters.

Pielou index (J) was used to estimate the degree of evenness among the isolates (Pielou, 1966):

$$J = {}^{H'}/_{ln\, S} \qquad [3.19]$$

Where H' is the Shannon–Weaver index and S is the total number of clusters per soil at 80% similarity level.

CHAPTER FOUR

RESULTS

4.1 Soil characterization

Results on selected chemical, physical and biological properties are presented in Table 4.1.

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

Soil Characteristics	Level			
	Adenta	**Akuse**	**Haatso**	**Toje**
pH_w (H_2O)	5.7	6.5	6.4	5.4
pH_s (0.01M $CaCl_2$)	4.9	5.2	5.2	4.3
Organic carbon (g kg^{-1})	4.8	8.7	3.0	8.7
Total N (g kg^{-1})	4.5	0.9	1.2	1.5
Available P (mg kg^{-1})	4.08	7.6	3.8	5.3
C/N ratio	0.88	9.67	2.5	5.8
Bulk Density (Mg m^{-3})	1.32	1.46	1.34	1.25
Sand (g kg^{-1})	680	514	755	515
Silt (g kg^{-1})	80	63	65	127
Clay (g kg^{-1})	240	423	180	358
Textural Class	SCL	SC	SL	SCL
CEC (cmolc kg^{-1} soil)	7.1	33.2	7.4	5.8
Rhizobia Population (cells g^{-1} soil)				
African yam bean	170	200	140	93
Cowpea	680	200	280	120

SCL = Sandy Clay Loam **SC**=Sandy clay **SL**=Sandy loam

The pH in water (1:1) was moderately acidic in Adenta and Toje and near neutral in Haatso and Akuse, ranging from 5.4 to 6.5 (Table 4.1). The pH values for all soils determined in 0.01M $CaCl_2$ were lower than those determined in water, and ranged from 4.3 in Toje to 5.2 in Akuse and Haatso series'. The organic carbon (OC) contents (Table 4.1) of the soils were low and ranged between 3.0 g kg^{-1} in the Haatso soil to 8.7 g kg^{-1} in the Akuse and Toje soils.

Total N contents were generally low in the soils and followed a similar trend as OC contents in the soils.

Available P values ranged from 3.8 mg kg^{-1} and 4.1 mg kg^{-1} in Haatso and Adenta series to 5.3 mg kg^{-1} and 7.6 mg kg^{-1} in Toje and Akuse soils (Table 4.1).

Bulk density of the soils ranged from 1.47 Mg m^{-3} to 1.25 Mg m^{-3}. Particle size distribution using Calgon as dispersing agent exhibited higher sand values and lower clay and silt values. Sand content of the soils varied from 514 to 755 g kg^{-1} while the clay and silt contents ranged from 180 to 432 g kg^{-1}, and from 63 to 127 g kg^{-1}, respectively.

The populations of indigenous rhizobia specific to African yam bean (*Sphenostylis stenocarpa)* and cowpea *(Vigna unguiculata)* were estimated using the Most Probable Number (MPN) plant infection technique (Vincent, 1970) in the four soil series'. Although all the soils investigated contained indigenous rhizobia capable of nodulating African yam bean, there was variability in their numbers. The estimated total rhizobia population of African yam bean ranged from as low as 9.3×10 cells g^{-1} soil in Toje series to 2.0×10^2 cells g^{-1} soil in Akuse series (Table 4.1). That of cowpea ranged from as low as 1.2×10 cells g^{-1} soil in Toje series to 6.8×10^2 cells g^{-1} soil in Adenta series. About 75% of the soils tested contained more than 10^2 rhizobia cells per gram of soil, except for Akuse series in which the bradyrhizobia that nodulated cowpea had the same

population as African yam bean bradyrhizobia, all the soils harboured less compatible African yam bean bradyrhizobia than the cowpea (Table 4.1).

4.2 Nodulation Potential of African Yam Bean (*Sphenostylis stenocarpa)*

The nodulation capacity of African yam bean with native rhizobia was evaluated in four Ghanaian soils. Root nodules were formed on all the African yam bean plants grown in all the soils. However, significant differences ($p < 0.05$) occurred among the different soils (Table 4.2). Toje series with the lowest pH recorded the lowest number of nodules (14 nodules plant^{-1}) with those in Haatso forming the highest (39 nodules plant^{-1}) (Table 4.2). Nodulation in Adenta series like that in Haatso series was significantly ($p < 0.05$) higher than that in Akuse series, which was in turn higher than that in Toje series. Adenta series recorded about 76% higher nodulation compared to Toje series, and about 11% less compared to Haatso. Haatso series thus recorded about 78% higher nodulation compared with Toje series (Table 4.2). Haatso series recorded the highest amount of shoot total nitrogen, followed by Adenta. There were significant differences ($p < 0.001$) in the shoot dry weight of African yam bean among the soils tested.

Table 4.2: Nodulation, N accumulation and Shoot dry weight of African Yam Bean grown in four Ghanaian Soils.

Soil	Nodules plant^{-1}	Shoot Dry Weight (g plant^{-1})	Shoot N (%)	Shoot Total N (mg plant^{-1})
Adenta	37	2.0	3.0	61.1
Akuse	23	1.1	4.2	37.8
Haatso	39	1.9	3.1	73.1
Toje	14	1.2	3.1	38.4
LSD (0.05)	**8.95**	**0.34**	**0.55**	**11.3**
CV (%)	**21.30**	**14.40**	**10.40**	**13.9**

4.3 Response of African Yam Bean to N Fertilization

4.3.1 Effect of Nitrogen Application on Nodulation in African Yam Bean

Nitrogen fertilization decreased nodulation of African yam bean in both Adenta and Akuse soil series' (Table 4.3). Fertilization with 50 kg N ha^{-1} reduced nodulation in the Adenta series by more than half and was almost a quarter of the amount when 200 kg N ha^{-1} was applied in comparison to the control. Similar trends were observed in the Akuse series, with the application of 50 and 100 kg N ha^{-1} reducing nodulation to half of that obtained in the unfertilised control but with no significant ($p > 0.05$) differences among them. The addition of 200 kg N ha^{-1} further reduced nodulation to about a third of that recorded in the control (Table 4.3). The control recorded about 72% more nodules than at 200 kg N ha^{-1} in Adenta series ($p < 0.05$), whiles in Akuse series the unfertilized control recorded about 62% higher nodulation than at 200 kg N ha^{-1} ($p < 0.05$). Nodule number per plant correlated positively with tissue %N ($r = 0.34$) in Adenta series, and a lower positive correlation ($r = 0.25$) in Akuse series. Generally, the depressing effect of nitrogen fertilization on nodulation was less in Adenta soil compared to Akuse soil.

Similarly nodule mass decreased with increases in nitrogen fertilizer application. The nodule dry matter reduced drastically when nitrogen was increased from 50 to 100 kg N ha^{-1} (Table 4.3). The results showed that nodule dry matter accumulation was affected more by N fertilization more than the production of new nodules. Nodule number correlated positively ($r = 0.88$) with nodule dry weight in Adenta series, even though there was a high variability in the dry weight of nodules. Similarly, there was a significant positive correlation ($r = 0.91$) between nodule number and nodule dry weight in Akuse series with significant differences among treatments.

Table 4.3: Effect of N application on nodulation and nodule mass of African yam bean in two soils.

	ADENTA		AKUSE	
Fertilizer Rate (kg N ha^{-1})	**Nodule number per plant**	**Nodule dry wt. (mg plant^{-1})**	**Nodule number per plant**	**Nodule dry wt. (mg plant^{-1})**
0	37	39.2	20	20.9
50	15	10.0	11	3.5
100	14	6.8	10	7.0
200	10	3.5	8	2.0
LSD (0.05)	**8.9**	**10.6**	**6.2**	**9.9**

4.3.2 Effect of Nitrogen application on N_2 fixation in African yam bean

The effect of nitrogen fertilization on nitrogen fixation is presented in Table 4.4. The amount of N_2 fixed was calculated using the ^{15}N Isotope dilution method. The result shows a general decrease in N_2 fixation with increasing rate of nitrogen application in both soils even though there was high variability among treatments. Nitrogen fertilization of 200 kg N ha^{-1} recorded high amount of total N_2 fixed than the lower rates (Table 4.4). This could be due to the high dry matter yield observed with increase in plant available nitrogen. Generally nodule number correlated positively (r = 0.781) with total nitrogen fixed. Total N_2 fixed when 50 kg N ha^{-1} was applied was insignificant. Interestingly, nitrogen fixation increased at 200 kg N ha^{-1} in Adenta soil.

Table 4.4: Effect of Nitrogen on N_2 fixation in African yam bean in two Ghanaian soils.

	Ndfa* (%)		Total N fixed (mg plant^{-1})	
Fertilizer Rate (kg N ha^{-1})	**Adenta**	**Akuse**	**Adenta**	**Akuse**
0	34.6	12.9	23.7	4.5
50	0.0	0.0	0.0	0.0
100	13.4	35.9	12.4	28.9
200	32.9	5.5	41.7	79.1
LSD (0.05)	**5.2**	**4.7**	**5.9**	**8.8**

Ndfa* = Nitrogen derived from Atmosphere

4.3.3 Effect of Nitrogen application on N accumulation in African yam bean

The concentration of N in shoot of African yam bean increased significantly ($p < 0.05$) in both soils with increase in N rates. Shoot %N varied from 3.3% in the control to 5.5% at 200 kg N ha^{-1}. The percentage increases in shoot N concentration (%N) across the different rates of N are 24%, 46% and 65% for 50, 100 and 200 kg N ha^{-1} respectively, in comparison with the control in Adenta series. Similarly Akuse series also recorded significant ($p < 0.05$) increases in shoot N concentration across treatments (Table 4.5). The variations in the shoot N concentration with N fertilization were 13% to 46% in 200 kg N ha^{-1}. Secondly, increasing the rate of N applied, resulted in increment of shoot total N accumulation (Table 4.5).

Table 4.5: Effect of Nitrogen application on N accumulation and shoot total N of African yam bean in two soils.

	ADENTA		AKUSE	
Fertilizer Rate (kg N ha^{-1})	**Shoot N (%)**	**Total N in Shoot (mg plant^{-1})**	**Shoot N (%)**	**Total N in Shoot (mg plant^{-1})**
0	3.3	57.3	3.8	33.1
50	4.1	62.6	4.3	47.5
100	4.9	92.9	5.1	74.4
200	5.5	117.8	5.3	137.3
LSD (0.05)	**0.49**	**14.1**	**0.8**	**14.5**

4.3.4 Effect of Nitrogen Application on Dry Matter Yield of African yam bean

Nitrogen fertilization had a significant influence on above ground biomass of African yam bean in both soils (Figure 4.1). Shoot dry matter accumulation increased significantly ($p < 0.001$) with increases in N treatment in both soils (Figure 4.1). Shoot dry matter accumulation in the unfertilised control and 50 kg N ha^{-1} was significantly ($p < 0.05$) different from those of the 100 and 200 kg N ha^{-1} in the Adenta soil. Increasing the rate of nitrogen from 0 to 200 kg N ha^{-1} in Adenta resulted in about 2–fold increase in shoot dry matter accumulation ($p < 0.017$).

Shoot dry matter accumulation however showed about a threefold increase at 200 kg N ha^{-1} over the unfertilised control in Akuse soil. Increases in shoot dry weight in Akuse soil across the various N treatments showed that shoot dry weight approximately doubled at 200 kg N ha^{-1} compared to 50 kg N ha^{-1}. Although less pronounced, there were significant ($p < 0.05$) differences in the shoot dry weight between the 50 and 100 kg N ha^{-1} in Akuse. A strong

positive correlation was recorded between the shoot dry weight and nitrogen accumulation (% N) in both soils (r = 0.61 and r = 0.77 for Adenta and Akuse soils' respectively).

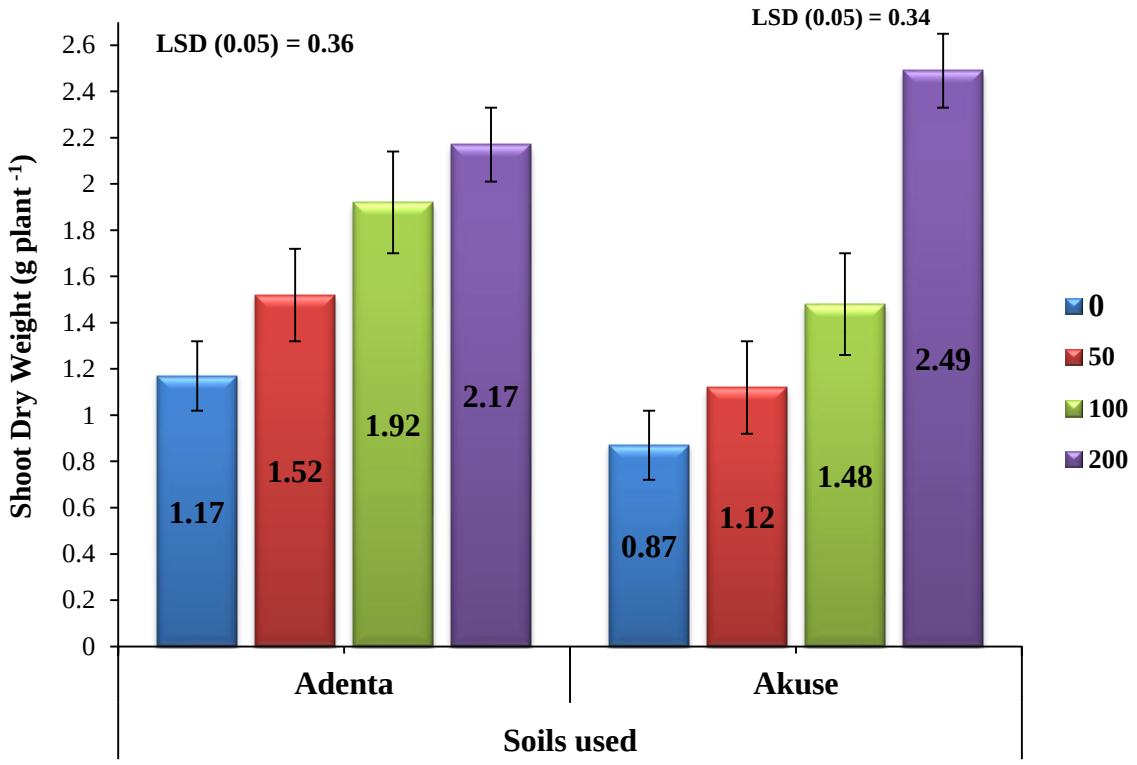


Figure 4.1: Shoot dry weight of African yam bean in Adenta and Akuse soils as affected by N application.

4.4 Response of African Yam Bean to Phosphorus Application

4.4.1 Effect of P application on nodulation and nodule dry weight of AYB

In contrast to nitrogen fertilization, increasing the rates of phosphorus application resulted in significant ($p < 0.001$) increases in nodule numbers in both soils over their respective controls (Table 4.6). Nodule numbers ranged from 34 nodules plant^{-1} in the control to 78 nodules plant^{-1}at 120 kg P ha^{-1} in Adenta soil, and 15 nodules plant^{-1} in the control to 94 nodules plant^{-1}

at 120 kg P ha^{-1} in Akuse soil. However, addition of phosphorus in excess of 80 kg P ha^{-1} in Adenta soil, did not translate into any significant increase in nodule numbers, while significant increases in nodule numbers were recorded in Akuse soil (Table 4.6).

Under low soil P conditions, both soils gave fewer and smaller nodules compared to the more numerous and heavier nodules produced under high P levels. This implies a positive response of nodule mass to increasing phosphorus rate. Comparison of Adenta and Akuse soils' dry nodule biomasses, across phosphorus rates, revealed that dry nodule mass was about 31% heavier in Adenta soil than Akuse, with more pronounced variations observed at lower rates of fertilization. There was a significant ($p < 0.05$) relationship between nodule number and nodule mass in both soils, with both parameters increasing linearly ($r = 0.81$ and $r = 0.94$ for Adenta and Akuse soils', respectively) with increase in P rate. In Adenta soil, there was a little over two–fold increase in nodule number with added P (between 0 and 120 kg P ha^{-1}) but the nodule dry weight increased by a little over seven folds (Table 4.6). Similarly in Akuse soil, nodule number increased by six fold while nodule dry weight increased by 15–fold. Thus the addition of P had a greater effect on nodule mass than on the numbers.

Table 4.6: Effect of P application on nodulation and nodule mass of African yam bean in two soils.

	ADENTA		AKUSE	
Fertilizer Rate (kg P ha^{-1})	**Nodule number per plant**	**Nodule dry wt. (mg plant^{-1})**	**Nodule number per plant**	**Nodule dry wt. (mg plant^{-1})**
0	34	39.2	15	20.9
40	43	189.5	42	176.9
80	75	265.7	71	240.8
120	79	289.8	94	300.8
LSD (0.05)	**16.8**	**87.7**	**16.1**	**60.6**

4.4.2 Effect of P application on N_2 fixation in African yam bean

The data on the effect of P fertilization on nitrogen fixation in African yam bean across treatments is presented in Table 4.7. Phosphorus fertilization from 40 to 80 and 120 kg P ha^{-1} did not result in any significant increase in the percentage of nitrogen derived from atmosphere (% Ndfa) in both soils (Table 4.7). Thus majority of the incremental effect of P on nitrogen fixation (% Ndfa) occurred between zero and 40 kg P ha^{-1} in both soils (Table 4.7).

Similarly, P had a positive effect on total nitrogen fixation in both soils (Table 4.7). Total N_2 fixation ranged from 23.7 mg N plant^{-1} in the unfertilised control to 117.8 mg N plant^{-1} at 120 kg P ha^{-1} in Adenta soil. A similar trend was observed in Akuse soil with values ranging from 4.5 mg N plant^{-1} in the control to 105 mg N plant^{-1} at 120 kg P ha^{-1} (Table 4.7). However, the increase in total N_2 fixed with increase in P was higher in Akuse soil than in Adenta soil. Interestingly in Akuse soil, even though there was no significant difference in %Ndfa among P treatments except for the control, the corresponding total N_2 fixation showed significant differences among treatments (Table 4.7).

Table 4.7: Effect of Phosphorus on N_2 fixation in African yam bean in two soils.

Fertilizer Rate (kg P ha^{-1})	Ndfa* (%)		Total N fixed (mg plant^{-1})	
	Adenta	Akuse	Adenta	Akuse
0	34.6	12.9	23.7	4.5
40	78.5	87.9	100.1	72.6
80	77.4	81.8	107.4	82.9
120	78.6	84.9	117.8	105.0
LSD (0.05)	**18.8**	**7.3**	**23.7**	**8.0**

Ndfa* = Nitrogen derived from Atmosphere

4.4.3 Effect of Phosphorus on N accumulation by African yam bean

Increasing the P fertility of the soils enhanced the accumulation of N in shoots. In Adenta soil, a greater percentage of the increase in shoot %N occurring from 0 to 40 kg P ha^{-1} than rates above 40 kg P ha^{-1}. Thus increasing the P fertility of Adenta soil in excess of 40 kg P ha^{-1} did not translate into any significant increases in the shoot N accumulation (Table 4.8). A similar trend was observed in Akuse soil.

Shoot total N accumulation also increased with increased P fertilization. The shoot total N uptake significantly ($p < 0.001$) increased from 68.2 mg plant^{-1} in the control to 144.6 mg plant^{-1} at 120 kg P ha^{-1} (Table 4.8) in Adenta soil. Similar trend was observed in Akuse soil. However, the increase in shoot total N at 120 kg P ha^{-1} was about three and a half times (352.2%) that of the unfertilized control in Akuse soil while Adenta soil recorded a percentage increase of a little over 100% (112.1%) in shoot total N at 120 kg P ha^{-1} in comparison to the control.

Table 4.8: Effect of P application on Percent Shoot N and shoot total N of African yam bean in two soils.

	ADENTA		AKUSE	
Fertilizer Rate (kg P ha^{-1})	**Shoot N (%)**	**Total N in Shoot (mg plant^{-1})**	**Shoot N (%)**	**Total N in Shoot (mg plant^{-1})**
0	3.3	68.2	3.8	33.1
40	4.14	126.6	4.2	74.7
80	4.1	138.7	4.4	113.8
120	4.1	144.6	4.7	146.3
LSD (0.05)	**0.4**	**12.3**	**0.7**	**29.3**

4.4.4 Effect of Phosphorus application on Dry Matter Yield of African yam bean

Shoot dry matter also showed a significant increase with increase in P rate (Figure 4.2). At 120 kg P ha^{-1}, shoot dry matter increased was about 75% ($p < 0.001$) relative to the unfertilized control in Adenta soil. On the other hand, a greater percentage increase was observed in Akuse soil, with 120 kg P ha^{-1} recording a shoot dry matter of as much as three times that of the control (Figure 4.2). Increasing the rate of P from 40 to 80 kg P ha^{-1} did not translate into significant increases in shoot biomass in both soils. However, there were significant ($p < 0.05$) differences observed between the unfertilised control and 120 kg P ha^{-1}.

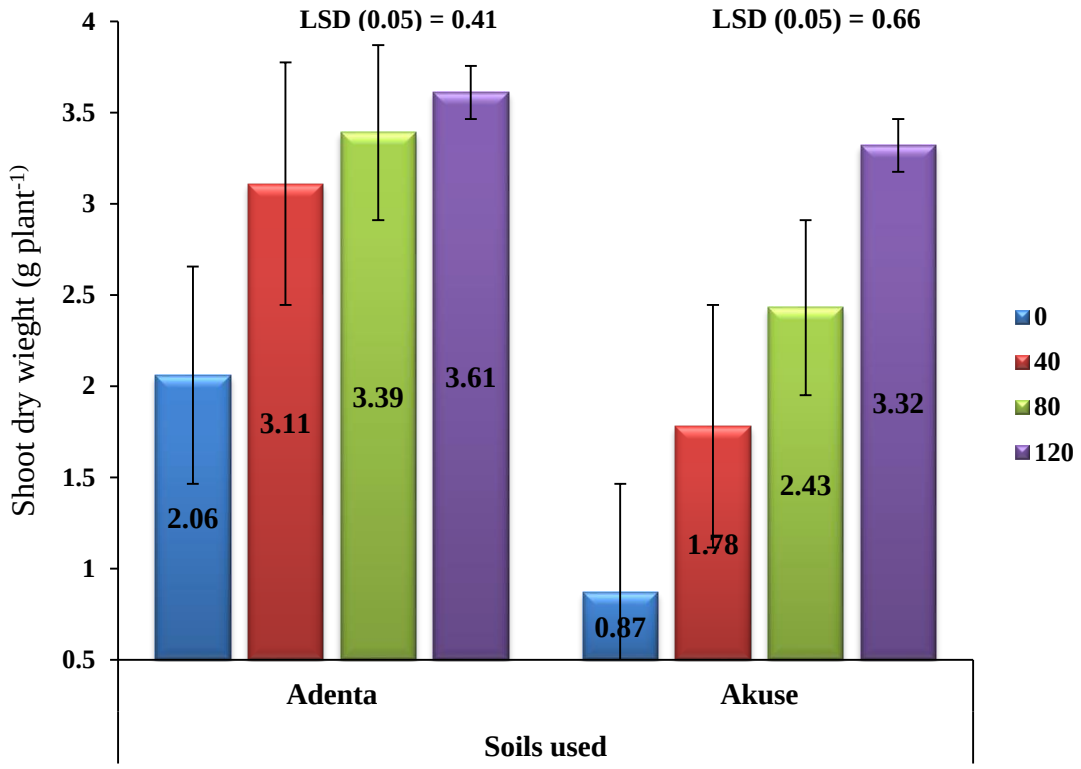


Figure 4.2: Shoot dry weight of African yam bean in Adenta and Akuse soils as affected by phosphorus application.

4.4.5 Effect of P application on P uptake and Phosphorus Utilization Efficiency (PUE) of African yam bean

Table 4.9 shows that there were significant ($p < 0.05$) increases in shoot P uptake with higher P rate. The increase in P uptake may be attributed more to the increased dry matter yield rather than increases in shoot P concentration (%P). The PUE is a derived technical expression of the performance of African yam bean, and reflects the effectiveness or otherwise of the legume to phosphorus fertilizer. The utilisation efficiency of the phosphorus applied was calculated for the different rates in terms of dry matter yield per unit of P taken up. The results showed that lower PUE was seen at higher P rates. There was a negative correlation between P uptake and PUE (r =

–0.80, R^2 = 0.65 and r = –0.46, R^2 = 0.212, respectively) on Adenta and Akuse soils'. The maximum PUE was observed at 40 kg P ha^{-1} and decreased significantly ($p < 0.05$) with increase in application rates (Table 4.9). Also, there was higher phosphorus utilisation efficiency (PUE) by plants grown in the Adenta soil compared to those grown in Akuse soil.

Table 4.9: Effect of Phosphorus on P uptake and P Utilisation Efficiency.

	P Uptake (mg $plant^{-1}$)		**PUE* (g DMY per mg P taken up)**	
Fertilizer Rate (kg P ha^{-1})	**Adenta**	**Akuse**	**Adenta**	**Akuse**
0	3.8	1.5	0.51	0.47
40	5.4	4.7	0.57	0.52
80	8.0	5.3	0.42	0.47
120	8.9	8.0	0.49	0.44
LSD (0.05)	**2.2**	**3.6**	**0.12**	**0.13**

PUE* = Phosphorus Utilization Efficiency

4.5 Diversity of Indigenous African Yam Bean Rhizobia

4.5.1 Cross Inoculation Studies (Host Range Analysis)

The degree of compatibility of indigenous African yam bean rhizobial isolates with some selected legume species was examined, to determine the level of promiscuity of African yam bean (Table 4.10). The results showed that there are differences in the type of host nodulated by the different isolates. Three (3) out of the six (6) host legumes tested were nodulated by 80% of the African yam bean bradyrhizobia isolates. Two host legumes from the genus *Vigna*; cowpea (*Vigna unguiculata*) and yardlong bean (*Vigna sesquipedalis*) were compatible with most (80%)

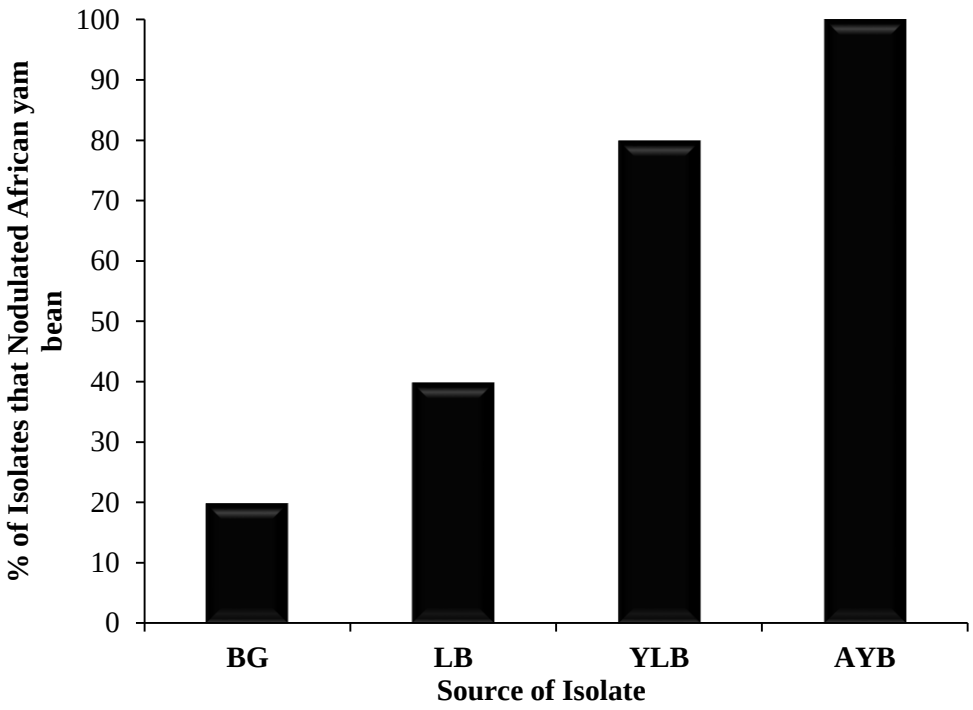
of the five isolates tested. However none of the isolates tested was capable of eliciting nodulation on bambara (*Vigna subterranea*), lima bean (*Phaseolus lunatus*) or crotalaria. Interestingly, most of the isolates tested formed nodules with *Pueraria spp*.

Table 4.10: Test of the abilities of five African yam bean Bradyrhizobia isolates to cross inoculate five host legumes.

	Nodulation Score						
Isolate	**AYB***	**Cowpea**	**Bambara**	**Lima bean**	**YLB****	**Pueraria**	**Crotalaria**
AYB 1	+	+	–	–	+	+	–
AYB 2	+	+	–	–	+	+	–
AYB 3	+	+	–	–	–	+	–
AYB 4	+	+	–	–	+	–	–
AYB 5	+	–	–	–	+	+	–

*AYB=African Yam Bean **YLB=Yard Long bean + = presence of nodules and – = absence of nodulation.

In a second experiment, rhizobia isolates from lima bean, yardlong bean and bambara groundnut were inoculated on African yam bean (Figure 4.3). In contrast to the earlier results, African yam bean was compatible to some (20%) of *rhizobia* isolates obtained from bambara groundnut, 40% of lima bean rhizobia and 80% of rhizobia isolated from yardlong bean.



BG = Bambara Groundnut, LB = Lima Bean, YLB = Yardlong bean, AYB = African yam bean

Figure 4.3: Abilities of *Rhizobium* isolates obtained from three different legume genera to form nodules on African yam bean.

4.5.2 Molecular Analysis

4.5.2.1 DNA Amplification using arbitrarily primed oligonucleotide (RPO4)

Random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of a DNA sequence. The arbitrarily primed PCR amplifies anonymous fragments of DNA from any genome (Welsh & McClelland, 1990).

The amplification patterns obtained using this primer revealed a high level of polymorphism among the 30 African yam bean rhizobial isolates tested. An example of RPO4 primer fingerprint is shown in Figure 4.4. The sizes of DNA bands that were produced in the PCR reactions ranged from >100 bp to ≈ 2100 bp, but most of the bands were between 100 bp and 1000 bp range. The dendrogram (Fig. 4.5) derived from RPO4 profiles clustered all the 30 African yam bean *Rhizobium* strains into two distinct groups (1 & 2) at 60% similarity level. Thus at 60% similarity level all the isolates were the same. The first major cluster (1) has nine isolates which diverge at 63% similarity level. Within the second major cluster (2), two groups of strains could be distinguished. Five isolates forming the first sub cluster (A) at 67% similarity level. The second sub cluster (B) at 65% similarity has 16 isolates. Identical RAPD patterns (100% similarity coefficient) using RPO4 primer were observed for two isolates (both from Adenta series'). Six isolates (5 from Haatso and 1 from Adenta) and 8 isolates (2 from Akuse, 3 from Adenta, 2 from Haatso and 1 from Toje soils') had 88% and 92% similarities, respectively (Fig. 4.5). Based on these results, it is evident that RAPDs are useful for isolate discrimination and identification in rhizobium populations collected from different soils.

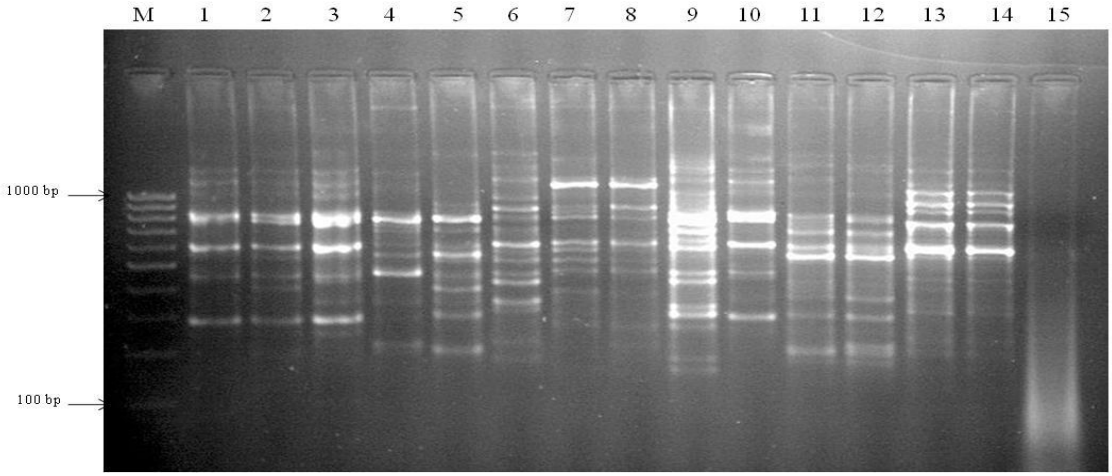


Figure 4.4: Sample of a horizontal Gel electrophoretic pattern of amplified DNA of 15 African yam bean bradyrhizobia isolates using RPO4 Primer. Lane M: 100 bp molecular maker and Lane 1–15 are rhizobia isolates.

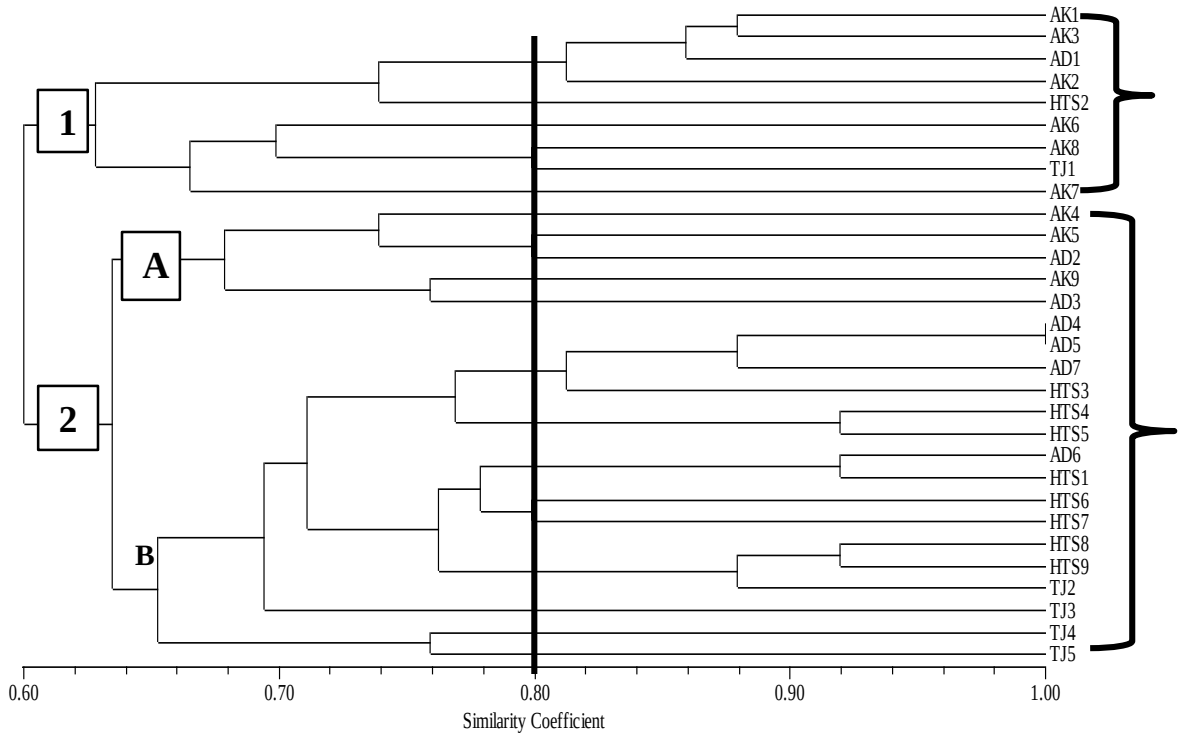


Figure 4.5: Dendrogram generated with Un–weighted pair group with mathematical average (UPGMA) showing genetic relationships among 30 African yam bean bradyrhizobia isolates identified by RPO4 Primer analysis. AK, AD, HTS and TJ implies isolates from Akuse, Adenta, Haatso and Toje soils respectively.

4.5.2.2 Direct DNA Amplification using *nif* gene directed primer (RPO1)

Direct amplified polymorphic DNA (DAPD) analysis, was performed using a single primer (RPO1) directed to a *nif* gene promoter consensus sequence (Richardson *et al.,* 1995). Based on the average cluster analysis, the isolates formed 2 major groups at 54% similarity level (Fig. 4.6). The first cluster had two distinct clusters with a mean similarity of 58%. The second major cluster had three isolates all from Toje soil with 65% similarity, with two isolates having a similarity of 72%. The RPO1 Primer showed a 95% similarity between two isolates from the Akuse series.

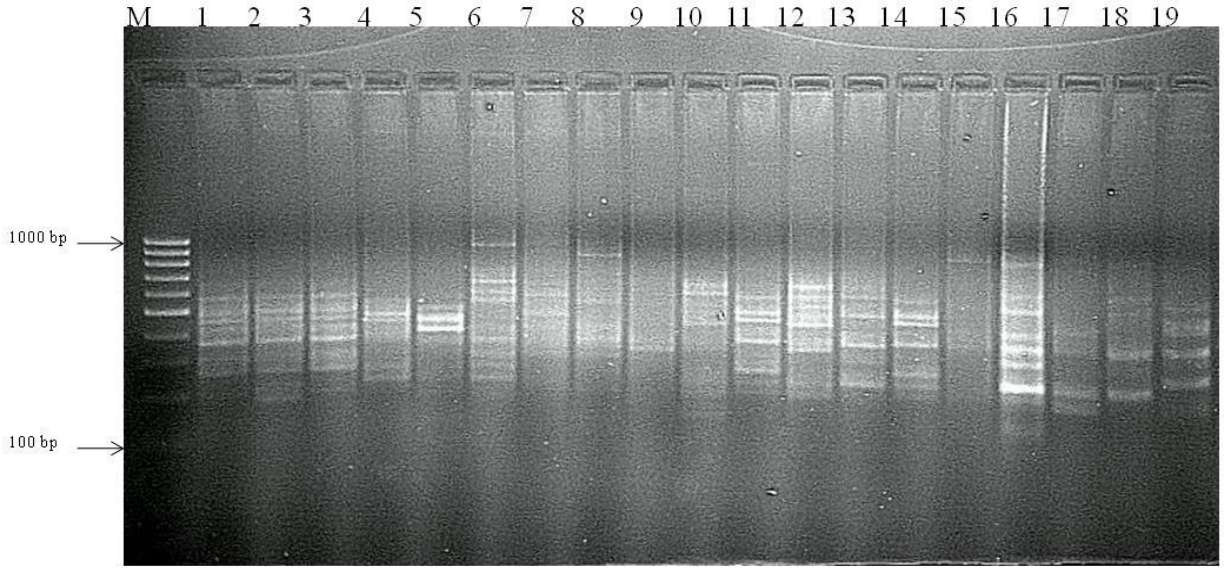


Figure 4.6: Sample of a Gel electrophoretic pattern of amplified DNA of African yam bean rhizobia using RPO1 Primer. Lane M: 100 bp molecular maker and Lane 1–19 are rhizobia isolates.

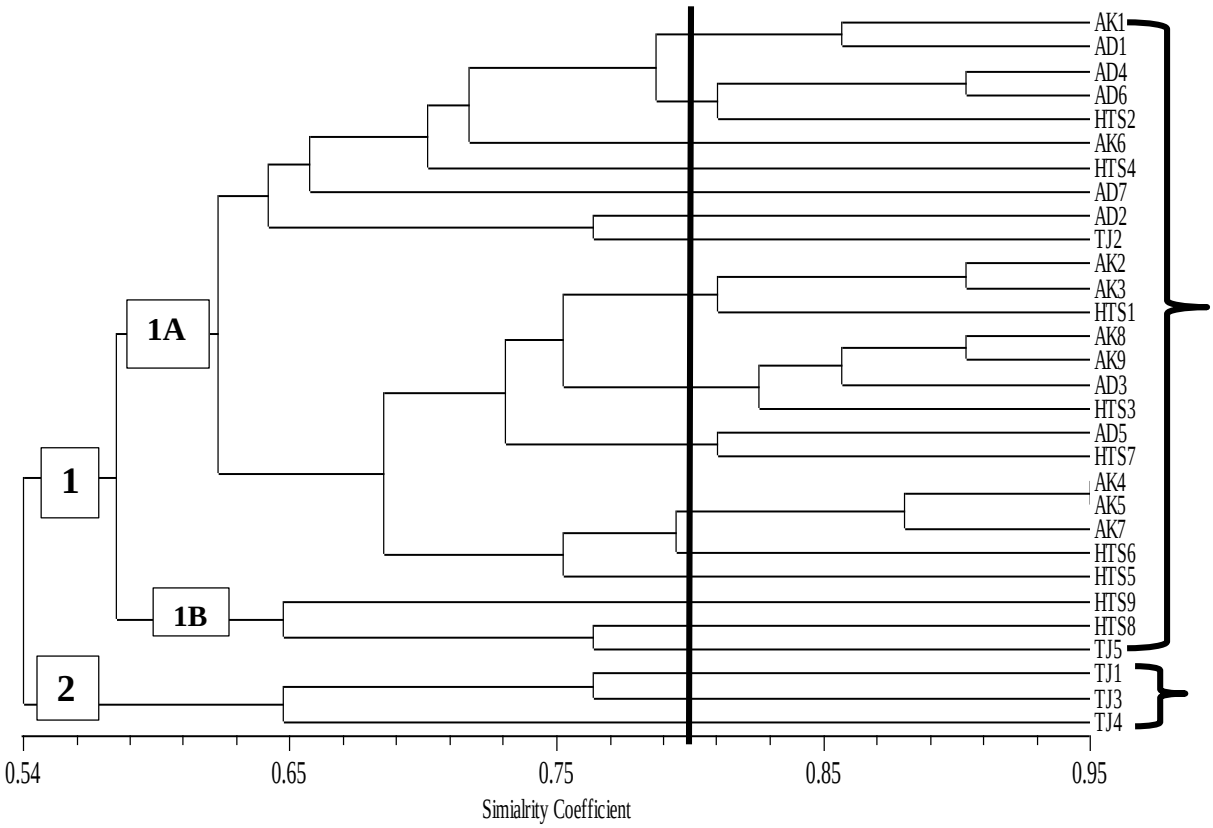


Figure 4.7: Dendrogram (UPGMA) of genetic relationships among 30 African yam bean bradyrhizobia isolates identified by RPO1 Primer analysis. AK, AD, HTS and TJ implies isolates from Akuse, Adenta, Haatso and Toje soil series' respectively.

4.5.2.3 Diversity Indices based on RPO1 and RPO4 genomic DNA Profiles

In order to quantify the diversity among isolates from each soil based on the amplification profiles generated by RPO1 and RPO4 primers, the Shannon–Weaver diversity index (*H'*) was calculated, maintaining the basis of 80% similarity level (Table 4.11). The Shannon–Weaver index calculates the uncertainty of predicting the type of another isolate from that population. The results revealed a high genetic diversity of bradyrhizobia isolated from the different soils.

The calculations showed higher diversity in Haatso series revealed by RPO1 (H′ = 2.03), followed by Adenta series (H′ = 1.55), and Akuse series (H′ = 1.52) soils (Table 4.11). However, RPO4 showed a higher diversity among rhizobia isolates in Akuse series (H′ = 1.67) than the RPO1 primer (H′ = 1.52). The diversity among rhizobia isolates revealed by RPO1 in Haatso soil like that of Adenta was higher than that revealed by the RPO4 primer, whiles that in Akuse soil revealed by RPO4 primer was higher than that revealed by RPO1. Diversity among the isolates in Toje soil revealed by both primers was the same (Table 4.11).

The evenness index (J) was almost the same in all soil populations studied for both primers (Table 4.11). The richness index (*S*) of the different soil types was generally higher for RPO1 in Adenta and Haatso soils than for RPO4 primer. However, there was a higher richness in Akuse soil revealed by the RPO4 primer than RPO1 (Table 4.11), confirming the trends in diversity. The mean indices determined across all soils for the African yam bean nodulating rhizobia gave a Shannon–Weaver diversity index of 1.68 and 1.47, an evenness of 0.97 and 0.95 and a richness of 5.24 and 4.24 for RPO1 and RPO4 primers' respectively.

Table 4.11: Shannon–Weaver diversity, Pielou evenness and Margalef richness indexes of RPO1 and RPO4 amplified DNA profiles of 30 Bradyrhizobium isolates of African yam bean obtained from four Ghanaian soils.

Source of isolates (soil)	Shannon–Weaver Diversity		Pielou Evenness		Margalef Richness	
	RPO1	RPO4	RPO1	RPO4	RPO1	RPO4
Adenta	1.55	1.27	0.96	0.92	4.49	3.49
Akuse	1.52	1.67	0.94	0.93	4.54	5.54
Haatso	2.03	1.31	0.98	0.94	7.54	3.54
Toje	1.61	1.61	1.00	1.00	4.38	4.38
Mean	**1.68**	**1.47**	**0.97**	**0.95**	**5.24**	**4.24**

4.5.2.4 RFLP analysis of the amplified 16S–23S rDNA Spacer (ITS).

Thirty (30) unclassified bradyrhizobia isolates from African yam bean were examined by restriction fragment length polymorphism (RFLP) analysis of the ITS gene amplified by polymerase chain reaction (PCR).

The electrophoretic analysis of the undigested PCR products of the ITS region showed, in most cases, bands of variable lengths, from 350 bp and 650 bp. The ITS PCR products were restricted with *Hind*III, *Hha*I and *EcoR*I. The combined *Hind*III and *Hha*I restriction patterns of the amplified ITS region were used for cluster analysis by UPGMA. The *EcoR*I endonuclease was unable to digest the ITS region. The restriction analysis revealed two groups (Figure 4.8), with a mean similarity of 56%, which is consistent with those obtained by the analysis of individual RFLP patterns (gel image not shown). The first cluster (1) with 60% similarity among members of the group had only three isolates. The second cluster (2) was composed of 27 isolates with a mean similarity of 58% among its members. This cluster further diverges at 65% similarity level into two sub units (Figure 4.8). Generally, the formation of sub groups within this cluster was influenced by the origin of the isolate.

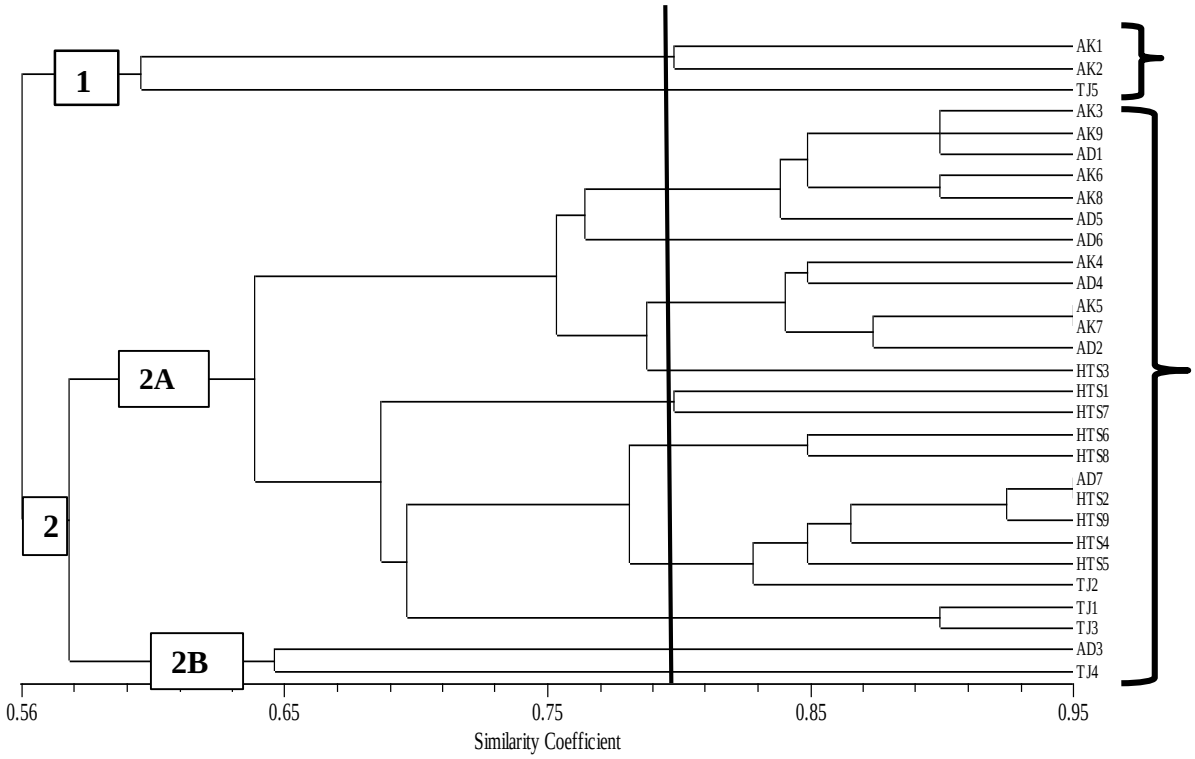


Figure 4.8: UPGMA dendrogram based on the combined *Hha*I and *Hind*III ITS RFLPs of 30 bradyrhizobia isolates of African yam bean strains. AK, AD, HTS and TJ implies isolates from Akuse, Adenta, Haatso and Toje soil series' respectively.

4.5.2.5 RFLP analysis of the amplified 16S rDNA region.

Thirty (30) unclassified bradyrhizobia isolates from African yam bean were examined by restriction fragment length polymorphism (RFLP) analysis of the 16S rDNA genes amplified by polymerase chain reaction (PCR). Gel electrophoresis of the amplified 16S rDNA gene revealed band length of nearly 1500 bp length of 16S rDNA of the isolates. Almost all the strains produced a single band and the resulting PCR product was digested separately with *Hae*III and *Hha*I restriction endonucleases. Cluster analysis of the combined restriction patterns resolved the isolates into two major clusters at a mean similarity level of 53%, (Figure 4.9). Cluster 1 was the largest, including 25 test strains. Five test strains (4 strains from Toje and 1 from Adenta soils'),

formed cluster 2. The first major cluster (1) further diverged at 57% similarity level into two sub clusters (A & B). Sub cluster (B) had only one isolate, whiles sub cluster (A) further diverged at 60% similarity level into two clusters (Figure 4.9). At 80% similarity level, 19 groups were formed based on the combined cluster analysis, with two strains from Akuse soil having a 100% similarity.

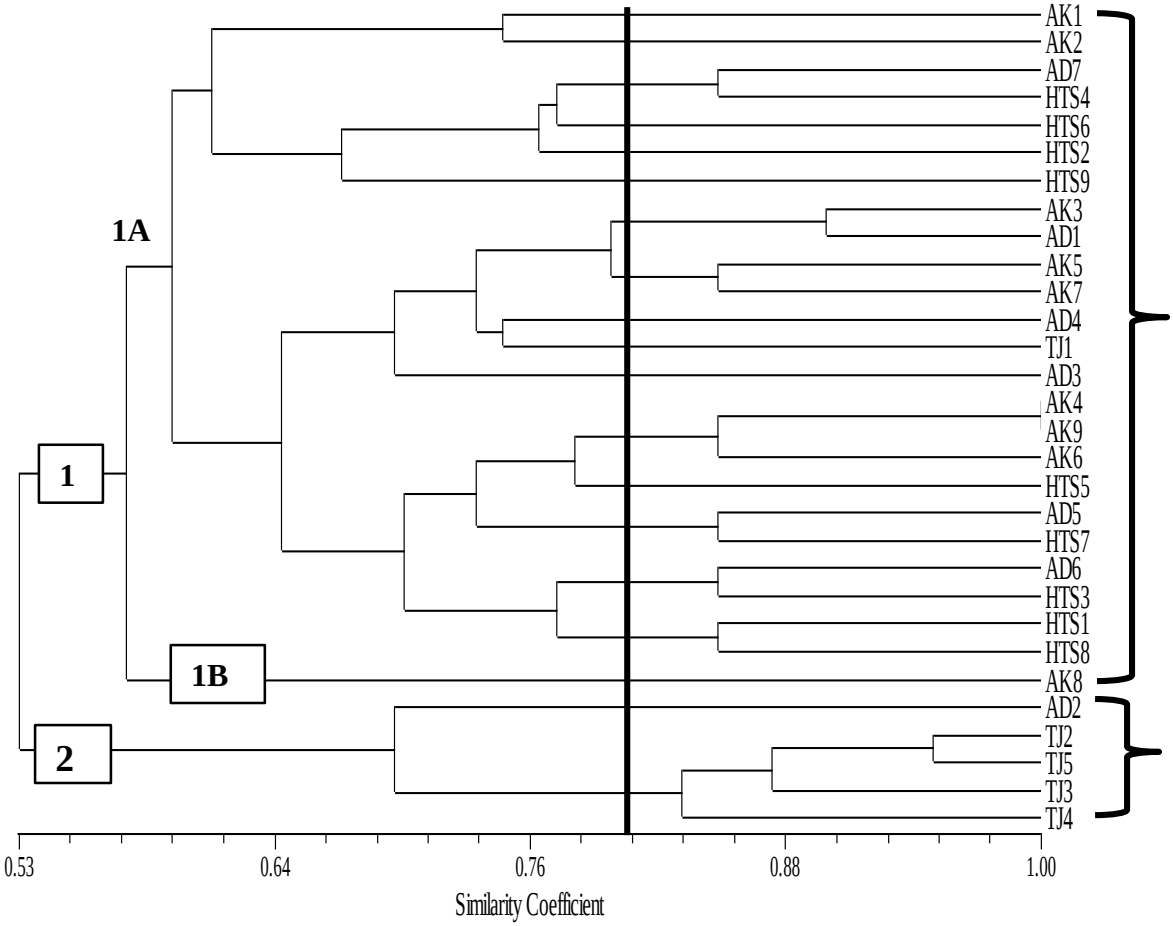


Figure 4.9: UPGMA dendrogram based on the combined *Hha*I and *Hae*III 16S RFLPs and of nodule isolates of African yam bean strains. AK, AD, HTS and TJ implies isolates from Akuse, Adenta, Haatso and Toje soil series' respectively.

CHAPTER FIVE
DISCUSSION

5.1 Soil Characterisation

The four soils (Adenta, Akuse, Haatso and Toje series') recorded higher pH in water than in salts solution, making the net change in pH negative. This suggests that the soils have a net negative charge that may serve as potential sites for adsorption of positively charged compounds. Secondly the negative change in pH value is indicative of the fact that the soils are deeply weathered (Abekoe & Sahrawat, 2001).

The low carbon content of the soils is characteristic of soils in semi–arid ecosystems, where the high rate of mineralization due to high temperatures reduces the accumulation of carbon (Dowuona *et al.*, 2012). Soil nitrogen levels were compared to general ratings defined by Landon (1984) as very high (>10 g kg^{-1}), high (5–10 g kg^{-1}), medium (2−5 g kg^{-1}), low (1−2 g kg^{-1}) and very low (<1 g kg^{-1}). Based on this, the results showed generally low nitrogen contents of the soils (Table 4.1). The low C/N ratio of the soils confirms the high rate of decomposition of organic matter. The textural characterization of the soils indicates that they are predominantly sandy clay loam, sandy clay, and sandy loam for Adenta and Toje soils, Akuse and Haatso soils respectively.

The high sand content and low clay content coupled with the average daily temperature of 28^{o}C prevailing in the Haatso environment would promote fast decomposition and low retention of added organic matter. Consequently organic matter and consequently organic carbon contents of the soils is lower (Table 4.1). Jones *et al.* (2006) stated that clay fraction has been found to accommodate more organic carbon, total N and P contents than silt and sand fractions. It is

therefore, not surprising that with its higher clay content, the Akuse series had comparatively higher organic carbon and available P contents'.

Available P was generally low in all the soils. The low level of this nutrient element is characteristic of soils in the savannah zones of Africa due to the low organic matter content (Brammer, 1967).

The medium bulk density of the soil (ranges between 1.25–1.5 Mg m^{-3}) reflects the sandy clay loam characteristic of soils. The higher bulk density (1.5 Mg m^{-3}) of the Akuse series could be due to the presence of high activity clays like montmorillonite in the soil. The presence of this mineral will make the soil harden on drying to increase the bulk density.

Cation exchange capacity (CEC) ranged from 5.8 $cmol_c$ kg^{-1} soil in Toje to 33.2 $cmol_c$ kg^{-1} soil in Akuse series. This observed trend in CEC could be attributed to the higher clay and organic matter contents associated with the Akuse soil.

The rhizobia capable of forming root nodules on African yam bean exist in Ghanaian soils, even though in varying populations. Akuse soil, followed by Adenta and Haatso soils' harboured the 200, 170 and 140 cells g^{-1} soil of African yam bean rhizobia respectively. Toje soil recorded the lowest population of 93 cell g^{-1} soil of African yam bean nodulating rhizobia. The abundance of naturally occurring rhizobia showed that the population density of indigenous bradyrhizobia capable of nodulating African yam bean was greatly influenced by the soil types.

5.2 Nodulation of African yam bean (*Sphenostylis stenocarpa)* in four Ghanaian Soils.

The ability of African yam bean to nodulate profusely with *Bradyrhizobium* strains indigenous to Ghanaian soils confirms observations made by Oagile (2005) that describes the African yam bean to be a promiscuous legume and have the ability to adapt to varying soil environments (Oagile *et al.,* 2012). Nodulation trends observed in the various soils indicate the diversity in

African yam bean rhizobia populations in Ghanaian soils. Also, rhizobia from woody legumes were found to promiscuously nodulate tropical pulse crops such as common bean *(Phaseolus vulgaris*), groundnut *(Arachis hypogeal),* soybean *(Glycine max)* and African yam bean (*Sphenostylis stenocarpa)* (Assefa & Kleiner, 1997). This suggests that the African yam bean has the ability to match other indigenous legumes in terms of plant growth and nodulation, indicating its potential as a component of local cropping systems (Assefa & Kleiner, 1997). Based on an intercropping trial conducted to assess the effect of some food legumes used as cover crops on the performance of cassava, yam and maize, Obiagwu (1995) reported that African yam bean nodulates profusely and also contributed to soil productivity and yield of the main crop without any inoculation. Oganale (2009) also described the crop as a profuse nodulator, compatible with many *Rhizobium* strains. He remarked that the African yam bean landraces that he studied obtained 79–97.6% of their nitrogen from the atmosphere through fixation. Moyin–Jesu and Adekayode (2010) concluded that the use of African yam bean plant as biological fertilizer source for yam *(Dioscorea rotundata L)* production could substitute for 250 kg ha^{-1} NPK fertilizer and 6 t ha^{-1} poultry manure.

Although Vincent (1980) reported the presence of rhizobia in virgin soils, the fact that all the soils tested, hitherto, were not cultivated with African yam bean, and contained large enough numbers or populations to enable them infect African yam bean is not unusual. This may imply that either African yam bean rhizobia naturally occur as part of the indigenous soil rhizobia population or that other native legumes grown previously on the soil belong in the same host group as African yam bean and thus serve as inoculant sources of African yam bean rhizobia.

The results from the MPN counts and the screening experiments seem to suggest some relationship between rhizobia numbers and nodulation. This is because the soils that recorded high nodulation generally had higher rhizobial numbers and vice versa. Thies *et al.* (1991) used the rhizobial population density of a given soil as an indirect means of predicting whether or not a legume will respond to inoculation. It is therefore most likely that these soils may not need to be inoculated, or inoculation responses may not be as high as those without inoculation. The ability of African yam bean to nodulate in different soil types implies that it is highly adaptable to varying soil conditions and thus is a good legume for soil improvement.

5.3 Nitrogen and Phosphorus Response Studies

5.3.1 Response of African Yam Bean to Nitrogen Fertilization

The response of African yam bean to nitrogen was assessed in two soils and the results indicated that nitrogen fertilization had an inhibitory effect on nodulation of African yam bean, with nodule numbers decreasing significantly from 37 nodules plant^{-1} in the unfertilised control to 10 nodules plant^{-1} at 200 kg N ha^{-1} in Adenta soil. A similar trend of N effect was observed in the Akuse soil, with 20 nodules plant^{-1} in the unfertilised control and 8 nodules plant^{-1} at 200 kg N ha^{-1} (Table 4.3). The dry weight of nodules also followed a similar trend. The results showed that the higher the number of nodules, the higher the dry weight. This result is substantiated by the assertion made by Oti and Agbim (2000a) that a simple relationship exists between nodule number and nodule dry weight, and they are indices of N_2 fixation.

The inhibitory effect of high inorganic nitrogen on nodulation and nitrogen fixation has been reported by many researchers (Eaglesham *et al.*, 1983; Becker *et al.*, 1986; Waterer, 1992). It has been suggested by Harper (1987) and Streeter (1988) that there are multiple effects of nitrate inhibition; such as decrease in number of nodules, nodule dry mass and N_2 fixation activity.

Furthermore factors such as acceleration of nodule senescence or disintegration as well as carbohydrate deprivation in nodules (Streeter, 1988; Vessy & Waterer, 1992), feedback inhibition by products of nitrate metabolism such as glutamine (Neo & Layzell, 1997), asparagine (Bacanamwo & Harper, 1997), and decreased O_2 diffusion into nodules which restricts the respiration of bacteroids (Gordon *et al.*, 2002) have also been documented. The generally poor N_2 fixed upon addition of mineral nitrogen suggests that African yam bean is very sensitive to mineral nitrogen and thus low levels or no supply of available nitrogen may cause the crop to fix more nitrogen for its use. However, the higher dry matter yield observed in N–fertilised plants than the unfertilised control plants is indicative that N_2 fixation was not supplying the plants with all the nitrogen it needed for maximum growth, or that the strains that nodulated were not highly effective in N_2 fixation. These observations were supported by conclusions made by Sanginga *et al.* (2000b) that the improved growth and yield observed in soybean line 1456–2E, indicated that N_2 fixation induced by indigenous bradyrhizobia community supplied less than optimal amounts of nitrogen.

The inhibitory effect of available nitrogen on nodulation was more prominent in Akuse than in Adenta. With soils low in mineral N, a moderate dose of starter–N has been demonstrated to stimulate seedling growth and subsequently N_2 fixation (Herridge *et al.*, 1984). Becker *et al* (1986) stated that nodulating legumes prefer easily absorbable forms of N to nodulation and subsequent fixation of atmospheric N because of the "cost of energy" involved in the later. The available N inhibitory effect was more severe in Akuse soil than in Adenta soil. This could also be due to the slightly high pH (6.5) of Akuse soil. At pHs' near neutral (near pH 7), the microbial conversion of ammonium (NH_4^+) to nitrate (NO_3^-) is rapid, and crops generally take up nitrate

(Rosen *et al.*, 2004). This must have contributed to the low performance (in terms of nodulation) of African yam bean on Akuse soil compared to Adenta soil.

Dry matter accumulation is one of the measures of plant growth (Noggle & Fritz, 1983), a function of crop species and soil fertility (Oti & Agbim, 2000b) and reflects the relative growth rate with regards to the net assimilation rate. The result in the present study (Figure 4.1) showed that increasing the rate of mineral N resulted in a corresponding improvement in shoot biomass accumulation of African yam bean in both Adenta and Akuse soils. The significant positive relationship between dry matter and N accumulation (% N) is in agreement with work done by Oikeh *et al.* (2008) who also observed a direct relationship between dry matter and N accumulation in legumes. This confirms the assertion made by Becker *et al.* (1986) that root nodulating legumes prefer the uptake of available or mineral nitrogen to atmospheric nitrogen fixation, in order to conserve energy. The high N accumulation and its subsequent increase in shoot dry weight recorded in response to higher rates of nitrogen, supports the conclusions made by Mae (1997) that larger amounts of absorbed nitrogen increases leaf weight and N content in leaves, which leads to enhancement of photosynthetic capacity and promotion of carbohydrate accumulation in leaf. Sage and Pearcy (1987) also reported that photosynthetic capacity and total amount of leaf N per unit leaf area are usually correlated. Dordas and Sioulas (2009) stated that the shortage or excess of nitrogen can affect assimilate partitioning between the vegetative and reproductive organs as well as dry matter partitioning. This may have been responsible for the low dry matter yield recorded in the low N fertilised treatment.

The results of nitrogen influence on N uptake indicate that nitrogen uptake increased significantly with increasing rate of application (Table 4.4). The relationship that exists between N and dry matter yield, as well as N concentration and N uptake in African yam bean led to the

conclusion that the high N uptake recorded is due to high dry matter yield of the African yam bean recorded at higher rates of nitrogen and not necessarily due to high nitrogen concentration (%N) in the plant biomass or tissue. Plant biomass and tissue N (%N) were linearly correlated to nodule mass while correlation to nodule number was low, suggesting nodule mass to be a better indicator of symbiotic efficiency than nodule number.

5.3.2 Response of African Yam Bean to Phosphorus Fertilization

The influence of P on symbiotic nitrogen fixation in leguminous plants has received considerable attention. Results from this study indicate that P application significantly improved nodulation in both soils; with nodule numbers ranging from 34 nodules plant^{-1} in the control to 78 nodules plant^{-1} at 120 kg P ha^{-1} in Adenta soil and 15 nodules plant^{-1} in the control to 94 nodules plant^{-1} at 120 kg P ha^{-1} in Akuse soil (Table 4.6). This increase in nodulation upon P addition, is supported by conclusions that, in legumes, phosphorus deficiency particularly, affects symbiotic nitrogen fixation by limiting the growth and survival of rhizobia (O'Hara *et al.*, 1988), nodule formation (Drevon & Hartwig, 1997), nodule functioning (Tang *et al.*, 2001a), host plant growth (Tsvetkova & Georgiev, 2003), root development and N_2 fixation (Danso, 1992). However some contradictory reports on P requirements for nodule formation and functioning have also been recorded. For instance, phosphorus deficiency has been reported either increase, decrease or not affect the nodule number per unit of shoot mass (Tang *et al.*, 2001; Vadez *et al.*, 1996), as well as the specific nitrogenase activity and the amount of N_2 fixed per nodule mass (Tang *et al.*, 2001b). Secondly, Sa and Israel (1991) also concluded that the importance of P for optimal growth and symbiotic N_2 fixation processes can be assessed by nodule development and functioning. Robson and O'Hara (1981) concluded that P nutrition increased symbiotic N_2

fixation in subterranean clover (*Trifolium subterraneum L.)* by stimulating host plant growth rather than by exerting specific effects on rhizobial growth or on nodule formation and function. Higher nodule mass with increasing P rates was observed (Table 4.6). Thus lower rates of P resulted in fewer and lighter nodules compared to higher P rates. Miller *et al.* (1982) reported that nodule weight was a contributing factor to nitrogen fixation activity while nodule number was important in its relationship with nodule weight. Phosphorus deficiency might have caused a negative effect on the processes of nitrogen fixation by decreasing nodule capacity to fix atmospheric N_2 as a result of lower nodule size. The possibility that ATP synthesis was limited by deprivation in inorganic P (Sa & Israel, 1991) could also be considered.

Dry matter accumulation also increased significantly with increase in phosphorus rates. The significant performance of African yam bean on both soils in terms of dry matter accumulation may be due to the high level of available P which promoted early development of root, higher P uptake and subsequently better growth of the crop. The results from this study showed a positive relationship between shoot dry matter yields and P application in both soils (Figure 4.2). The increase in plant dry matter accumulation could be due to the fact that P is an important element that significantly affects plant growth and metabolism (Brady & Weil, 2004). Phosphorus is a component of DNA and RNA, involved in cell division and is important for plant growth (Brady & Weil, 2004). Thus the expansion (growth and development) of plants under P stress conditions decreased by the number of cell divisions, which implies control of cell division by a common regulatory factor (Chiera *et al.*, 2002). Phosphorous also encourages vegetative growth and formation of root system which efficiently absorbs nutrients from soil (Shukla *et al.*, 2010).

The control in the Adenta soil performed better in P uptake than the counterpart in the Akuse soil (Table 4.9). This may be due to the higher level of available P in the original Adenta soil which

may have enhanced early development of roots of the crop. This effect resulted in higher shoot dry matter yield in the Adenta soil and it confirms the important role that P availability plays in terms of P uptake and dry matter yield of crops (Marschner, 1993).

The significant increase of nitrogen fixation (Table 4.7) in African yam bean upon P addition was also supported by increases in the number of nodules and nodule dry weight (Table 4.6). These observations agree with conclusions made by Ibeawuchi *et al.* (2004), who reported that nodule weight and nodule numbers are all indices of nitrogen fixation since they have either direct or indirect relationship. The high nodule dry weight and number of nodules of African yam bean and its ability to fix nitrogen in this study are an indication of the importance of African yam bean in sustainable agriculture. Also, the positive response to P fertilizer observed in this study may be due to the low native available P of the soils coupled with the specific role of phosphorus in nodule initiation, growth and functioning.

Richardson *et al.* (2011) defined phosphorus utilization efficiency as the ability of a plant species to produce higher dry matter per unit of P absorbed or taken up. The phosphorus utilization efficiency of African yam bean was computed and the results showed that, P uptake increased with an increase in P application, but the P utilization efficiency decreased (Table 4.9). This implies that the utilisation of nutrients decreases with increasing rate of nutrient application, as stated by Liebig's law of minimum. This high PUE at lower rates of P, could reduce fertilizer input cost and also decrease the rate of nutrient losses and enhance crop yields. Efficient utilization of acquired P is also considered an important adaptation for plant growth on low P soils. According to Raghothama (1999) the mechanism of higher internal P utilization efficiency may be related to the ability of a plant in releasing inorganic P from the storage pool (vacuole) to the cytoplasm. Also selective allocation of P between cytoplasm and vacuole, if in favour of

cytoplasm ensures sufficient Pi concentration in metabolically active compartments for normal functioning of plant metabolism (Raghothama, 1999).

The higher P utilization efficiency recorded in low P treatments may be due to the fact that, during P starvation, plants increase the efficiency of P use. Plaxton and Tran (2011) explained this phenomenon as a regulation of a wide array of P–starvation inducible hydrolases that scavenge and recycle P from intra and extracellular organic P compounds.

5.4 Diversity of Indigenous African Yam Bean *Rhizobium* Isolates

5.4.1 Cross Inoculation Studies (Host Range Analysis)

The cross inoculation group concept is based on the ability of rhizobia to specifically nodulate a group of legume host species (Fred *et al.*, 1932). It is based on this concept that species of *Rhizobium* have been classified as either promiscuous or specific. The concept was therefore applied in this study to determine the symbiotic specificity or otherwise of native African yam bean bradyrhizobia obtained from the screening experiment. The results showed that isolates of rhizobium obtained from the African yam bean were compatible with cowpea, yardlong bean, and pueraria, but did not form nodules on lima bean, Bambara and crotalaria. This may mean that African yam bean could be in the same cross inoculation group with *Pueraria spp* and yardlong bean.

Lie *et al.* (1987) stated that population diversity among rhizobia compatible with a particular legume is likely to be greatest in the center of origin of that host. However, varied rhizobial populations can arise in symbiosis with species that are not indigenous to a particular region (Sadowsky & Graham, 1998). Further experimental studies conducted in the frame of this project, where African yam bean was cross inoculated with rhizobia isolates from different

legumes species, showed that although rhizobium isolated from the African yam bean were unable to nodulate lima bean, bambara and crotalaria isolates from each of these three legumes formed nodules on African yam bean. This observation is confirmed by similar results obtained by Assefa and Kleiner (1997) who cross inoculated African yam bean with *Bradyrhizobium* sp. AUEB20, isolated from *Erythrina brucei* (an Ethiopian tree legume). They reported that African yam bean plants were nodulated profusely and the nodules were observed to be active in nitrogen fixation. Oagile (2005) also found African yam bean landraces to form nitrogen fixing nodules when inoculated with strains of *Rhizobium spp*, ORS 302 (broad host range), and *Bradyrhizobium spp*, CP 279 (broad host range). The ability of African yam bean to nodulate profusely with *Rhizobium* indigenous to Ghanaian soils confirms conclusions made by Oagile (2005) that describes African yam bean to be a promiscuous legume, being able to form nodules with three of the five strains tested and belonging to both *Rhizobium* spp. and *Bradyrhizobium* spp. The large numbers of nodules formed in African yam bean landraces reported by Oagile (2005) were also found to be comparable to numbers recorded on cowpea inoculated with *Bradyrhizobium* strains indigenous to Ghanaian soils (Obiagwu, 1995).

Three out of the six host legumes tested were nodulated by 80% of the African yam bean bradyrhizobia isolates tested. This agrees with assumption made by Halliday (1985) that most tropical legumes are non–selective in the rhizobia they require for effective symbioses. However, contrasting result was observed when rhizobia isolated from lima bean and bambara nodulated with African yam bean. Similar results of one rather than dual–way nodulation has been observed in studies on tree legume rhizobia (Boakye, 2014).

This result on host range studies reflects the promiscuity of the tested isolates and differences between these findings may be due to the particular isolate(s) tested. It is well recognized that

strains within a rhizobial population show great variation in their specificity and symbiotic effectiveness on many hosts. It is, therefore, not surprising to observe similar results in this study whereby all the isolates exhibited some variations in interaction with other host plants

5.4.2 Molecular Analysis

The genetic diversity of 30 bradyrhizobia isolated from root nodules of African yam bean, cultivated in four Ghanaian soils was studied by RAPD (Random amplified polymorphic DNA) (RPO4 primer), DAPD (Direct amplified polymorphic DNA) (RPO1 primer) and restriction fragment length polymorphism (RFLP) analysis of PCR–amplified ITS and 16S rDNA techniques.

The amplification profiles generated by RPO4 differentiated between the African yam bean bradyrhizobia isolate tested. The primer grouped the rhizobia isolates into different clusters (Figure 4.5) at different levels of similarity. The results from the RAPD analysis showed that PCR using short arbitrary oligonucleotide primers of random sequence is an effective means of differentiating complex DNA genomes (Welsh & McClelland, 1990). Closely related taxa had similar fragment distributions, while distantly related ones were more divergent. This confirms conclusions made by Borowsky *et al.* (1995) that arbitrarily primed oligonucleotide (RPO4) amplifications contain considerable phylogenetic information.

Direct amplification of polymorphic DNAs (DAPD) fingerprinting also proved to be highly discriminatory and fast in strain identification (Figure 4.7). These results are supported by the conclusions that DNA fingerprinting with RPO1 primer is a global technique, as the sequence of the RPO1 primer is reiterated in rhizobial genomes, including plasmids and chromosomes (Alexandre *et al.,* 2006). Also the analysis of *Mesorhizobium loti* genome revealed more than 60

regions with homology to RPO1 primer sequence (Laranjo *et al.,* 2002). This confirms the discriminatory power of RPO1 primer. The dendrogram generated from the RPO1 amplification profiles shows some similarity with the clustering revealed by RPO4 primer. However in contrast to RPO4 primer, RPO1 primer clustered most (27) of the isolates instead of nine by RPO4 into the first major cluster with only three rather than 21 isolates in the second major cluster (Figure 4.6).

The use of the *nif*–directed RPO1 primer for amplifying the Rhizobium strains enable the direct differentiation of the strains tested. This may be due to the fact that the amplification profiles generated by the RPO1 primer were not affected by the presence of contaminating DNAs. However, though not investigated, it is assumed that this may not be achieved or possible with 10–*mer* arbitrary primers such as RPO4 and RPO5, etc, which are expected to amplify a wide range of target DNA (Welsh & McClelland, 1990).

The Shannon–Weaver diversity index calculated for the various soils using RPO1 and RPO4 primers showed *Bradyrhizobium* diversity among the four soils. The large diversity of rhizobia–nodulating African yam bean in the different soils may indicate that African yam bean accepts many different microsymbionts and indicates broad host range ability. This could be supported by the assertion that, the use of techniques targeting more specific functional symbiotic genes such as *nod* and *nif* genes could reveal more information on rhizobia diversity.

The restriction analysis of the ITS and 16S gene regions revealed additional heterogeneity in the bradyrhizobia isolates. Variations in length of the ITS region recorded in this study have also been observed in other bacteria. ITS band sizes of 303–305 bp have been reported for *Staphylococcus aureus* (Gurtler & Barrie, 1995), 100–700 bp for *Pseudomonas aeruginosa*

strains (Hartman *et al.*, 1986). The ITS band size ranges recorded within the African yam bean bradyrhizobia population studied, demonstrates that the 16S–23S rDNA spacer region could be of taxonomic importance. Furthermore, digestion of this region with *Hha*I and *Hind*III restriction enzymes was also useful for fingerprinting the rhizobia isolates, all of which were obtained from the same legume host (African yam bean), and with the potential for different nodules to harbour the same or different strains. The general level of similarity revealed by the ITS PCR–RFLP analysis are consistent with both that of RPO1 and RPO4 primers. The results of this experiment further showed that there is a strong tendency of isolates from the same location or soil to be grouped together. This suggests that restriction analysis of amplified DNA may serve as an efficient tool for rhizobial population studies across different locations or soil.

The 16S rDNA gene PCR–RFLP pattern has been used to detect potential taxa of new isolates (Laguerre *et al.*, 1994). The 16S rDNA gene is one of the most conserved genes of a DNA, implying that it has undergone very little change throughout time, or it varies very little from cell to cell. Even organisms that are distantly related, or that evolved a long time ago, have 16S rDNA sequences that are very similar. Scientists use this gene, as well as other ribosomal genes, to measure taxonomy, phylogeny and the rate of divergence. It is also known that a longer stretch of the 16S rDNA gene (e.g. 800 instead of 300 bp) contains a conserved region that is sufficient to show the variation within groups of root rhizobia, hence its frequent use to infer the phylogenetic affiliation of isolates (Odee *et al.*, 2002; Bala *et al.*, 2003). In this study, by using 16S rDNA gene PCR–RFLP, several subgroups were identified (Figure 4.6). The groups produced by the RFLP analysis confirmed the groupings established by RAPD and DAPD analyses.

The wide genetic diversity of the *Bradyrhizobium* isolates that were obtained from the same legume specie (African yam bean) has further strengthened the views of workers such as Odee *et al.* (2002) that the sub–Saharan region might be an important centre of rhizobial biodiversity.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY AND CONCLUSION

The primary purpose of this study was to assess the nodulation, nitrogen fixation abilities and growth of uninoculated African yam bean, how these parameters are influenced by the N and P levels, and the diversity of the indigenous rhizobia that nodulate African yam bean in each of four Ghanaian soils.

The results obtained indicate that rhizobia capable of forming root nodules on African yam bean exist in Ghanaian soils, even though in varying populations. Akuse soil, followed by Adenta and Haatso soil harboured the highest number of African yam bean rhizobia (200, 170 and 140 cells g^{-1} soil, respectively). Toje soil recorded the lowest population of 93 cell g^{-1} soil of African yam bean nodulating rhizobia.

Interestingly, screening for nodulation of African yam bean on these soils showed that African yam bean plants grown on Haatso and Adenta soils formed the highest number of nodules (39 and 37 nodules per plant, respectively), even though the MPN count reported lower populations than were present in the Akuse soil.

The application of nitrogen and phosphorus affected various parameters such as nodulation, nodule mass, nitrogen fixation, nutrient uptake and shoot dry matter yield. Increasing the mineral N fertility of the soils, resulted in lower nodulation, reduced nodule masses, with corresponding lower nitrogen fixation, but in increased shoot dry matter yield in both Adenta and Akuse soils. The shoot total nitrogen (N uptake) was also increased upon N addition.

In contrast to N application, P fertilization enhanced nodulation, nodule mass, nitrogen fixation, increased N and P uptake, increased shoot dry matter yield and improved growth.

Cross inoculation studies revealed African yam bean to be compatible with rhizobia from lima bean, bambara groundnut, and yardlong bean. Also African yam bean *Bradyrhizobium* was able to nodulate cowpea, yardlong bean and pueraria spp.

Further genetic diversity studies of African yam bean bradyrhizobia isolates collected from four Ghanaian soils suggests that random and direct amplification of the genomic DNA and PCR–RFLP analysis of the ITS and 16S regions can be used whenever necessary in evaluating collections of rhizobial isolates.

Moreover, random and direct fingerprinting proved to be fast and reproducible methods for rhizobial isolate differentiation. Ranges of discriminating powers were also equivalent between the two approaches. These markers have found a wide range of application in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with previous methods.

Cluster analysis of RAPD and DADP–generated genomic fingerprint yielded results that are in close agreement with ARDRA analysis as well as by direct comparison of primary similarity values. The observed correlation between RAPD, DAPD and ARDRA genomic fingerprint analyses suggests that genomic fingerprint methods can function as core techniques in polyphasic taxonomy, regardless of the statistical model employed.

However, since different numbers of bands may be generated with each primer set, the annealing conditions used, and the prevalence distribution of the target repetitive elements in question may

vary. An example of a similar variation may occur in RFLP, ARDRA and AFLP, depending on the restriction that is used.

6.2 RECOMMENDATIONS

Based on the results obtained from this study, the following recommendations are made:

1) The study should be conducted in various areas with different soil types to establish the validity of these conclusion that African yam bean nodulating rhizobia are common in most if not all Ghanaian soils.

2) African yam bean should be allowed to grow to maturity to observe the effect of nodulation and nitrogen fixation on seed yield.

3) Further taxonomic study of indigenous African yam bean *Rhizobium* isolates in different soils should be carried out by taking as many morphological, biochemical and genetic characters as possible to have a more complete picture of their taxonomy and the evolutionary relationship of the African yam bean rhizobia.

4) Finally, although the African yam bean appears to be a relatively permissive host, this character does not guarantee effectiveness in nitrogen fixation and may lead to formation of less effective symbioses with strains not highly evolved to fix nitrogen with African yam bean. Because little has been done to measure potential benefits of rhizobial diversity, nitrogen fixation, and African yam bean productivity, I believe that research in this area may be useful in the future.

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APPENDICES

Appendix A: 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	1.0
Agar	15.0
Sterile distilled water	1 L

Appendix B: Composition and Preparation of N–free Nutrient Solution (Broughton & Dillworth, 1971).

Stock solution	**Composition**	**Quantity (g L^{-1})**
Solution 1	$CaCl_2.2H_2O$	249.1
Solution 2	KH_2PO_4	135.1
Solution 3	Fe-citrate	6.70
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
Solution 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

For each 10 litres of full strength culture solution, take 5.0 mL each of solutions 1 to 4, then add to 5.0 litres of water, then dilute to 10 litres. Use 1N NaOH to adjust the pH to 6.6–6.8.

Appendix C: ANOVA table for Nitrogen effect on some growth parameters of African yam bean in Adenta and Akuse soils'.

Variate: Nodule number plant^{-1} **(Adenta).**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	1505.05	501.69	28.67	<.001
Residual	8	140.00	17.50		
Total	11	1645.06			

Variate: Nodule number plant^{-1} **(Akuse).**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	287.08	95.69	8.92	0.006
Residual	8	85.83	10.73		
Total	11	372.92			

Variate: Nodule dry weight (mg plant^{-1}) **(Adenta)**.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	24327	8109	1.55	0.25
Residual	26	41738	5217		
Total	29	66065			

Variate: Nodule dry weight (mg plant^{-1}) **(Akuse)**.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	669.13	223.04	8.00	<.001
Residual	8	223.09	27.89		
Total	11	892.22			

Variate: Shot total N (mg/plant) **(Adenta).**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	7155.05	2385.02	42.34	<.001
Residual	8	450.62	56.33		
Total	12	7605.67			

Variate: Shoot total N (mg/plant) **(Akuse).**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	19322.83	6440.94	108.58	<.001
Residual	8	474.57	59.32		
Total	12	19797.40			

Variate: Shoot dry weight (g plant^{-1}) **(Adenta)**.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	0.683	0.229	6.21	0.017
Residual	8	0.293	0.037		
Total	12	0.976			

Variate: Shoot dry weight (g plant^{-1}) **(Akuse)**.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	4.549	0.5162	46.07	<.001
Residual	8	0.263	0.033		
Total	11	4.812			

Appendix D: ANOVA tables for Phosphorus effect on some growth parameters of African yam bean in Adenta and Akuse soils'.

Variate: Nodule number plant^{-1} in Adenta soil

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	4722.92	1574.31	17.83	<.001
Residual	8	706.33	88.29		
Total	12	5429.25			

Variate: Nodule number plant^{-1} in Akuse soil

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	12615.73	4205.24	108.70	<.001
Residual	8	309.50	38.69		
Total	11	12925.23			

Variate: Nodule dry weight (mg/plant) **(Adenta)**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	11693	37231	17.16	<.001
Residual	8	17357	2170		
Total	11	129050			

Variate: Nodule dry weight (mg/plant) **(Akuse)**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	132563	44188	43.43	<.001
Residual	8	8140	1017		
Total	11	140703			

Variate: Shoot total N (mg/plant) **(Adenta)**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	11048.62	3682.87	86.32	<.001
Residual	8	341.33			
Total	11	1389.95			

Variate: Shoot total N (mg/plant) **(Akuse)**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	24356.3	8118.8	33.61	<.001
Residual	8	1932.6	241.6		
Total	11	26288.9			

Variate: Shoot dry weight (g/plant) **(Adenta)**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	4.24937	1.4165	30.06	<.001
Residual	8	0.7693	0.0471		
Total	11	4.62631			

Variate: Shoot dry weight (g/plant) (**Akuse).**

Source of variation	d.f	s.s.	m.s.	v.r.	F pr
Treatments	3	11.2379	3.7460	29.89	<.001
Residual	8	1.0026	0.1253		
Total	11	12.2405			