THE OCCURRENCE AND DIVERSITY OF INDIGENOUS BRADYRHIZOBIA THAT NODULATE AND FIX NITROGEN IN SOYBEAN AND PIGEONPEA IN THREE GHANAIAN SOILS UNDER DIFFERENT LEVELS OF N AND P FERTILIZERS

By

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

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DECLARATION

I hereby declare that, this thesis has been written by me and that it is the record of my own research work. To the best of my knowledge and belief, it has neither in whole nor in part been presented for another degree elsewhere. Works of other researchers have been cited and duly referenced. Also all assistances received have been duly acknowledged.

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Prof. E. Owusu-Bennoah (Co-supervisor)   Date
DEDICATION

This thesis is dedicated to the bright memory of Professor S. K. A. Danso, for the advice, encouragement and immense support both financially and socially that he gave me in pursuance of my second degree. Unfortunately, he passed on in about a month to the final submission. May his gentle soul rest in peace.
ACKNOWLEDGEMENTS

I am most grateful to God Almighty without whose providence and faithful guidance, I would not have been able to come this far in my academic career.

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ABSTRACT

The need to feed the projected world population of 9.7 billion by 2050 has received much attention and increased food production has been recommended to address the problem. Soybean and pigeonpea are important global commercial and food legumes that have been recommended for food security and sustainable agriculture particularly in tropical and subtropical regions where the majority of the world’s resource poor live, and where agricultural production is constrained by low soil fertility, primarily by soil N and P deficiencies. It is therefore necessary to enhance the legumes’ ability to access more and more of their N from biological nitrogen fixation, so as to decrease the need for expensive inorganic N fertilizers especially for these poor farmers. There is, however, limited information on biological nitrogen fixation and factors that affect this process in Ghana. In this study, the effects of phosphorus and nitrogen on nodulation, dry weight of nodules formed and growth of soybean and pigeonpea were determined. Except at excessive rates of P (160 kg P/ha and 200 kg P/ha) which decreased nodulation and growth of nodules formed on pigeonpea in the Haplic Acrisol from the coastal savannah, application of P increased nodulation and growth of nodules in both pigeonpea and soybean. In contrast to P, N application to soil inhibited nodulation in the legumes. Application of 80 kg/ha P and above in combination with an inhibitory rate of N (100 kg N/ha) revived nodulation and nodule growth in all cases. The conclusion from this study is that, for a given legume, the amount of N in a soil’s solution that may induce toxicity and subsequently inhibit nodulation under low P conditions would not be enough to support the growth and nodulation of that same legume when P is applied to the soil. Such conditions become favourable for the growth of the legume as well as nodulation in the presence of compatible bradyrhizobia.
The effect of the application of P on N acquisition and symbiotic N\textsubscript{2} fixation in soybean plants grown in a coastal savannah Haplic Acrisol was also determined in another study. Application of P enhanced both % N\textsubscript{dfa} and total N fixed. Highest increase in BNF occurred between the 0 and 40 kg P/ ha. However, highest % N\textsubscript{dfa} (54.7 %) and total N\textsubscript{2} fixed (51.5 kg N/ha) occurred when 120 kg P/ ha was applied to the soil. The conclusion from the study is that nodules were more efficient at fixing N at lower P rates compared to higher P rates.

In this study, the indigenous populations of bradyrhizobia for cowpea and soybean were estimated using the most probable number (MPN) plant infection assay. Except for the Ferric Acrisol which contained very low populations (less than 10 cells/ g soil) of indigenous soybean \textit{Bradyrhizobium}, the Haplic Acrisols from the coastal savannah and semi-deciduous forest both contained satisfactory number of indigenous bradyrhizobia that were capable of nodulating both Soybean and Cowpea. The diversity of 120 bradyrhizobial strains isolated from cowpea, soybean and pigeonpea root nodules was investigated using DAPD and RAPD fingerprinting with primers RPO1 and RPO4, respectively. Based on the combined RPO1-PCR and RPO4-PCR patterns, a high diversity existed within and between indigenous bradyrhizobial isolates that nodulated cowpea, pigeonpea and soybean grown in the soils from the different agro-ecological zones. Phosphorus application had varying effects on the diversity of isolates from the different soils that nodulated soybean pigeonpea and cowpea. The conclusion is that, the increased number of nodules formed on legumes with P application is not always associated with increased diversity of the compatible bradyrhizobia. Thus bradyrhizobia differ in their soil P tolerance.
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CHAPTER ONE

1.0 INTRODUCTION

The current 7.1 billion (2013) and projected 9.7 billion by 2050 global population statistics (PRB, 2013) have resulted in an increased need for food production and distribution (food security) to support the continuity of the human race beyond 2050 (Nellemann et al., 2009). Statistics show that, the greater proportion of the population increase will come from the regions where the resource poor live (PRB, 2013).

The need for combating hunger (promoting food security) through soil fertility replenishment in sustainable agriculture have received much attention and have yielded good results as can be seen in the decline of the global hungry people from 1 billion to 842 million in the past two decades (FAOSTAT, 2013). The decline in the number is obviously not due to increase in death tolls but as a result of more food being produced and made available to the hungry (Waggoner, 1994; Trewavas, 2001).

The attempts made to increase food production seem paradoxical as efforts in the past two decades have resulted in a decline in the fertility status of soils under cultivation, especially those in the tropics and sub-tropics (Koning and Smaling, 2005; Muchena et al., 2005), where majority of the world’s resource-poor live.

The use of chemical fertilizers (Sanchez et al., 1997) has therefore been recommended as a first step approach in addressing the problem of soil fertility depletion (Sanchez and Jama, 2002) to correct primarily for deficiencies in soil nitrogen (N) and phosphorus (P), the two most limiting plant macronutrients in tropical soils (Bieleski, 1973; Sanchez and Salinas, 1981; Nyemba, 1986; Mengel and Kirkiby, 1987; Smil, 1999; Socolow, 1999; Graham and Vance, 2000; Mamo et al., 2002).
The high cost of these fertilizers has, however, deterred poor peasant farmers from using fertilizers (Sanchez et al., 1997; Vance, 2001). As such, the problem of low soil fertility persists in tropical and sub-tropical farmers' fields giving low crop yields after cultivation (Kang, 1989; Mwangi, 1996; Ibeawuchi et al., 2009). In an attempt to increase food production, farmers in tropical and subtropical regions abandon degraded lands to cultivate new areas (Lobell et al., 2009). The soils within the areas that are being relied upon for increased food production in most cases are marginal and as such, often get depleted of plant nutrients in no time, consequently, becoming degraded in many cases (Scherr and Hazell, 1994; Scherr and Yadav 1996). The bigger problem is that, the area of degraded lands in the tropical and sub-tropical regions is always increasing (Hatermink, 2006) warranting an urgent need for restoration. However, the cost of reclaiming degraded lands and the restoration of soil fertility is very high (FAOSTAT, 2013) and not all governments are willing to commit resources to that effect.

Whereas phosphorus replenishment strategies are mainly through non-renewable mineral-fertilizer supplementation (Sanchez et al., 1997; Ezawa et al., 2002), nitrogen replenishment may include biologically based strategies such as biologically fixed nitrogen, particularly through the symbiosis between legumes and rhizobia (Bejiga, 2004) as a supplement to non-renewable mineral-fertilizer application (Sanchez et al., 1997).

Biological nitrogen fixation (BNF) is a natural process far cheaper than chemical fertilizers and also environmentally friendly and has therefore been recommended for tropical farmers to correct for soil N deficiency (Mugwe et al., 2007). Because of their ability to grow in poor soils or on their lower dependence on the soil’s nitrogen supply, legumes in general and grain legumes in particular have been suggested in sustainable farming (Jemo et al., 2010). The symbiosis between these legumes and compatible rhizobia is of greatest agricultural importance (Anjum et al., 2007; Chianu et al., 2011). In addition, these legumes contribute organic matter to soils (Valenzuela, 2011).
Cowpea, soybean and pigeonpea are examples of grain legumes commonly cultivated in Ghana (Adjei-Nsiah, 2012, Monitor group, 2012). Of these, soybean and pigeonpea are important commercial and food legumes recommended for food security and sustainable agriculture (Asgar et al., 2010; Mula and Saxena, 2010; Qiu et al., 2013). Although there is some information on BNF on legumes such as soybean and pigeonpea in cropping systems in Ghana (Karbo et al., 1998; Yeboah et al., 2004; Abunyewa and Karbo, 2005; Adjei-Nsiah et al., 2007; Fening et al., 2009; Adjei-Nsiah, 2012), there is still room for more studies.

Soybean has been demonstrated to give potential yields of about 4.5-6 MT/ha in Ghana (Lawson et. al., 2008; MiDA, 2010). However, the current average yield of soybean in Ghana (0.8 MT/ha) is very low (MiDA, 2010). Although soybean was introduced in Ghana as far back as 1909 (Snow, 1961; Mercer-Quarshie and Nsowah, 1975), soybean cultivation is mainly concentrated in the northern sector of the country (Plahar, 2006) despite the vast areas of arable land (forests and coastal savannahs) available (SRID, 2001) in the southern sector. Consequently, soybean production in Ghana is very low.

Pigeonpea, another food security legume has a great potential for soils low in both N and P and an ability to tolerate a wide range of environmental conditions including drought. Pigeonpea is known to fix large amounts of N and has the ability to release bound sources of P in soil by its root exudates (Ae et al., 1990). In Ghana, the legume has been found to have a great potential in the semi-deciduous forest agro-ecological zone, where it increases soil fertility through nutrient cycling and its ability to grow in the low P soils (Adjei-Nsiah et al., 2007). However, the potential of pigeonpea as a soil fertility improvement crop through nitrogen fixation (Peoples et al., 1995) has not been exploited to any appreciable extent and the amount of land cultivated to pigeonpea in Ghana is very negligible (Adjei-Nsiah, et al., 2007).
In general little work has been done on the different soils (agro-ecological zones) in Ghana with regards to; (a) Nutrient requirements of soybean and pigeonpea, (b) Establishing the need for soybean and pigeonpea inoculation and (c) Isolation and preservation of high quality strains of rhizobia for inoculant production, and these have also contributed to the low production of these grain legumes in Ghana.

Phosphorus has been recognized as a key nutrient element in the growth of crops in general and legumes in particular (Poehlman, 1991; Parvez et al., 2013). Phosphorus serves as a key constituent of ATP for powering nitrogen fixation and plays significant roles in energy transformations (Sankar, 1984; Mullins 2001). Gentili and Huss-Danell (2003) have reported that nodule initiation and growth of legumes have a high P demand and that phosphorus is necessary for optimizing nitrogen fixation. However, P requirements for maximum nodulation and nitrogen fixation may differ in different soils (Shu Jie et al., 2007) and for different legumes. This may be influenced by the different populations of native rhizobia in the different soils, especially across different agro-ecological zones (Fening, 1999; Klogo, 2006; Boakye, 2013) as well as the difference in soil physical and chemical characteristics across the agro-ecological zones and also the genetic differences among legume types (Boateng, 2012). In a recent study, Boateng (2012) reported that, application of phosphorus (TSP) significantly increased nodulation by cowpea and pigeonpea in a soil from the semi-deciduous rainforest and also increased nodulation by cowpea, soybean and pigeonpea in a soil from the coastal savannah agro-ecological zone of Ghana. Soybean did not nodulate at all in the Nzima soil series from the semi-deciduous rainforest of Ghana.

It is therefore necessary to study the N and P requirements for the successful establishment of soybean and pigeonpea under low N and P soils and the response of the symbiotic process and the diversity of the compatible bradyrhizobia to different levels of soil P.
The research therefore aimed at studying the nodulation and nitrogen fixation of soybean and pigeonpea under N and P fertilization and the diversity of compatible *Bradyrhizobium* in Ghanaian soils under different phosphorus levels. Specifically, the following objectives were investigated:

- To determine the indigenous populations of bradyrhizobia that nodulate soybean, and cowpea in a Ferric Acrisol from the semi-deciduous forest of Ghana and two Haplic Acrisols each from the coastal savannah and the semi-deciduous forest zones of Ghana.

- To determine the effects of phosphorus and nitrogen levels on nodulation, nodule growth and growth of soybean and pigeonpea in the Ferric Acrisol and the coastal savannah Haplic Acrisol.

- To determine the effect of Phosphorus on nodulation, total N uptake and symbiotic N\textsubscript{2} fixation in soybean plants grown in the Haplic Acrisol from the coastal savannah zone.

- To investigate the diversity of indigenous populations of cowpea, pigeonpea and soybean *Bradyrhizobium* in the Ferric Acrisol as well as the Haplic Acrisols with or without phosphorus fertilization.

The following hypotheses were tested:

1. **Ho**: The population of indigenous soybean and cowpea bradyrhizobia in Ghanaian soils is low and insufficient to initiate nodulation in these legumes.

   **Ha**: The population of indigenous soybean and pigeonpea bradyrhizobia in Ghanaian soils is high enough to initiate nodulation in these legumes.

2. **Ho**: The addition of N and P will not affect the nodulation, nodule growth and growth of soybean and pigeonpea plants.
HA: The addition of N and P will affect the nodulation, nodule growth and growth of soybean and pigeonpea plants.

3. Ho: The addition of P will not enhance the nodulation, total N uptake and symbiotic N₂ fixation for meeting the total N requirement of soybean grown in the coastal savannah Haplic Acrisol.

HA: The total N requirements of soybean grown in the coastal savannah Haplic Acrisol soil could only be met by the symbiosis with P addition.

4. Ho: The genotypic characteristics of the indigenous bradyrhizobia nodulating a particular legume are not diverse.

HA: There is great diversity among the genotypic characteristics of the indigenous bradyrhizobia nodulating a particular legume.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

This Chapter presents a review of studies conducted on Biological Nitrogen Fixation (BNF) in legumes in general, and more specifically on soybean and pigeonpea.

2.2 Nitrogen fixation

Nitrogen is a mineral element that plays a very significant role in sustaining life. Till date, nitrogen still remains the soil nutrient element needed in greatest quantity by crops (Panwar and Laxmi, 2005; Crosby et al., 2008). Nitrogen occurs in the atmosphere as dinitrogen (N$_2$) gas constituting about 79% of all gases, and in a form that is not readily available to vascular plants (Bundy, 1998; Nolte, 2010). As such there is the need to convert the atmospheric form of nitrogen into usable forms that are also readily available to these plants for their growth and development.

Nitrogen fixation is the reduction of the atmospherically stable nitrogen (N$_2$) to a biologically useful, combined form (NH$_3$) (Giller and Wilson, 1991). Nitrogen fixation is an important processes for plant growth and development and the process is second only to photosynthesis in terms of importance to plants (Hernández, 2002). However, the industrial conversion (also known as Haber–Bosch process) of the very stable N$_2$ in the atmosphere into usable forms is costly energy wise (Giller and Wilson, 1991), as it requires 350-550°C of temperature and a pressure of 150-350 atmospheres. The energy cost alone comprises 70-90% of the total cost of producing nitrogen fertilizers (Plewes and Smith 2009). As energy is becoming ever scarcer; the cost of industrial N will continue to increase posing a great challenge to peasant farmers who are unable to afford inorganic nitrogenous fertilizers even at today’s prices.
The equation for the Haber–Bosch reaction (modified from Modak, 2011) is given below.

\[
N_2(g) + 3H_2(g) \xrightarrow{400^\circ C, 200 \text{ atm}} 2NH_3(g) + \Delta H^0 = -90 \text{ kJ}
\]

Nitrogen fixation also occurs through lightning (Noxon, 1976). However, this is generally insufficient for extensive crop production as it constitutes only about 10% of the world's supply of fixed nitrogen (Zahran, 1999).

Microorganisms through a process termed biological nitrogen fixation (BNF) are able to convert the atmospherically stable form of nitrogen (N\(_2\)) into plant usable forms. This microbial conversion of atmospheric N\(_2\) into plant usable forms is highly recommended for sustainable agriculture (Zahran, 1999). Biological nitrogen fixation is carried out by prokaryotic organisms that possess the enzyme nitrogenase and use energy in the form of adenosine triphosphate (ATP).

### 2.2.1 Biological Nitrogen Fixation (BNF)

Biological nitrogen fixation is a complex biochemical reaction resulting in atmospheric N\(_2\) being enzymatically reduced to NH\(_3\) by prokaryotic organisms that possess the enzyme nitrogenase (Cabello \textit{et al.}, 2012). The reduction (fixation) process can be achieved by diazotrophic organisms both in the free-living state (free-living fixers) and also in symbiotic association with plants (Hirsch \textit{et al.}, 2001).

Many cyanobacteria are found in nature as free-living N\(_2\) fixing species (Cabello \textit{et al.}, 2009). Other free living fixers include Klebsiella and Azotobacter. The actinomycetous Frankia species, cyanobacteria living in association with plants, suitably ferns and rhizobia constitute the symbiotic fixers which are dominated by the latter (Cabello \textit{et al.}, 2009). Among the symbiotic fixers, that which is of greatest agricultural importance and as such has received much attention is the symbiosis between rhizobia and legumes (Cabello \textit{et al.}, 2009). Symbiotic
nitrogen fixation that occur between legumes and rhizobia involve different hosts and microsymbionts, respectively (Zahran, 1999; Simon et al., 2007). Biological nitrogen fixation as used in this thesis refers to the symbiotic nitrogen fixation between legumes and rhizobia. In order for the nitrogen fixation process to occur, leguminous host plants in the presence of their compatible rhizobia must enter into a symbiotic or mutually beneficial partnership. The complex processes and mechanisms involved in the establishment of the partnership results in the formation of an organ called nodule (Soyano et al., 2013). After a successful partnership has been established, nitrogen (N\(_2\)) in the air of soil pores around the nodules is combined with hydrogen (H\(_2\)) to form (NH\(_3\)). The NH\(_3\) is further protonated to form NH\(_4^+\) (Baker and Hall, 1988) which is the actual form of nitrogen assimilated by plants (Schubert, 1995).

The reaction equation below (Cooper and Scherer, 2012) depicts the energy demand of the biological nitrogen fixation process;

\[
N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi
\]

2.3 Legumes

Legumes refer to all flowering plants that belong to the family *Leguminosae* which is the third largest of flowering plants, comprising slightly under one twelfth of all known flowering plants (Allen and Allen, 1981; Sprent, 2001). They are found on all continents, except Antarctica (Sprent, 2001). Many members of the *Leguminosae* due to their ability to form nitrogen-fixing symbiosis with rhizobia (Doyle and Luckow, 2003) are of ecological and economic significance. The *leguminosae* are classified into three subfamilies (Sprent, 2008), namely, *Caesalpinioideae, Mimosoideae* and *Papilionoideae* which was previously called *Fabaceae* (Lewis et al., 2005). The subfamily *Papilionoideae* includes grain legumes and species of trees, shrubs, herbs and climbers while the *Caesalpinioideae* and the *Mimosoideae* include species of trees and shrubs and rarely herbs (de Faria et al., 1989, Batello et al., 2013). Among the
subfamilies, the *Papilionoideae* are the most diverse and the most economically important (Somasegaran and Hoben, 1994; van Berkum and Eardly, 1998). Currently, there are approximately 750 genera and around 20,000 species in the *Leguminosae* ranging in habit from small herbs to huge trees (Elkan, 1992; Lewis *et al*., 2005). Among the *Leguminosae*, variability in nodulation is known to exist (de Faria *et al*., 1989; Sprent, 2008).

### 2.3.1 Importance of Legumes.

Legumes, by virtue of their capacity to fix nitrogen, are widely distributed, occupying habitats ranging throughout the world from rain forests to arid zones, displaying a wide range of tolerance to acidity, alkalinity, water logging and drought, mineral deficiencies (Rengel, 2002) among others. As such, they are seen to colonize marginal lands and soils that are impoverished. In so doing legumes offer several advantages in the rehabilitation of marginalized lands and degraded soils (Fassil, 1993; Arianoutsou and Thanos, 1996) to promote ecological diversity. Legumes are known to be environmentally friendly in that, the plants assimilate all the nitrogen that is fixed by their association with rhizobia with no leaching and ground water pollution (Reetz, 1989).

### 2.3.2 Uses of legumes

Throughout history human beings have used legumes in many ways, including as sources of food, forage, fuel, shelter and traditional medicines (Duke, 1981; Saxena, 1988; Graham and Vance, 2003; Howieson *et al*., 2008). This comprises of the not more than 200 species cultivated. Majority of legumes particularly grain legumes have been incorporated into agricultural systems to replenish soil N (Adjei-Nsiah *et al*., 2007).
2.3.3 Grain legumes

Economically important species of the *Leguminosae* include grain legumes (pulses and oil seeds) which belong to the tribe *Phaseoleae* and pasture legumes. Whilst grain legumes provide high protein food for humans, both grain and pasture legume species provide high quality feed for cattle and other small ruminants (Minson *et al.*, 1993; Baker and Dynes, 1999; Howieson, 1999). Additionally, deep-rooted pasture legume species can assist in reducing rising water tables in areas prone to secondary salinity (Howieson *et al.*, 2000).

Grain legumes together with *Arachis* (which belongs to *Aeschynomeneae*) are crops of most importance in the tropics and account for approximately 20 % of global food production (Broughton *et al.*, 2003). More than 35 % of the world’s processed vegetable oils and 33 % of the dietary protein needs of humans come from grain legumes (Broughton *et al.*, 2003).

Grain legumes provide nutritious seeds that are valuable and upon effective nodulation, they can give promising yields in nitrogen-deficient soils where cereals and other non-leguminous crops would barely survive (Eaglesham and Ayanaba, 1984).

Among the legumes soybean is the most important globally (Herridge *et al.*, 2008). Below are short notes on soybean and pigeonpea.

2.3.3.1 Soybean

Soybean also known as *Glycine max* (L) Merrill is an annual grain legume that is predominantly found in the tropics (Flaskerud, 2003). The crop is strictly a self-pollinating legume having maturity dates that range between 90-115 days depending on the variety (Sidibe *et al.*, 1999). The leading soybean producers in the world are USA, Brazil, Argentina and China (Song *et al.*, 2006).

Soybean seeds are composed of roughly 22 % lipids, about 30.16 g of carbohydrates, 7.33 g sugar, 2.88 g saturated fat and many other nutrients (Song *et al.*, 1999).
2.3.3.1 Uses

Oil (including lecithin) and protein (meal or flour) are two major components of soybean seeds that are used as raw materials for manufacturing printers’ ink, cosmetics, plastics, glue, soap, shampoo and many more (Endres, 2001). Other products made from soybean include detergents and gasoline (mainly in the USA) (Adu-Dapaa et al., 2004; Kamara, 2013). Soybean is also used industrially for making paints, water-proofings for textiles, and a sizing for paper (Shurtleff and Aoyagi, 2009) and also for the production of margarine, cheese, and others (Shurtleff and Aoyagi, 2012).

Soybean has a great potential for solving the protein energy malnutrition problems in West Africa and the World at large (Asgar et al., 2010). Soy milk and soy flour/powder are products of unfermented soybean (Mariansky and Mariansky, 2011; Viska et al., 2013). Fermented soybean foods include soy sauce, fermented bean paste, natto and tempeh, among others (Mariansky and Mariansky, 2011; Viska et al., 2013).

2.3.3.1.2 Importance

Fixed atmospheric nitrogen in soybean improves soil fertility and subsequently enhances sustainable crop production especially when in rotation with cereals (Mohammad et al., 2008). Soybean plants provide good soil cover that reduces soil erosion and suppresses weed growth (Moncada and Sheafer, 2010). It also breaks pest and disease cycles when grown in rotation with cereals (Aikins et al., 2011; Kamara, 2013). Soybean is known to cause suicidal germination of Striga seeds and hence reduce the population of Striga for a successful cereal production in the subsequent year of a rotational cropping (Adu-Dapaa et al., 2004; Kamara, 2013).

Soybean contains significant amounts of essential amino acids which are of great importance to both human and animal health (Symolon et al., 2004). Isoflavones from soybeans have
recently been suggested to reduce the risk of cancer and serum cholesterol (Guha et al., 2009; Shu et al., 2009) while phytoestrogen from soybean and soy foods have been suggested as a possible alternatives to hormone replacement therapy for post-menopausal women (Graham and Vance, 2003).

Soybean has a great potential towards developing some key sectors of a nation’s economy such as Health, Agriculture and Industry (Plahar, 2006; World Initiative for Soy in Human Health, 2006). The economic benefits derived from soybean production have been documented extensively by Egbe (2010).

2.3.3.1.3 Soybean Cultivation in Ghana

The earliest known cultivation of the soybean crop in Africa was in Algeria in 1896, (Shurtleff and Aoyagi, 2009). Soybean was first grown in Ghana in 1909 (Mercer-Quarshie and Nsowah, 1975; Snow, 1961). However, there was no serious attempt to establish the production of the crop in Ghana until the early 1970s (Mercer-Quarshie and Nsowah, 1975).

2.3.3.2 Pigeonpea

Pigeonpea also known as Cajanus cajan (L.) Millsp is a tropical grain legume that is grown in a wide range of cropping systems and environments (climates and soils) (Nene and Sheila, 1990), notable exceptions being those areas that are excessively wet or that experience frost (Troedson et al., 1990). Although pigeonpea is a woody perennial, the legume is grown mainly as an annual (Giller, 2001). Depending on the variety, pigeonpea can take from 90-260 days to produce seed (Van de Maesen, 1985; Ali, 1990). The crop although can tolerate a wide pH (4.5-8.4) range, grows best at soil pH 5.0-7.0. It can withstand a temperature of 35°C or more (Valenzuela and Smith, 2002). The crop was probably domesticated in India. However, just before 2000 BC, the legume spread to Africa, a second centre of diversity (Van de Maesen,
A wild species of pigeonpea (*Cajanus* kerstingii) still exists in West Africa (Giller, 2001). The legume was later carried from Africa to the West Indies (Giller, 2001).

### 2.3.3.2.1 Uses

Pigeonpea is a protein-rich pulse crop that has growing demand in Asia, especially in India because it can provide high quality protein in the diet of humans (Lee *et al*., 2006). The plant has got immense medicinal value and is also used as fodder for cattle and other small ruminants (Singh and Diwakar 1993; van der Maesen, 2006; World Initiative for Soy in Human Health, 2006).

### 2.3.3.2.2 Importance

As a multipurpose leguminous crop, pigeonpea can provide food, fuel wood and fodder for small-scale farmers in subsistence agriculture (Tabo *et al*., 1995; Egbe, 2005). The legume is deep-rooted and also drought tolerant and as such adds organic matter to the soil (Egbe, 2005). Pigeonpea in addition can fix up to 235 kg N/ha (Peoples *et al*., 1995) thus producing more N per unit area from plant biomass than many other legumes.

Pigeonpea is nutritionally well balanced and is an excellent source of proteins (20–30 %) (Snapp *et al*., 2003). In addition to proteins, pigeonpea provides carbohydrates and high levels of vitamins A and C.

Farmers who grow pigeonpea can derive benefit in the form of income which is usually by computing the monetary advantage (Rafey and Prasad, 1992) or by estimating the net returns (Ramakrishna *et al*., 2005; Guedes and Araujo, 2010). The importance of intercropping pigeonpea with cereals are available (Egbe, 2005; Egbe and Adeyemo, 2006; Egbe and Bar-Anyam, 2011).
2.3.3.2.3 Pigeonpea Cultivation in Ghana

Pigeonpea is mostly cultivated in India and eastern Africa. In Ghana, the crop is mainly grown on pastures (Adjei-Nsiah et al., 2007). Being the major pigeonpea producing country, India accounts for over 93% of the global pigeonpea production (Rani, 2011). Other researchers have reported Nigeria (Aiyeloja and Bello, 2006), Niger, Mali, Benin (Versteeg and Koudokpon, 1993), Ethiopia, Zimbabwe (Kamanga and Shamudzarira, 2001), Zambia (Boehringer and Caldwell, 1989), Botswana (Amarteifio et al., 2002), and South Africa (Swart et al., 2000) to be other pigeonpea producing countries in Africa.

Pigeonpea derives between 36.10-114.04 kg N/ha from fixation when intercropped with maize and 35.94-164.82 kg N/ha under intercropping with sorghum (Egbe, 2007). However, yields in Nigeria have been reported to be as low as 0.5-1.0 t/ha under traditional cropping systems (Egbe and Idoko, 2012). The story is the same for Ghana, where yield of pigeonpea ranges from 129-1872 kg/ha with an average of 946 kg/ha. This is very low compared to an average yield of 2600 kg/ha by the same pigeonpea lines grown in India (Marfo et al., 1997). The low yield could be as a result of production being concentrated in the semi-deciduous forest zones where other legumes are preferred to pigeonpea in intercropping. Adjei-Nsiah (2012) reported that pigeonpea seed consumption is low and is caused by low knowledge on pigeonpea meals making farmers place a much less priority on the legume for intercropping.

2.4 Rhizobia

Rhizobium in Latin means ‘root living’ which is why they are also referred to as root nodule bacteria (RNB). Rhizobia are facultative microsymbionts (Provorov, 1998) that can infect roots of most legumes (Hirsch et al., 2001; Matiru and Dakora, 2004) and transform atmospheric N₂ into forms usable by plants (Phillips, 1999; Bala and Giller, 2001; Sessitsch et al., 2002).
Rhizobia are Gram negative, motile, rods that are pleomorphic under adverse growth conditions (Jordan, 1984). They usually accumulate granules of poly-β-hydroxybutyrate when carbon is in excess and are aerobic, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor (Jordan, 1984).

### 2.4.1 An overview of rhizobial taxonomy.

Early classification of rhizobia was based on the cross inoculation group concept, which grouped rhizobia on the basis of their ability to infect and fix nitrogen with a discrete group of legumes (Jensen, 1958). The concept of cross inoculation was disproved by Zakhir and Lajudie (2001).


Extensive study and classification of rhizobia (Broughton, 2003) has brought about changes in the taxonomy. This has led to revisions and additions to the taxonomy of rhizobia (Jordan, 1984). The revisions have revealed progress in rhizobial taxonomy and systematics notably in the last decade which is mainly due to the characterization of new isolates together with the general use of 16S rRNA sequencing and polyphasic taxonomic approaches (van Berkum and Eardly, 1998; Zakhir and Lajudie, 2001).

Currently, there are 44 species of RNB that have been accepted and they are distributed in 12 genera in the class *α-Proteobacteria* (Sawada *et al.*, 2003) and the *β-Proteobacteria* (Chen *et al.*, 2001).
The α-Proteobacteria class consists of five families: Rhizobiaceae (including the genera Allorhizobium/shinella, Rhizobium and Sinorhizobium/Ensifer), Phyllobacteriaceae (including the genus Mesorhizobium), Bradyrhizobiaceae (including the genus Bradyrhizobium), Hyphomicrobiaceae (including the genera Azorhizobium and Devosia) and Methyllobacteriaceae (including Methyllobacterium) as defined by their 16S rDNA sequence analysis (Garrity et al., 2003; Sawada et al., 2003; Rengel, 2002). Those in the β-Proteobacteria class are contained in two families: Burkholderiaceae (including the genera Burkholderia and Wautersia) (Garrity et al., 2003; Sawada et al., 2003) andRalstoniaceae (including the genus Ralstonia) (Chen et al., 2001; Moulin et al., 2001).

Soybean is nodulated by Bradyrhizobium japonicum, Bradyrhizobium elkanii (Kuykendall et al., 1993), Bradyrhizobium liaoningense (Xu et al., 1995) and Sinorhizobium fredii (Chen et al., 1988 Young et al., 2001).

Pigeonpea is nodulated by Rhizobium leguminosarum bv viceae and Bradyrhizobium spp. (Nautiyal et al., 1988; Singh et al., 1997). Below are notes on the main rhizobia genera from which soybean and pigeonpea are nodulated.

2.4.1.1 The genus Rhizobium

Rhizobium refers to the fast growing, acid-producing strains of rhizobia. There are three species within this genus. They are Rhizobium phaseoli, Rhizobium trifolii and Rhizobium leguminosarum. Rhizobium leguminosarum consists of three biovars named to distinguish their plant affinities: biovar viciae as it nodulates Vicia spp.; biovar trifolii as it nodulates Trifolium spp. (clover) and biovar phaseoli as it nodulates Phaseolus vulgaris (common bean) (Jordan, 1984). The host ranges of the three biovars are quite distinct and seem to be mutually exclusive.
2.4.1.2 The genus Bradyrhizobium

The genus *Bradyrhizobium* refers to bacteria that are extremely heterogeneous and different from other legume symbionts by their slow growth and production of alkaline reactions in growing media (Jordan, 1984). The species of this genus include *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium liaoningense* and *Bradyrhizobium spp*.

2.4.1.3 The genus Ensifer/Sinorhizobium

Until recent times, all fast-growing rhizobia were put under the genus *Rhizobium* including *Rhizobium fredii* a fast-growing soybean Rhizobium. Continues research later on lead to the renaming of *Rhizobium fredii* as *Sinorhizobium fredii* and a second species, *Sinorhizobium xinjiangense* was proposed Chen *et al*. (1988). The genus *Sinorhizobium* is now widely accepted and currently has 11 valid species. There is genetic evidence to support the separation of *S. xinjiangense* and *S. fredii* (Peng *et al*., 2002). Recent comparisons using the 16S rDNA has revealed that *Ensifer adhaerens* is a phylogenetic member of the *Sinorhizobium* lineage (Balkwill, 2005). In a 16S rDNA dendrogram, *Ensifer* and *Sinorhizobium* form a single group which belongs alpha-Proteobacteria and the two may therefore be regarded as a single genus. Efforts to choose the appropriate name *Sinorhizobium adhaerens* or *Ensifer adhaerens* (Willems *et al*., 2003, Young, 2003) for the genus are still ongoing. As of now *Ensifer adhaerens* is preferred and remains the correct name.

2.4.2 Rhizobial diversity

The variation in the DNA sequences between strain types in a rhizobial population is called genetic diversity (McInnes, 2002). The strains of RNB inhabiting a particular soil may be diverse in symbiotic as well as phenotypic and genetic characters (Pinto *et al*., 1974). Cropping history, the degree of disturbance of an environment and the range of legume species of an area can influence the diversity of rhizobial strains in that area (Brockwell *et al*., 1995) as well as
strain population in the area. In measuring diversity, it is important to note that measuring the diversity of RNB using a trap host only resembles the diversity of RNB able to nodulate that particular trap host and not the total diversity of RNB resident in the soil. Many studies have assessed the diversity of RNB strains nodulating a particular legume species or the diversity of RNB that exist in a particular soil (Laguerre et al., 1996; Lafay and Burdon, 1998; Wang et al., 1999; Zhang et al., 2000).

Many attempts have been made to determine the actual composition and characteristics of indigenous rhizobia using strains isolated from different cultivated legumes (Laguerre et al., 1996; Carelli et al., 2000). There increased attention in the assessment of diversity within rhizobial natural populations in various regions of the world (Chen et al., 2000; Zhang et al., 2000) in the last few years gives an indication that the subject has not been fully exhausted.

The development and availability of several sensitive and accurate PCR-based genotyping methods (Jensen et al., 1993; Jude et al., 1993; Selenska-Pobell et al., 1995) have helped in the differentiation of closely related bacterial strains even among natural field populations. There has also been detection of higher rhizobial diversities compared to previous times (Vinuesa et al., 1998; Doignon-Bourcier et al., 2000; Tan et al., 2001). It is important to note, however, that the discriminatory power of individual strain typing methods varies and this can give rise to different diversity assessments for the same field site tested (Schwinghamer and Dudman, 1980; Barnet, 1991; Bottomley, 1992) even for a particular legume species.

At present there is a substantial array of techniques used for detecting and describing rhizobial diversity and they are discussed in the next section.

**2.4.2.1 Methods used to investigate rhizobial diversity**

Phenotypic and physiological characters such as host range, comparative growth in culture, serological relatedness, bacteriocin production, intrinsic antibiotic resistance and bacteriophage
resistance (Schwinghamer and Dudman, 1980) were previously used to determine rhizobial diversities. The recent molecular era has enabled the inclusion of more prominent methods such as substrate utilization, protein profiling, Multilocus Enzyme Electrophoresis (MLEE) and Fatty Acid Methyl Ester (FAME) analysis in assessing rhizobial diversity (Graham et al., 1995; Van Rossum et al., 1995). The phenotypic and physiological methods used in times past had some limitations, particularly, low discriminatory power compared to molecular methods (Jenkins and Bottomley 1985; Barret, 1991; Bottomley, 1992) and also an often poor correlation between strain groupings (Kleczkowsky and Thornton, 1994; Roughley et al., 1992; van Rossum et al., 1995) which were due to the instability of strain characters over time (Lindström et al., 1990).

In recent times, there are large numbers of genotypic (molecular based) methods used for rhizobial diversity studies and the most common methods comprise:

1. Plasmid profiling (Broughton et al., 1987; Young and Wexler, 1988; Laguerre et al., 1992; Louvrier et al., 1996; Wernegreen et al., 1997)
2. Restriction Fragment Length Polymorphism (RFLP) (Schofield et al., 1987; Young and Wexler, 1988; Laguerre et al., 1993; Bromfield et al., 1995; Kishinevsky et al., 1996; Lafay and Burdon, 1998; Vinuesa et al., 1998; Odee et al., 2002)
3. Polymerase Chain Reaction based techniques (PCR) (de Bruijn, 1992; Richardson et al., 1995; Louvrier et al., 1996; Laguerre et al., 1997; Gao et al., 2001)

Genotypic methods generally have high discriminatory power and the majority of these methods are rapid compared to most phenotypic methods (Handley et al., 1998). However, it is important to note some of their limitations. Most of the genotypic methods used in diversity studies, especially the PCR based techniques (BOX PCR, ERIC PCR, Rep PCR and RPO1 PCR) are reported to be of low reproducibility. Their success is mostly dependent on the DNA extraction protocol, colony age, source of reagents, concentration and purity, and thermal
cycling conditions (Welsh and McClelland, 1990; Coutinho et al., 1993; Kay et al., 1994; Richardson et al., 1995; Laguerre et al., 1996; Vachot et al., 1999). These disadvantages can however be overcome by rigorously standardising the protocols, using many repeats, replicates and including appropriate controls.

2.4.3 Host Range

A proper definition of host range is to consider the diversity of the symbiotic genes rather than the diversity of the species that carry these genes (Laguerre et al., 2001). The success of clearly defining the host range of newly identified strains was due to the recent development molecular techniques. The phylogenetic classification of genes is now used for the description of new rhizobia as previously proposed for the 16S rRNA gene sequences (Graham et al., 1991).

2.5 The BNF Process

2.5.1 The root nodule formation

The process of nodulation (root nodule formation) involves the transfer of signals between a legume host plant and compatible rhizobia. The process is commenced by the release of organic compounds from the roots of host legumes (Gualtieri and Bisseling, 2000) into the rhizosphere most of which supports the growth of rhizosphere microorganisms (Barran and Bromfield, 1997; Perret et al., 2000). The rhizosphere environment must be appropriate prior to the successful exchange of the signal molecules that precede infection (Leibovitch et al., 2001; Zhang et al., 2002). The released compounds (exudates) consist mainly of carbohydrates, organic acids, vitamins, amino acids and flavonoids (2-phenyl-1, 4-benzopyrone derivatives) (Rélić et al., 1994; Broughton et al., 2000; Perret et al., 2000).
In nodulating plants, flavonoids specifically trigger the expression of the rhizobial genes required for nodulation (\textit{nod}). The importance of flavonoids is seen in the complete inhibition of nodule initiation whenever these flavonoids are absent (Perret \textit{et al.}, 2000).

The \textit{nod} genes are responsible for the synthesis of the nodulation factors, which are involved in the establishment of the symbiotic relationship with the legume host (Perret \textit{et al.}, 2000). The types and functions of these \textit{nod} genes have been documented extensively (Triplett and Sadowsky, 1992; Van Rhijn \textit{et al.}, 1993; Downie, 1998; Hirsch \textit{et al.}, 2000; Perret \textit{et al.}, 2000; Zhang \textit{et al.}, 2000). The \textit{nod} genes are organized in several operons, located on the chromosome (Kaneko \textit{et al.}, 2000) or on a mobile symbiosis island which is integrated into the chromosome (Sullivan \textit{et al.}, 2002) or on large symbiotic (\textit{sym}) plasmids (Hynes and MacGregor 1990; Brom \textit{et al.}, 1992; Triplett and Sadowsky, 1992; Barnett \textit{et al.}, 2001; Finan \textit{et al.}, 2001).

The rhizobial \textit{nod} genes often produce and secrete nodulation (Nod) factors as return signals in response to the flavonoids secretion from host plants. Each rhizobial strain produces a characteristic spectrum of Nod factors that is generally unique for a given isolate (Downie, 1998; Schultze and Kondorosi, 1998). Nod factors are the signals required for the entry of rhizobia into the leguminous plants acting like ‘keys’ for the invading rhizobia to the legumes root hair ‘doors’ (Broughton \textit{et al.}, 2000; Gualtieri and Bisseling \textit{et al.}, 2000; Parniske and Downie, 2003). Additional signals, or ‘keys’, which normally proceed the entry of rhizobia are necessary for later steps of the infection process (Perret \textit{et al.}, 2000).

The root hairs of the host legumes are induced to branch upon contact with the Nod-factors. The branched roots then after deform and curl thereby preventing further root hair cell growth. Bacterial cells are trapped in pockets of the host cell wall at the curled regions. The root hair cell walls undergo hydrolysis at certain sites where bacteria entry of the roots of host legumes occurs. The entry process is described as ‘penetration through an invagination of the plasma
membrane’. The plant host reacts to bacterial entry by depositing new cell wall material around the lesion. This is usually in the form of an inwardly growing tube that later becomes what is called infection thread (Vanderleyden and Van Rhoij, 1995; Hirsch and LaRue, 1997; Gage and Margolin, 2000; Gualtieri and Bisseling, 2000). The infection thread is the medium through which rhizobia penetrate root tissues (Sprent, 1989, 2001; Brewin, 1991; Gage et al., 1996; Hadri et al., 1998; Gage, 2004; Oldroyd and Downie, 2004; Maunoury et al., 2008). The rhizobial infection of the roots of some legumes is through a wound or crack (Gonzalez-Sama et al., 2004). At the time a Rhizobium penetrates the root tissue, there is cell division in the outer or inner root cortex which results in the production of a nodule primordium. Bacteria are released from the infection threads into the host cytoplasm within the growing nodule primordium where they are differentiated into bacteroids and the nitrogen-fixing nodule develops (Roth and Stacy, 1989; Gualtieri and Bisseling, 2000). As the nodule develops in the presence all other requirements, there is fixation of atmospheric N₂ in the air surrounding the nodule (Long and Ehrhardt, 1989). The inside colour of a nodule with active nitrogen fixation is usually red or pink. The red colour is due to leghaemoglobin, an iron-containing pigment associated with active nitrogen fixation (Bergersen, 1982). The number of nodules and the rate of nitrogen fixation will increase with time after emergence and normally reaches a maximum just before the legume blooms (Imsande, 1988).

Ineffective rhizobia often produce nodules, but these nodules are small and white, grey or green on the inside (Starker et al., 2006). Where legumes are ineffectively nodulated, the plants show symptoms of nitrogen deficiency, i.e. progressive yellowing of the leaves and generally poor growth is observed (Meyer et al., 2007).
2.5.2 Control of nodule development

Host plants possess the genetic information for symbiotic infection and nodulation (Vanderleyden and Van Rhijn, 1995). This means what the bacteria does is to switch these host plant genes on. The positive as well as negative (expression of the *nod* genes) actions of these plant genetic factors (Suzaki *et al.*, 2013), are what control the development of nodule. The regulation of the symbiotic process in most cases is influenced by external factors such as nitrogen in the soil solution (Caetano-Anollés, 1997). The plant host limits the number of nodules and this regulation might be integrated in the mechanisms that control lateral root development (Stougaard, 2000).

2.5.3 Host specificity

The amounts of Nod factors released by rhizobia are important in determining the host range (Perret *et al.*, 2000) and specificity. During the formation of symbiotic associations, there appears to be communication between the symbiotic partners, leading to their recognition of each other (Schultze and Kondorosi, 1998). Specificity is not only confined to nodulation but also extends to the ability to form effective, nitrogen-fixing nodules. Only certain combinations of host plants and effective rhizobia are compatible with each other to form a nitrogen-fixing symbiosis (van Rhijn *et al.*, 1998). Various ‘*nod*’ gene inducers, Nod factors and polysaccharides are all involved in determining host specificity (van Rhijn *et al.*, 1998). Certain rhizobial isolates are capable of forming effective nodules on some host legumes (*Nod*+, *Fix*+) whilst forming ineffective nodules on others (*Nod*+, *Fix*-) (Valverde *et al.*, 2005). Specificity among compatible partners minimizes the chance of pathogen infection and the formation of ineffective associations (Perret *et al.*, 2000).
2.5.3.1 Molecular basis of host specificity

The specificity in the symbiotic interaction between legumes and rhizobia is controlled at many levels. The type of NodD protein present in the RNB, the type of the flavonoid produced by the legume host, the type of Nod-box in the promoter region of nodulation genes and the type(s) of Nod-factor produced by the RNB are the four important levels in that order at which the symbiosis establishment is controlled (Sadowsky and Graham, 1998).

That different species produce different NodD proteins which respond to different types of plant flavonoids (Downie, 1994) buttresses this point. NodD1 of the broad host range *Rhizobium* sp. strain NGR234 recognises a wide range of flavonoids and transfer of the *nodD1* of strain NGR234 to other strains of restricted host range has been shown to extend the host range (Bender *et al.*, 1988). Thus, the initial level in symbiotic specificity is controlled by *nodD*.

When conditions in the soil results in the release of flavonoids from plant roots, the bacterial NodD or SyrM (Barnett and Long, 1990; Schlaman *et al.*, 1992) proteins activate the transcription of other *nod* genes (Roche *et al.*, 1996; Downie, 1998). The activation of the transcription of other nod genes regulates the initial infection process. NodD and SyrM proteins act as both plant signal sensors and transcriptional activators (Perret *et al.*, 2000) triggering the transcription of the *nodABC* operon in RNB by binding to the Nod-box in the promoter region of this operon. *Rhizobium* sp. strain NGR234, which can nodulate a broad range of legumes, contains 19 different homologous sequences for Nod-box, thereby providing many possibilities for fine-tuning *nod* gene expression (Perret *et al.*, 2000).

The specificity between legume hosts and RNB can range from the highly specific, *i.e.* where only a single species of RNB nodulate a given legume host (Nour *et al.*, 1994, 1995; Martinez-Romero, 2003), to being very promiscuous (Michiels *et al.*, 1998). The specificity is often is governed by a single recessive gene, *sym-2* (Holland, 1975; Lie, 1984).
Symbiotic promiscuities usually exist in two forms.

1. The promiscuity of RNB (broad host-range RNB): Where a single RNB strain enters into symbiosis with a range of different host plants (Perret et al., 2000).

2. The promiscuity of the host plant: A single legume may be nodulated by a range of RNB belonging to different species (Bromfield and Barran, 1990; Laguerre et al., 1993; Ezura et al., 2000; Aguilar et al., 2001; Howieson and Ballard, 2004).

Promiscuity in RNB is a valuable trait for elite inoculants selected for commercial use (Howieson et al., 2000). Such strains must form highly effective symbiosis on a wide range of host legume species (Howieson, 1999; Howieson et al., 2000). Ineffective nodulation by promiscuous RNB that are indigenous or resident in agricultural soils reduces the benefits of legume inoculation to agriculture (Demezas and Bottomley, 1984; Barran and Bromfield, 1997; Ballard and Charman, 2000; Denton et al., 2002).

Promiscuous legume species may face reduced productivity due to nodulation by a range of ineffective or less effective RNB (Trinick and Hadobas, 1989; Hungria and Vargas, 2000). As such commercial legume species with the ability to form effective nodules with many different soil rhizobia (Abaidoo et al., 2000; Sessitsch et al., 2002; Howieson and Ballard, 2004) should be considered.

2.6 Importance of BNF

Biological nitrogen fixation is undoubtedly of greatest agricultural importance as it occurs in all known ecosystems. On a global scale, nitrogen derived from biological nitrogen fixation may reach 175 million metric tons per year (Graham et al., 1994) of which approximately 90 million tonnes come from agricultural areas. Undeveloped land and forests (Bezdicek and Kennedy, 1988) contribute approximately 50 million tonnes of N from BNF.
The importance of biological nitrogen fixation is seen in the nitrogen nutrition of leguminous plants and their associated crops in natural and agricultural systems. Most grain legumes can obtain between 50 to 80% of their total nitrogen requirements through biological nitrogen fixation, although some, like faba bean can fix up to 90% (Herridge et al., 2008; Salvagiotti et al., 2008).

The input of nitrogen through biological nitrogen fixation allows for increased soil fertility, which helps to maintain soil nitrogen reserves (Graham and Vance, 2000) without having much adverse effect on the environment compared to chemical N fertilisers.

2.7 Factors Affecting Nodulation and BNF

The establishment of an effective rhizobia-legume symbiosis requires colonization and survival of rhizobia in the soil as saprophytes competing with other indigenous microbes and genetic compatibility with the host legume under a favourable environment to allow maximum nodulation and N$_2$ fixation (Bordeleau and Prevost, 1994).

2.7.1 Environmental factors that affect BNF

Typical environmental stresses that affect legume nodulation and the subsequent symbiotic nitrogen fixation include photosynthetic deprivation, soil moisture, salinity, soil nitrate and phosphate levels, soil acidity and alkalinity and temperature (Walsh, 1995).

2.7.1.1 Soil Moisture content

The rhizobial population density in a soil (Tate, 1995), rhizobial migration, nodule number and size (Williams and De Mallorea, 1984) is negatively affected by soil moisture. The effect is more pronounce on N$_2$-fixation as nodule initiation, growth and activity are more sensitive to water stress (Bordeleau and Prevost, 1994) than are root and shoot metabolism (Albrecht et al.,
In dry soil, infection of root hairs is restricted because roots become short, stubby and inadequate for rhizobial infection (Lie, 1981).

Excess soil water (a case of saturated soils) is also 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 even at low concentrations) (Eaglesham and Ayanaba, 1984).

When the surface of soils are dry at the time of planting, most of the bacteria on inoculated seeds are killed thereby decreasing the nodulation of the plant. A common observation with many strains of all species of *Rhizobium*, is the 99 % reduction in the viable population upon a single exposure of the soil to drying. Several cycles of soil wetting followed by drying, a common phenomenon of nature, reduces the population still further (Alexander, 1985). Although more than 99 % of the cells of other strains die under identical circumstances (Osa-Aftana and Alexander 1982), it appears, that means can be devised to obtain cultures not seriously affected by the drying of soil.

**2.7.1.2 Temperature**

Biological nitrogen fixation is affected under high soil temperatures (Michiel *et al.*, 1994) since rhizobial survival in very warm soil is so low. Soil temperatures above 20°C at seeding time will kill many rhizobia and greatly decrease nodulation. In some cases high temperatures may cause the symbiotic process to cease completely (Bordeleau and Prevost, 1994). Low temperature also delays root hair infection and decreases nodulation as well as nitrogenase activity (Waughman, 1977). Extreme temperatures affect root hair infection, bacteroid differentiation, nodule structure and functioning (Roughley, 1970). Thus, inoculated seed should be sown on cool days, if possible. Ammendment of soil with 5 % (w/vol) montmorillonite, fly ash or haematite has been reported to protect rhizobia from lethal effects of high temperatures (Lowendorf, 1980).
The optimum temperatures for growth in culture vary among strains and species; values between 27-39°C have been noted. The maximum temperatures are generally 35-39°C, but proliferation may take place up to 42°C (Munevar and Wollum, 1981). Differences in growth and colonizing abilities probably explain why some strains are more active in nodulating grain legumes at low temperatures and others are more active even at higher temperatures (Weber and Miller, 1972).

2.7.1.3 Soil pH (Acidity and Alkalinity)

Most legumes require neutral or slightly acidic soils (Bordeleau and Prevost, 1994) for normal growth and development. Under acidic-soil conditions legumes fail to nodulate and this is common, especially in soils with pH less than 5 (Raza et al., 2001).

Approximately 25% of the world's agricultural soils are acidic (Munns, 1986). Most of the naturally acidic soils in the world are found in the tropics. This is due to the high rainfall, low evaporation rates, leaching of cations and high biological activity (Jayasundara et al., 1998) associated with the tropics.

Acid soils reduce plant growth and are often not nutritionally balanced. Acid soils are mostly associated with deficiencies in phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), Molybdenum (Mo), copper (Cu) and cobalt (Co) (von Uexkull and Mutert, 1995), and also toxic concentrations of aluminum (Al), hydrogen (H), and manganese (Mn) (Helyar, 1987; O'Hara et al., 1988; Sadowsky and Graham, 1998).

2.7.1.3.1 pH effects on free-living rhizobia in the soil

The survival and persistence of rhizobia in soils is affected by soil acidity (Bottomley 1992; Ibekwe et al., 1997). Slow growing Bradyrhizobium strains are because they produce alkali reactions in soils (Correa and Barneix, 1997), are generally more acid tolerant than fast-growing strains of rhizobia. Thus the alkali they produce modifies the pH of their immediate
surroundings (Sylvester-Bradley et al., 1988). However, different levels of acid tolerance among the different *Bradyrhizobium* species exist (Graham et al., 1994; van Rossum et al., 1994; Bayoumi et al., 1995; Raza et al., 2001).

Low soil pH decreases rhizobial survival, growth, population size and even strain diversity (Munns, 1965; Sadowsky and Graham, 1998; McInnes et al., 2004). According to Munns and Keyser (1981), low levels of *rhizobium* now *Bradyrhizobium* and in some cases complete absence may occur at pH below 4.5. Many active nitrogen fixers fail to survive in a sterile soil at pH 5.2 (Lowendorf et al., 1980). Low soil pH affects the spatial distribution of indigenous rhizobia as well (Munns and Keyser 1981). The lower number of rhizobia that are present under low soil pH reduce the possibility of successful infection and ultimately reduce nodulation (Whelan and Alexander, 1986).

Low soil pH can affect aspects of the nodulation process other than rhizobial population growth (Evans et al., 1980) of RNB.

In some low pH soils, viable populations of rhizobia are known to exist (Graham and Parker, 1964; Dilworth et al., 2000; Howieson et al., 2000) in protected niches (Thornton and Davey, 1984; Wood et al., 1984; Lindstrom and Myllyniemi, 1987; Wood and Shepherd, 1987; Slattery et al., 1992; Gemell et al., 1993b; Howieson et al., 2000; Watkin et al., 2000).

### 2.7.1.3.2 pH effects on symbiotic communication, attachment and infection thread formation

These can be from indirect effects such as metal toxicity (Reeve et al., 2002). Under acidic conditions of e.g. pH < 5.0, capacity of root exudates from seedlings to induce *nodA* is reduced. Richardson et al. (1988) reported that expression of *nod* genes is inhibited by high aluminium ions in solution. However, some legumes are able to tolerate Al toxicity (Howieson et al., 1992).
The decreased growth rate of rhizobia in acid soils is likely to contribute to delayed colonisation of the root hairs. This is critical, since the size of the rhizobial population directly influences the concentration of Nod factors (Richardson and Simpson, 1989), and the root hairs are only receptive to infection (Bhuvaneswari et al., 1981; Bowra and Dilworth, 1981; Wood et al. 1984; Richardson et al. 1989) only for a brief moment.

Low pH affects the stability of rhizobia binding to roots as this causes desorption of those previously bound (Caetano-Anolles et al., 1989). The adsorption of rhizobia to roots of legumes dependends on the presence of divalent cations such as Ca\(^{2+}\) which are mostly present at neutral to near neutral pH (Caetano-Anolles et al., 1989). Most of these cations are absent under low soil pH conditions.

Two strategies that have been adapted to solve the problem of soil acidity in tropical soils are liming of acid soils and selecting acid tolerant varieties of legumes and strains of rhizobia. Very few data have been documented on the effects of high pH on rhizobial growth, nodulation or legume growth (Bordeleau and Prevost, 1994).

Mengel and Kamprath, (1978) reported on the effect of liming acid soils (pH 3.4-4.25) on nodule initiation and development in soybean. Soils with pH below 4.5-4.8 after liming were found not to nodulate. The researchers therefore concluded that pH in the range 4.5-4.8 is critical for nodule initiation and development in the soils. Lime application increases the nodulation of some rhizobial species more than the nodulation some other species. In acid soils lime application is usually more beneficial than mineral nitrogen application (Andrew, 1976). However, high levels of lime application that results in raising the pH of a soil by 1, can have deleterious effects on plant growth (Kennelly et al., 2012).
2.7.1.4 Phosphorus content and availability.

Phosphorus is the second most limiting nutrient for plant growth in tropical soils (Schachtman et al., 1998). Phosphorus is needed by plants right from germination to seed maturity (Marschner, 1993). During flowering and seed formation, most of the absorbed P is translocated from leaves to the fruits and seed regions depending on the availability of the nutrient (Marschner, 1993). Although tropical soils have total P, phosphorus deficiency is a major fertility problems in tropical agriculture (Miller and Ohlrogge, 1957; Bieleski, 1973; Fox and Kang, 1977). Most tropical soil solutions contain less than 0.1 ug P mL⁻¹ (Bieleski, 1973; Fox and Kang, 1977). Factors and mechanisms that affect phosphorus availability in tropical soil solutions have been discussed (Uehara, 1977; Fox and Searle, 1978; Velayutham, 1980; White, 1981; Mokwunye et al. 1986; Torrent, 1987; Owusu-Bennoah and Acquaye, 1989; Pena and Torrent, 1990; Warren, 1992; Owusu-Bennoah et al. 1997; Abekoe and Sahrawat, 2001).

There are two different forms of available phosphorus existing in soils which are dependent on the soil’s pH. These are H₂PO₄⁻ and HPO₄²⁻. At near neutral pH, both forms of the orthophosphate ions are equally represented in terms of proportionality. In acid soils, H₂PO₄⁻ is the dominant ion in solution (almost 100 % at pH 4-6) whereas in alkaline soils, the HPO₄²⁻ dominates (about 80 % at pH 8 with the remaining 20 % being H₂PO₄⁻) (Black, 1968). A continuous renewal of P in the solution that is in contact with the roots of plants is needed for P supply to meet the total P demand of growing plants.

2.7.1.4.1 Addressing the constraint of phosphorus availability in Tropical soils

Increasing the pH of the soil by liming reduces the concentration of Al and Fe in the soil solution thereby decreasing P adsorption (Fox and Searle., 1978). Although liming increases pH in soils, agricultural liming usually involves a consideration of the pH range that plants are normally grown. Thus agricultural liming may neither increase the solubility and hence the availability of P nor does it decrease adsorption of P (Fox and Searle., 1978). Lime application must always
be accompanied with adequate phosphorus fertilization to achieve maximum results. Therefore, application of substantial amounts of P fertilizer (Fox and Kang, 1977) is required for optimum plant growth and adequate food production (Sanchez and Buol, 1975; Cassman et al., 1981; Date et al., 1995) in tropical agricultural systems.

2.7.1.4.2 Phosphorus requirements for nodulation and nitrogen fixation

Nodulation and the rate of N₂-fixation are largely dependent on the availability of phosphorus (Singleton et al., 1985; Leung and Bottomley, 1987; Saxena and Rewari, 1991). As soil organic matter reserves and hence the supply of available nitrogen is reduced, nodulation and the potential rate of N₂-fixation will increase as long as other factors mostly phosphorus are not limiting (Kahindi et al., 1997).

Soybean depending on nitrogen fixation for N has 47 to 75 % higher P demand than when fertilizer nitrogen had been supplied (Cassman et al. 1981). Actively growing nodules have been found to have high phosphorus content (Bonetti et al., 1984) and has therefore been suggested that the amount of phosphorus required by the nodules probably forms a significant sink in relation to the rest of the plant (Qin et al., 2012).

It has been widely reported in literature that high soil nitrogen delays or inhibits nodulation and nitrogen fixation (Franco, 1977). However, there are indications adequate P in soils induces nodulation even in the presence of inhibitory nitrogen levels (Gentili and Huss-Danell, 2002; Gates and Wilson, 1974). Gentili and Huss-Danell (2002) have, therefore, concluded that N: P ratio is important for nodulation (Wall et al., 2000) just as concentrations of N and P.

2.7.1.5 Nitrogen Availability

In soils low in available nitrogen, nodules of pigeonpea and soybean are normally initiated within 6 days of planting and are visible by 9 days within a few centimetres of the soil surface (Eaglesham et al., 1983). High rates of mineral nitrogen have been documented to substantially
reduce nodule formation and inhibit nitrogen fixation eventually (Danso et al., 1992; Peoples and Griffiths, 2009). According to Schilling et al. (2006) and Gavrichkova and Kuzyakov (2009), nitrate (\(\text{NO}_3^-\)) reduction and assimilation is high and costly when carried out in plants. Thus compared to the only 14% of total respiratory energy (15 moles ATP) needed for assimilating N supplied as \(\text{NH}_4^+\), as much as 23% (5% for absorption, 15% for reduction and 3% for actual assimilation) of total respiratory energy (20 moles ATP) is needed for assimilating N supplied as \(\text{NO}_3^-\) (Salsac et al., 1987; Bloom et al., 1992). However, the 28-32 moles of ATP required by nitrogenase to reduce 1 mole \(\text{N}_2\) or the 12 g of glucose that is required for fixing 1 g \(\text{N}_2\) to \(\text{NH}_3\) (Evans et al., 1980) indicates that the \(\text{N}_2\) fixation process together with the assimilation of the end product is high and costly in terms of energy (Al-Niemi et al., 1997).

Considering the energy requirements, plants will prefer mineral N to fixing \(\text{N}_2\) from the atmosphere when presented with these two options. When the N supplied by mineral N is enough to meet the total N requirement of the legume, nodulation is completely inhibited. Several researchers have reported that the inhibition process is systemic.

According to Harper (1987) and Streeter (1988) the effects of mineral N inhibition is evident in several stages of the symbiotic process resulting in decreased number of nodules, dry weight of nodules and \(\text{N}_2\) fixation activity. In actively growing well nodulated legumes the supply of mineral N to meet the total N requirement of a legume results in feedback inhibition of nitrogen fixation by products of nitrate metabolism such as glutamine (Neo and Layzell, 1997), asparagine (Bacanamwo and Harper, 1997), reduced supply of carbohydrate to nodules for nitrogenase activity (Streeter, 1988; Vessey and Waterer, 1992) as well as decreased \(\text{O}_2\) diffusion into nodules which often restricts the respiration of bacteroids (Gordon et al., 2002). Under such conditions nitrogenase activity is completely halted and no further nitrogen fixation occurs resulting in the senescence of the nodules formed.
2.7.2 Biological Factors

2.7.2.1 Presence of rhizobia

Rhizobia are facultative symbionts that have adapted to persist for long periods in soils, in a free-living state, in the absence of a suitable legume (Sanginga et al., 1994; Graham, 2008). Their populations generally increase in upon the introduction of a host legume.

The ability of rhizobia to survive in, grow and eventually colonise a soil depends substantially on the physical and chemical characteristics of the soil (Bushby, 1982). To enable their survival and growth, rhizobia need to access adequate concentrations of mineral and organic nutrients from the soil to sustain their metabolic processes (O'Hara, 2001; Poole et al., 2008).

Agricultural soils often contain established populations of RNB and many common cultivated legume species achieve nodulation without inoculation (Thies et al., 1991; Mpepereki et al., 1996, 2000; Wang et al., 1999; Ballard and Charman, 2000; Sessitsch et al., 2002) though these legumes may fix nitrogen poorly (Ballard & Charman, 2000; Denton et al., 2002; Zdor and Pueppke, 1988).

Although many legumes can be nodulated by several species of rhizobia (Mateos et al., 2011), the fact that a high degree of host-specificity exists between legume hosts and rhizobial species (Thrall et al., 2000) means that loss of a single rhizobial species can result in loss of N₂-fixation by that legume.

Also, not all of the indigenous rhizobia are able to effectively nodulate cultivated legumes (Baraibar et al., 1999). In situations where the indigenous rhizobia are not effective in nodulating legume hosts, there is the need to introduce effective strains that can grow under the given soil conditions and compete for nodule occupancy with the indigenous strains (Naeem et al., 2004).
Aside from the abiotic factors, challenges associated with living in soil include tolerance to many biotic factors, such as grazing fauna and antagonistic micro-organisms (Bottomley, 1992).

2.7.2.2 Plant factors

Nodulation and the amount of nitrogen fixed vary according to the legume species and variety (Zahran, 1989). Within a plant species, the amount of nitrogen fixed is directly related to (dry matter) yield (Zahran, 1989). Leguminous plants that are adapted to extremely harsh soil conditions can be nodulated and subsequently fix nitrogen in regions where other legumes will not even survive. In terms of varieties, high yielding varieties are able to exhaust the soil of its nutrients (mostly N) in no time and then enter into symbiotic association with compatible rhizobia in order to benefit from the association (Yokota et al., 2009). On the other hand, even in the presence of compatible rhizobia, low yielding varieties of host legumes may not enter into any symbiotic association since a considerable portion of soil nitrogen may be taken up (Yokota et al., 2009). Where nodulation and nitrogen fixation occur in both high yielding and low yielding legumes, the high yielding variety will be more nodulated and derive more nitrogen from fixation than the low yielding variety (Yokota et al., 2009).

Selecting legume species that are compatible with the indigenous soil rhizobia is very important if success of nodulation and nitrogen fixation are to be attained.

2.8 Inoculation

Inoculation refers to the introduction of effective *Rhizobium* bacteria into soils with the intention that, the introduced bacteria will infect the root hairs of seedlings leading to the formation of nodules for nitrogen fixation. Inoculation of legume with compatible and competent bacteria results in a large benefit-cost ratio (Hardy, 1997). The success of inoculation is however dependent on the populations of native RNB (Mpepereki et al., 2000; Wang et al.,
1999; Ballard and Charman, 2000; Sessitsch et al., 2002; Keyser and Li, 1992). According to Thies et al. (1991), response to inoculation can be attained in the presence of rhizobia cells below 10 per gram of soil.

2.9 Assessment of Nodulation and Nitrogen Fixation.

The criteria used most frequently to evaluate and compare treatments in legume experiments are nodulation, dry matter production, amount of nitrogen and phosphorus accumulated in shoots (Cassman, 1981; Osman, 2002).

2.9.1 Nodulation

Nodule number and nodule dry weight are used as criteria for assessing nodulation. The reliability of this method is indicated by the fact that nodule mass is highly correlated to indices of growth such as dry matter and nitrogen content (Brockwell et al., 1982). The use of odulation as an indicator of effective nitrogen-fixation must be done with caution as the results can be misleading.

2.9.2 Dry Matter Yield

Nitrogen accumulation in shoot is strongly correlated with plant growth (Legget, 1971), and the dry matter produced by legumes has therefore been recommended for use as an index to assess effectiveness of nodulation.

Dry matter production, is often linked with the vegetative and early reproductive growth phases of plants which are dependent on total nitrogen uptake (Brockwell et al., 1982). In soils with depleted N, dry matter production is mainly reliant on nitrogen fixation (Brockwell et al., 1982). The relationship between dry matter production and nitrogen fixed is indicated by the significantly greater dry matter accumulation of effectively nodulated plants, compared to the
lower dry matter accumulation by ineffectively nodulated or non-nodulated plants (Singleton et al., 1986).

### 2.9.3 Nitrogen

Nitrogen is essential for the growth and developments of legumes. The total nitrogen in shoots is a commonly used index of nitrogen fixation. Correlations between total foliage nitrogen and acetylene reduction have been reported (Jardin Freirre, 1977; Singleton and Stockinger, 1983). For two legumes with different nitrogen fixing capabilities, there will be more N in the shoot of the legume with a high N fixing capability than in the shoot of the legume with a low N fixing capability. Thus, at harvest, the N content of shoots should be a good parameter to differentiate between legumes with different N$_2$-fixing capabilities.

### 2.9.4 Phosphorus content of shoot

A major factor that limits nitrogen fixation and symbiotic interactions is the availability and supply of phosphorus (Aono et al., 2001; Sadowsky, 2005). Phosphorus has been found to be of higher demand by legumes depending on fixation for N nutrition than legumes receiving nitrogen fertilisation (Graham and Vance, 2000). This could be due to the strong influence on nodulation and nitrogen fixation by availability of P (Leung and Bottomley, 1987). Total foliage phosphorus is commonly used as an index for estimating nitrogen fixation. There correlations between total phosphorus and acetylene reduction (Jardin Freirre, 1977; Singleton and Stockinger, 1983) as increased phosphorus uptake often leads to vigorous plant growth and subsequently higher demands for fixed nitrogen.

### 2.10 Measurement of BNF

In order to evaluate the nitrogen contribution of legumes to agricultural systems, it is important to accurately measure the amount of N$_2$ that these legumes derive from the atmosphere. There are many proposed methods for measuring the amount of nitrogen fixed by legumes in
symbiotic association with RNB. The methods that have been commonly used for estimating nitrogen fixation in plants are total nitrogen difference (TND), acetylene reduction assay and $^{15}$N isotope dilution method. The different methods have their advantages and limitations as well (Rennie and Rennie, 1983; Danso, 1985). The method used to measure the amount of nitrogen fixed in this study was $^{15}$N Isotope Dilution Technique
CHAPTER THREE

3.0 MATERIALS AND METHODS.

3.1 Soil sampling

The soils used in the study were Adenta, Bekwai and Nzima series. Bekwai and Nzima series, classified as Ferric Acrisol and Haplic Acrisol, respectively (Owusu-Bennoah et al., 2000), were sampled from the catena at the Forest and Horticultural Crops Research Centre (FOHCREC) Okumaning-Kade whilst Adenta series, classified as Haplic Acrisol (Dowuona et al., 2012) was sampled from the School Farm at the University of Ghana campus, Legon. The soils were sampled from uncultivated sites. The soils were dug from the 0-20 cm depth, bagged and labelled. The bagged soils were later air dried, crushed to pass through a 2 mm sieve to remove any organic debris, gravels and concretions. The sieved soil was mixed uniformly and portions were sampled for routine analyses in the laboratory. Bekwai and Nzima series are found in the Semi-deciduous forest agro-ecological zone, with an annual rainfall of 1400-1700 mm. The soils have a granitic parent material as they are developed from the lower Birimian. Adenta series on the other hand, is found in the Coastal Savannah agro-ecological zone of Ghana with an annual rainfall of less than 800 mm. Soils in these zones are developed on red, brown sandstone, shale. Adenta series occurs extensively on gentle middle slopes with site gradient of 1-2%.

3.2 Physical Analyses

The physical analyses carried out on the soils are particle size analysis, bulk density and field capacity determinations.
3.2.1 Particle Size Analysis

The hydrometer method of Bouyoucos modified by Day (1965) was used to determine the silt and clay contents of the soils used. Forty grams (40.0 g) of the sieved soil samples was weighed into a dispersing bottle and 100 mL of 5 % Calgon (Sodium Hexametaphosphate) solution was added to the weighed sample to form a suspension. The suspension was agitated using a mechanical shaker for 2 h to disperse the various soil particles into sand, silt and clay. The suspension was then transferred into a 1000 mL graduated sedimentation cylinder and made up to the 1000 mL mark with distilled water. A plunger was lowered into the cylinder and moved up and down, about 5 times to stir the suspension vigorously. Hydrometer readings were taken by lowering the hydrometer in the suspension and the readings taken at the meniscus. The readings were taken after 5 min (i.e. silt plus clay), and thereafter 5 h (i.e. clay). The sand content was determined by decanting the suspension directly onto a 47 µm sieve. The decant was discarded and the residue was washed thoroughly with tap water and then poured into a moisture can with known weight for oven drying at 105°C for 24 h. The weight of the dried particles (sand) was determined after oven drying and the particle distributions for the various soil series were then computed as follows:

Clay content = hydrometer reading at 5 h = \( A \) g  

Silt content = hydrometer reading at 5 min - hydrometer reading at 5 h = \( B \) g  

Sand content (weight of oven dried sample i.e. Dry weight) = \( C \) g

\[
\frac{A}{40} \times 100 \quad [1]
\]

\[
\frac{B}{40} \times 100 \quad [2]
\]

\[
\frac{C}{40} \times 100 \quad [3]
\]

Where 40 = weight of soil sample in grams
The distribution values were used to determine the textural class of the soils using the USDA textural triangle presented in Appendix 1.

### 3.2.2 Bulk density

Bulk density was determined using the core sample method of Blake and Hartge, (1986). Core samples were taken from locations previously selected to be representative of the entire area where the soils for the experiments would be taken. The soil surface was cleared and a cylindrical core sampler was gently driven into the soil far enough to fill the volume of the core with the help of a mallet. The soil surrounding the core sampler was gently removed so that the sampler could be removed from the soil without disturbance. The ends of the sampler were levelled with a knife edge and thereafter, the content was emptied into labelled polybags. The soils were then taken to the lab for bulk density determination.

In the laboratory, the content of the polybag was emptied into a clean moisture can with known weight \( W_1 \). The moisture can together with its contents were oven dried for 72 h at 105°C and thereafter, the weight was taken \( W_2 \). Bulk density was calculated using the formula by Blake (1965).

\[
\rho_b \text{ (kg/m}^3\text{)} = \frac{M}{(\pi d^2/4)h}
\]

[4]

Where

\( \rho_b \) = Bulk density of soil

\( M \) = mass of soil = \( W_2 - W_1 \)

\( W_2 \) = Weight in grams taken after oven drying the moisture can and its contents.

\( W_1 \) = Weight in grams of empty moisture can.
\[ \Pi \frac{d^2}{4} = \text{area of core base} \]

\[ d = \text{diameter of core} \]

\[ h = \text{height of core} \]

\[ \Pi = \text{constant} = 3.142 \]

\[ (\Pi \frac{d^2}{4}) h = \text{volume of core} = \text{volume of soil} \]

### 3.2.3 Field Capacity

Five hundred (500) grams each of the 2 mm sieved soil was weighed into a pot in triplicates and saturated with distilled water. The saturated soil was then allowed to drain for 48 h in open air. Thereafter, sub samples of the soil were taken, weighed and oven dried at a temperature of 150 °C for 24 h. The dry weight of the soil samples was also taken after oven drying the percentage water content at field capacity was computed as follows:

\[
\% \text{ water content} = \frac{\text{weight of wet soil} - \text{weight of oven dried soil}}{\text{Weight of the wet soil}} \times 100 \quad [5]
\]

### 3.3 Chemical analysis

#### 3.3.1 Soil pH

The pH of each soil was measured electrochemically (Peech, 1965) both in water and salt with ratios of 1:1 for soil: distilled water and 1:2 for soil: salt (CaCl2). Twenty (20) grams of sieved soil was weighed into a 50 mL beaker and 20 mL distilled water was added to form a suspension. The suspension was then stirred vigorously for about 30 min. The stirred suspension was allowed to stand for 1 h to allow for the entire suspended particles to settle. The pH meter (Pracitronic M.V 88) was standardised with standard aqueous solutions of pH 4 and pH 7. The pH of the soil was measured after carefully and gently inserting the glass electrode of the pH meter into the supernatant and recorded as pH in water (pHw). The process was
repeated with 20 g of soil and 40 mL of 0.01 M CaCl$_2$. The reading on the pH meter was taken and recorded as the soil pH in salt (pH$_3$)

### 3.3.2 Organic Carbon

The soil organic carbon content was determined using the wet combustion method of Walkley and Black (1934). Half a gram (0.5 g) of 0.5 mm sieved soil was weighed into a 250 mL Erlenmeyer flask. Ten (10) millilitres of 1N potassium dichromate (K$_2$Cr$_2$O$_7$) solution and 20 mL of concentrated sulphuric acid (H$_2$SO$_4$) were added to the content of the flask. The flask was swirled to ensure full contact of the soil with the solution after which it was allowed to stand for 30 min for an efficient combustion. Two hundred (200) millilitres of distilled water and 10 mL of orthophosphoric acid were also added. The unreduced K$_2$Cr$_2$O$_7$ remaining in solution after the oxidation of the oxidizable organic material in the soil sample was titrated with 0.2 N ammonium ferrous sulphate using 3 mL of barium diphenylamine sulphate as indicator. A sharp change to green signified the end point of the reaction. The normality of the Fe (NH$_4$)$_2$(SO$_4$)$_2$ was standardised using a prepared blank solution. The percent organic carbon was calculated as:

\[
\% \text{ C} = \frac{0.3 \times (10 - XN) \times 1.33}{W} \times 100
\]  

[6]

Where $\% \text{ C} = \text{Percent organic carbon}$

- $X =$ Titre value (mL)
- $N =$ Normality of Fe (NH$_4$)$_2$(SO$_4$)$_2$
- $W =$ Weight of soil sample
- $0.3= 0.003 \times 100$
- $0.003 =$ Milliequivalent weight of carbon (g)
- $1.33 =$ correction factor ($f$)
3.3.3 Available Nitrogen

A 10.0 g soil sample that has passed through a 2.0 mm sieve was weighed into a 100 ml extraction bottle and 50 ml of 2 M KCl was added. The soil suspension was shaken for 20 min, after which it was filtered through a No 42 Whatman filter paper into a clean empty plastic bottle. A 10 mL aliquot of the filtrate was taken into a 250 mL Kjeldahl flask and 0.2 g MgO powder was added after which 100 mL of distilled water was added to distil for ammonia (NH₃). The NH₃ was distilled into 5 mL of 2 % boric acid (containing a methylene blue and methyl red indicator mixture) in a 150 mL conical flask. Fifty mL of the distillate was collected. The solution left in the 250 mL Kjeldahl flask was allowed to cool and 0.2 g of Devarda’s alloy was added to reduce the NO₃-N to NH₄⁺-N. Nitrite in the sample was destroyed by the addition of 1 mL of sulphamic acid. Fifty millilitres of the distillate was collected into 5 mL of 2 % boric acid indicator mixture in a separate conical flask. The distillate was titrated against 0.01M HCl. The concentration of NH₄⁺ mg L⁻¹ soil was calculated as follows:

\[
NH₄^+ \text{ kg}^{-1}\text{soil} = \frac{M_{\text{HCl}} \times V_{\text{HCl}} \times 10^{-3} \times 18 \times V_{\text{KCl}} \times 1000 \text{ mg}}{\text{Volume of Aliquot} \times \text{Weight of soil (g)}}
\]  

Where:

\(M_{\text{HCl}}\) = Molarity of the HCl

\(V_{\text{HCl}}\) = Titre of the HCl

\(V_{\text{KCl}}\) = Volume of KCl extractant

18 = Molecular weight of NH₄⁺
3.3.4 Total Nitrogen

The Kjedahl method was used in the determination of total N. Half a gram (0.5 g) of 2 mm sieved soil was weighed into 250 mL Kjedahl flask and a tablet of a digestion accelerator, selenium catalyst was added. This was followed by addition of 5 mL of concentrated H$_2$SO$_4$. The mixture was digested until the digest became clear. The flask was then cooled and its content transferred into a 100 mL volumetric flask. The content was made to the 100 mL mark with distilled water. An aliquot of 5 mL of the digest was taken into a Markham distillation apparatus. Five (5) mL of 40 % NaOH was added and the mixture distilled. The distillate was collected in 5 mL of 2 % boric acid (H$_3$BO$_3$) 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:

\[
\% \text{ N} = \frac{0.01 \times \text{titre volume} \times 0.014 \times \text{volume of extract} \times 100}{\text{Sample weight (g)} \times \text{volume of aliquot (mL)}}
\]  

Where 0.01 = Normality of HCl

0.014 = Milliequivalents of Nitrogen

3.3.5 Available Phosphorus

Available phosphorus was determined using the method of Bray and Kurtz (1945). Five grams of 2 mm sieved soil was weighed into an extraction bottle. Fifty millilitres of Bray 1 solution (0.03M NH$_4$F in 0.025M HCl) was added. The suspension formed was shaken for 3 min on a reciprocating shaker, allowed to settle and filtered through a No. 42 Whatman filter paper into a 100 mL volumetric flask and made up to the volume. Phosphorus in the filtrate was
determined using the molybdate-ascorbic acid colour development method of Watanabe and Olsen (1965) as follows:

Five millilitre aliquots of the supernatant were pipetted in duplicate into a 100 mL volumetric flask and the pH adjusted with para-nitrophenol indicator. Then after, the solution was neutralized with a few drops of ammonium hydroxide (4M NH₄OH) until the colour changed to yellow. This was followed by addition of distilled water till a colourless solution was observed. Reagent A was prepared by dissolving 12 g of ammonium molybdate and 0.2998 g of antimony potassium tartrate in 250 mL of distilled water. Reagent B was prepared by dissolving 1.056 g of ascorbic acid in 200 mL of Reagent A. The dissolved reagents were added to 1000 mL of 2.5 M H₂SO₄, mixed thoroughly and made to volume in a 2000 mL volumetric flask. Eight millilitres of Reagent B was then added to the sample solution and made to volume in a 100 mL volumetric flask. A blank was also prepared using 5 mL of distilled water and 8 mL of reagent B. The Philips PU 8620 spectrophotometer was calibrated using 25 mg L⁻¹ standard P solution prepared in the same manner as above. Phosphorus in the solution was determined by reading the resultant colour intensity on 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

\[
P (\%) = \frac{\text{spectrophotometer reading (mgL}^{-1}) \times \text{total volume of extract}}{\text{volume of aliquot} \times \text{weight of soil sample} \times 10^6} \times 100\quad [9]
\]

**3.3.6 Total Phosphorus**

Total phosphorus was determined by digesting 2 g of 0.5 mm sieved soil with 25 mL of a mixture of concentrated HNO₃ and 60 % HClO₄ in the ratio of 2:3. The digestion was continued until white fumes of HClO₄ ceased. The digest was cooled, diluted with distilled water and then filtered into a 100 mL volumetric flask using a No. 42 Whatman filter paper. The volume
was brought to the 100 mL mark with distilled water. Phosphorus in the filtrate was measured by colour development and read on Philips PU 8620 spectrophotometer as described in section 3.3.5

3.3.7 Exchangeable bases and Cation Exchange Capacity (CEC) Determination

3.3.7.1 Extraction of exchangeable bases.

Ten (10) grams of soil was weighed into a 200 mL extraction bottle and 100mL of 1N ammonium acetate (NH₄OAc) solution buffered at pH 7.0 was added. The bottle and its content were placed on a mechanical shaker and shaken for 1 h, and thereafter centrifuged at 3000 rpm for 20 min. The supernatant solution was then filtered through a No. 42 Whatman filter paper. The filtered solutions (aliquots) were used for the determination of Ca, Mg, K and Na.

3.3.7.2 Calcium.

To a 10 mL aliquot of the sample solution, 10 mL of 10% KOH and 1mL triethanolamine (TEA) were added. Three drops of 1M KCN solution and a few crystals of cal-red indicator were then added after which the mixture was titrated with 0.02N EDTA solution from red to blue end point. The titre value was used in the calculation of calcium as shown below.

\[
Ca \text{ cmol}_c kg^{-1} = \frac{\text{Titre value} \times N \times \text{Vol of extract} \times 100}{\text{Aliquot} \times \text{Weight of soil}}
\]  

[10]

Where N = Normality of EDTA

3.3.7.3 Magnesium.

To a 10 mL aliquot of the sample solution, 5 mL of ammonium chloride-ammonium hydroxide buffer solution was added followed by 1mL of triethanolamine. Three drops of 1M KCN solution and a few drops of Erichrome black T solutions were added after which the mixture was titrated with 0.02 N EDTA solution from red to blue end point. The end point titre value
determines the amount of calcium and magnesium in the solution. The titre value of magnesium was then determined by subtracting the value obtained for calcium above from the new titre value obtained. The titre value of magnesium was then used for the calculation of the concentration of magnesium (Mg) as shown below.

\[
\text{Mg } \text{cmol}_c \text{kg}^{-1} = \frac{\text{Titre value } \times \text{N } \times \text{Vol.of extract } \times 100}{\text{Aliquot } \times \text{Weight of soil}} \tag{11}
\]

Where \( N \) = Normality of EDTA

3.3.7.4 Potassium

The flame photometer was standardized such that 10 mg/kg of K gave 100 full scale deflections. The flame photometer after standardization was used to determine the concentration of potassium in the aliquot. The result was used in the calculation of the amount of potassium present in the soil as shown in the formula below.

\[
\text{K } \text{cmol}_c \text{kg}^{-1} = \frac{R \times \text{Vol.of extract } \times 100}{\text{Weight of soil } \times 39.1} \tag{12}
\]

Where,

\( R \) is the flame photometer reading (ppm)

39.1 = Atomic weight of K

3.3.7.5 Sodium

The flame photometer was standardized in a way that 10 mg/kg of Na gave 100 full scale deflections. After the standardization of the photometer, the concentration of sodium in 10mL aliquot was determined. The result was then used in the calculation of the amount of sodium (Na) present in the soil as shown by the formula below.
\[ \text{Na cmol} \cdot \text{kg}^{-1} = \frac{R \times \text{Vol. of extract} \times 100}{\text{Weight of soil} \times 23} \]  

Where,

- \( R \) (ppm) = flame photometer reading
- 23 = Atomic weight of Na

### 3.3.7.6 Cation Exchange Capacity (CEC)

The residue after filtration in section 3.3.7.1 was immediately leached with 25 mL portions of methanol into empty plastic bottles. The soil was leached again with 25 mL portions of acidified 1M KCl. Each portion was added at a time and allowed to pass through, before adding the next portion. Ten millilitres of the leachate was transferred into a Kjedahl flask and 10 mL of 40% NaOH was added and then distilled. The distillate was collected into 5 mL of 2% boric acid and an aliquot was titrated against 0.01M HCl. The ammonium ion concentration in the filtrate was determined and the CEC of the soil in cmol\( _c \) kg\(^{-1} \) soil estimated.

### 3.3.8 Exchangeable acidity and Effective Cation Exchange Capacity (ECEC) Determination

#### 3.3.8.1 Extraction of exchangeable acidity (H\(^+\) and Al\(^{3+}\)).

Ten (10) grams of soil was weighed into a 100 mL extraction bottle and 50 mL of 1M KCl solution was added. The bottle and its content were placed on a mechanical shaker and shaken for 30 min. The soil suspension was then filtered through a No. 42 Whatman filter paper into an empty clean bottle. Twenty five millilitre aliquot was pipetted into a 100 mL conical flask and 2-3 drops of phenolphthalein indicator was added for titration to a permanent pink end point against 0.01M NaOH. The titre value was recorded as titre for both H\(^+\) and Al\(^{3+}\). Ten millilitres
of NaF was added to the solution at the endpoint and back titrated against 0.01M HCl until a colourless end point was reached. The titre was recorded as the titre for Al$^{3+}$.

3.3.8.2 Effective Cation Exchange Capacity (ECEC)

The Effective Cation Exchange Capacity is equal to the sum of the exchangeable Ca$^{2+}$, Mg$^{2+}$, Na$^+$, K$^+$, H$^+$, and Al$^{3+}$. (i.e. Ca$^{2+}$+ Mg$^{2+}$+ Na$^+$+ K$^+$+ H$^+$+Al$^{3+}$)

3.4 Biological Analysis

3.4.1 Estimation of the populations of rhizobia in the soils using the Most Probable Number (MPN) plant infection technique.

The populations of indigenous bradyrhizobia in the soils capable of nodulating the test legumes were estimated by the Most Probable Number (MPN) plant infection assay (Vincent, 1970) using a modified Leonard jar assembly by Ferreira and Marques (1992). The assembly was composed of a plastic cup tapered to a similar cup at the bottom. The cup containing the rooting medium (6 M HCl washed sand) was inserted into a similar plastic cup containing 100 mL of N free nutrient solution (Somasegaran and Hoben, 1994). The rooting medium was irrigated with a cotton wick connecting the upper and the lower units (containing the nutrient solution). Thereafter, the whole assembly was autoclaved to get rid of microorganisms. The legume seeds were surface sterilized in 70% ethanol for 3 min and rinsed thoroughly in several changes of sterile 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 pick up the sterilized seeds and plant at two seedlings seed per growth pouch with the radicle facing downwards. The holes were deep enough to accommodate pre-germinated seeds 0.5 cm below the surface. The assemblies were randomly arranged in a greenhouse. Ten fold serial dilutions up to level 5 ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$) were prepared for each of the soils using yeast extract mannitol broth as diluent, and used as inoculum. One millilitre of the
inoculum was used to inoculate the seedlings in triplicates per every dilution level. 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 was 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:

\[ \text{MPN} = \frac{m \times d}{v} \]  

Where: \( m \) is the most likely number from MPN table (Alexander, 1965).

\( d \) is the lowest dilution in the series and

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

3.5 Greenhouse Experiment.

3.5.1 Test crops used

The test crops used were soybean (\textit{glycine max} cultivar Anidaso), pigeonpea (\textit{Cajanus cajan} cultivar ICPL 88034), cowpea (\textit{Vigna unguiculata} cultivar black eye) and maize (\textit{Zea maiz} Cultivar Obaatampa used as a reference plant).

3.5.2 Nitrogen and Phosphorus Response Experiment

In all, three greenhouse experiments were conducted in this study. The experiments were conducted in plastic pots (14.5 cm high, 16.3 cm wide at the top and 11.2 cm wide at the base). Three openings were created at the base of the pots and covered with cotton wool. The pots were filled with 2 kg of sieved soil and then after, subjected to the field bulk densities of the soils. Water was applied at field capacity and then the pots were allowed to stay for two days before seeds were sown into them. The pots were placed in basins with water serving as reservoir. Water supplied in the basins was taken up through the openings created at the base.
of the pots. The first experiment was conducted to assess the response of soybean and pigeonpea to nitrogen and phosphorus fertilizer application in the Bekwai and Adenta soils. The counteracting effect of P on N inhibition of nodulation as well as N toxicity on plant growth were also assessed. In one treatment, \((NH_4)_2SO_4\) was applied alone at rates 0, 40, 80, 120, 160 and 200 kg N/ha. In another treatment, triple super phosphate (TSP) was first applied alone at rates 0, 40, 80, 120, 160 and 200 kg P/ha then in combination with 100 kg N/ha as another treatment. The second experiment was conducted to assess the effect of P on nitrogen fixation in the Adenta soil. Phosphorus was applied at rates 0, 40, 80, and 120 kg P/ha. Nitrogen in the form of \((^{15}NH_4)_2SO_4\) which contained 10.93 \(^{15}\text{N}\) % natural abundance was applied at a rate of 10 kg N/ha in two splits (5 days after germination and three weeks after germination) to the pots to supply 1.5 mg N/pot. The last experiment was carried out to investigate the effect of P on the diversity of the indigenous bradyrhizobia that nodulated soybean, pigeonpea and cowpea in the Adenta, Bekwai and Nzima soils. Phosphorus was applied at 0 and 80 kg P/ha rates. The experiments were carried out one at a time. All the experiments were arranged in a completely randomised design with 4 replications. Four seeds were planted into each pot and later thinned to two, 4 days after emergence. The plants were kept in a greenhouse and watered daily (such that soil moisture was kept close to field capacity) for eight (8) weeks after which they were harvested for observations and further investigations.

3.5.3 Harvesting

The plants were allowed to grow for 8 weeks after which they were harvested. In most cases, nodules were selected for rhizobia isolation and characterisation. Nodule number and dry weight of nodules were taken as well. The harvested shoots were oven dried at 78\(^{\circ}\)C for 72 h to attain constant weight and thereafter the dry weights were taken. The dried shoots were ground using an electric grinder after which samples were taken for total nitrogen and phosphorus and \(^{15}\text{N}\) analyses.
3.5.4 Isolation of N\textsubscript{2}-fixing bacteria (rhizobia) from root nodules.

Nodules were carefully detached from washed roots of healthy green legumes as described by (Vincent, 1970). The detached nodules were carefully blotted dry with tissue and immersed momentarily in 75 % ethanol followed by another 3 min exposure to 0.1 % acidified HgCl\textsubscript{2} solution. After rinsing for 7 times with sterile de-ionized water, each nodule was dissected and the coloration of the internal bacteroid tissue noted. The dissected nodule was crushed in a drop of sterile de-ionized water, and a cooled sterile loop was used to pick from the turbid suspension formed and streaked unto yeast-mannitol agar (YMA) plates as described by Beck \textit{et al}. (1993). The plates were incubated at 28°C and the growth was monitored after day one for fast and slow growing rhizobia. Isolated single colonies were selected and re-streaked unto Congo red YEM for purification and thereafter authenticated (Vincent, 1970; Dakora and Vincent, 1984).

3.5.5 Estimation of N\textsubscript{2} fixed

The \% \textsuperscript{15}N atom excess in the fixer and non-fixer were analysed from KULEUVEN research and development laboratory in Belgium. From comparisons of the \textsuperscript{15}N contents of the fixer and the reference crop, the \% Nd\textsubscript{fixer} in fixing plants were calculated based on the formula established by Fried and Middelboe (1977).

The total N in shoots was analysed for \textsuperscript{15}N, and the percentage of N derived from the atmosphere (%Nd\textsubscript{fixer}) by the legume was calculated using the equation:

\[
\% \text{Nd}\textsubscript{fixer} = 1 - \frac{\% \text{Nd}(\text{fixer})}{\% \text{Nd}(\text{non-fixer})} \times 100
\]  

Where:

\% Nd\textsubscript{fixer} = percent N derived from the \textsuperscript{15}N labelled fertilizer by the fixing plant

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

Actual nitrogen fixed = % Ndfa x shoot total nitrogen of the fixing plant \[16\]

3.6 Statistical Analysis

The data collected from the various experiments were subjected to general Analysis of Variance and the means obtained were compared by LSD 5 % level of significance using GenSTAT (9th edition) software. Microsoft Excel program was used to generate graphs for data presentation.

3.7 Molecular Characterisation

Nodules were selected to be representative of the total number of nodules collected from soybean, pigeonpea and cowpea in Adenta, Bekwai and Nzima soils. In all about 150 strains of Bradyrhizobium were isolated from the selected nodules. Total genomic DNA was extracted for 120 isolates and also the reference strain USDA 110. The extracted genomic DNAs were used for DNA finger printing. Two sets of primers were used for the PCR amplifications. One set of the Primers for Bradyrhizobium was RPO4 (random) and RPO1 (specific, targeting the conserved nif gene promoter region) (Richardson et al., 1995). The other set of primers used were those targeting the 16S, 23S and ITS conserved regions of Bradyrhizobium. Isolates from fertilised treatments were designated F whereas those from unfertilised treatments were designated U.

3.7.1 DNA Extraction

Total genomic DNA was extracted using the Qiagen DNeasy plant mini DNA extraction kit. The rhizobial isolates were grown in YEM broth in an incubator shaker at 150 rpm at 28°C for 72 h. About 1-2 mL of rhizobial cultures were centrifuged at 8500×g (5950 rpm) for 10 min and the supernatant discarded. The cell pellets were re-suspended in 400 μL of preheated AP1
buffer (lysis buffer), vortexed and incubated at 65°C for 15 min in a water bath. The tubes were inverted every 5 min during the incubation period. The cell pellets were added with 130 µL of AP2 buffer (precipitation buffer), vortexed and incubated on ice for 5 min. The lysate was transferred into a DNA Mini spin column placed in a 2 mL collection tube and centrifuged for 5 min 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 650 µL of the mixture and transferred into a DNeasy Mini spin column in a 2 mL collection tube and then centrifuged for 1 min at 6000×g (4200 rpm), after which the flow-through was discarded. Five hundred microliters of AW buffer was added and centrifuged for 2 min at 20,000×g (14,000 rpm). 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 min, centrifuged at 6000×g (4200 rpm) for 1 min. The column was discarded and the DNA stored at 4°C for short term use and -20°C for long term use.

**3.7.2 PCR Amplification of Genomic DNA using RPO1 and RPO4 Primers**

Total genomic DNA of the bradyrhizobial isolates were amplified using the primers, RPO1 and RPO4. Amplification reaction was carried out in a 12.5 µL final reaction volume containing 1 µL of 12.5 mM MgCl₂, 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 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°C for 3 min. 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 min at 90 V and photographed under UV illumination in a GeneFlash (Syngene BIO Imaging) unit.
3.7.3 PCR Amplification of 16S-23S Intergenic Spacer (ITS) and 16S rDNA Gene

Polymerase chain reactions (PCR) of the 16S rDNA, 23S rDNA and the 16S-23S rDNA internally transcribed (ITS) conserved regions were performed with the primer sets; 16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SR (5′-AAGGAGGTGATCCAGCCGCA-3′) ITS 149072F (5′-TGCGGCTGGATCCCCTCCTT-3′), 23SF and ITS 13238R (5′-CCGGGTTTCCCCCATTCGG-3′). Amplification reactions were performed in a 25 µL volume, containing 2 µL MgCl₂(25mmolL⁻¹), 0.5 µL of dNTPs, 2.5 µL of 10× buffer, 0.5 µL Taq polymerase, 12.8 µL of H₂O, 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 1min, 35 cycles consisting of 1 min denaturation at 94°C, 1 min of primer annealing at 50°C and 2min of extension at 72°C, plus a 3min final extension at 72°C.

3.7.4 Cluster Analysis

Gel electrophoresed images were analysed and scored for presence (1) and absence (0) of band using the PyElph version 1.4 software. The patterns obtained from the PCR using oligonucleotide primers RPO1 and RPO4 were combined for cluster analysis. A simple matching coefficient was calculated to construct a similarity matrix and the Unweighted Pair Group Mean Arithmetic Method (UPGMA) algorithm was used to perform hierarchical cluster analysis and to construct a dendogram by using NTSYS-pc package V.2.1 (Rohlf, 1998) at a matrix of 1. Genetic comparisons for the isolates were carried out and clusters formed at 80 % similarity were considered different with isolates within a particular cluster being genetically similar.
3.7.5 Diversity Indices

The Shannon-Weaver (1949) diversity index and Pielou (1975) evenness index were calculated for every dendrogram generated and were compared to ascertain where a greater diversity among isolates occurred. These Indices of diversity (H') were estimated based on the number of clusters formed at 80% similarity.

3.7.5.1 Shannon-Weaver diversity index.

The Shannon-Weaver (1949) diversity index was calculated from the formulae below:

\[ H' = - \sum_{i=1}^{n} P_i \ln P_i \]  \[17\]

Where \( P_i \) is the proportion of individuals found in clusters i. For a well-sampled community, this proportion is given as \( P_i = n_i/N \), where \( n_i \) is the number of individuals in cluster i and \( N \) is the total number of individuals in the dendogram. 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.

3.7.5.2 Pielou evenness index

The Pielou (1975) evenness index was calculated from the formulae below:

\[ J = \frac{H'}{\ln S} \]  \[18\]

where \( H' \) is the Shannon–Weiner index and \( S \) is the total number of clusters per soil at 80% similarity level.
CHAPTER FOUR

4.0 RESULTS

4.1 Physical and chemical properties of soils used.

The Adenta soil had the highest proportion of sand (62 %) followed by the Nzima soil (33 %) and then the Bekwai soil (23 %) (Table 1). Among the three soils, the soils from the semi-deciduous forest contained higher silt (25 %) than the soil from the coastal savannah with 13 % silt. The Bekwai soil contained the most clay (52 %), while percent clay content in the Adenta series (25 %), was the lowest (Table 1). From the textural analysis, the Adenta soil was classified as a sandy clay loam using the USDA textural triangle (Appendix 1) with the Bekwai and Nzima soils being classified as clay. Bulk density was highest in the Bekwai soil (1.49 Mg m$^{-3}$) followed by the Nzima soil (1.42 Mg m$^{-3}$) and then the Adenta soil (1.35 Mg m$^{-3}$). The pH analyses conducted on the soils show that the Bekwai soil had the lowest pH in water (5.5) followed by the Nzima (5.6) and then the Adenta (6.2). A similar trend was observed for CaCl$_2$ determined pH. In all cases the pH determined in CaCl$_2$ was higher than the pH determined in water The Nzima soil contained the highest organic carbon (33.5 g kg$^{-1}$) followed by the Bekwai (31.9 g kg$^{-1}$) while the Adenta soil contained the lowest organic carbon (23.1 g kg$^{-1}$). The total nitrogen values obtained for the Bekwai and Nzima soils were higher than that for the Adenta soil with the Bekwai soil being the highest (Table 1). The Adenta soil, although recorded the lowest total nitrogen, had the highest nitrogen in soil solution. The Bekwai and Nzima soils recorded lower available phosphorus and available nitrogen compared to that for Adenta soil (Table 1). The cation exchange capacity (CEC) was highest in the Nzima soil (19.5 cmol$_c$ kg$^{-1}$) followed by the Bekwai soil (18.7 cmol$_c$ kg$^{-1}$) while the Adenta soil recorded the lowest CEC (7.0 cmol$_c$ kg$^{-1}$). The effective CEC (ECEC) was found to be highest in the Nzima soil (7.47) and lowest in the Adenta soil (5.25). The high clay content of the soils from the semi-deciduous forests together with a high organic carbon content accounted for the high differences in their
CEC and ECEC. The CEC and ECEC data from the coastal savannah soil with a lower organic carbon content and a lower clay content as well did not reveal such great difference compared to the forest soils (McCauley et al., 2003).

Table 1. Physical and Chemical properties of Adenta, Bekwai and Nzima soils.

<table>
<thead>
<tr>
<th>Soil Properties</th>
<th>Adenta</th>
<th>Bekwai</th>
<th>Nzima</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand (%)</td>
<td>62</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>13</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>25</td>
<td>52</td>
<td>42</td>
</tr>
<tr>
<td>Texture</td>
<td>SCL</td>
<td>Clay</td>
<td>Clay</td>
</tr>
<tr>
<td>Bulk Density (Mg m$^{-3}$)</td>
<td>1.35</td>
<td>1.49</td>
<td>1.42</td>
</tr>
<tr>
<td>pH H$_2$O (Soil: Water, 1:1)</td>
<td>6.2</td>
<td>5.5</td>
<td>5.6</td>
</tr>
<tr>
<td>pH CaCl$_2$ (Soil: CaCl$_2$, 1:2)</td>
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<td>4.3</td>
<td>4.4</td>
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<tr>
<td>Organic Carbon (g kg$^{-1}$)</td>
<td>23.1</td>
<td>31.9</td>
<td>33.5</td>
</tr>
<tr>
<td>Total N (g kg$^{-1}$)</td>
<td>0.98</td>
<td>1.96</td>
<td>1.62</td>
</tr>
<tr>
<td>Available N (mg kg$^{-1}$)</td>
<td>304.56</td>
<td>270</td>
<td>233.28</td>
</tr>
<tr>
<td>Available P (mg kg$^{-1}$)</td>
<td>8.75</td>
<td>2.79</td>
<td>2.54</td>
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<tr>
<td>Total P (mg kg$^{-1}$)</td>
<td>97.00</td>
<td>95.75</td>
<td>83.5</td>
</tr>
<tr>
<td>Cation Exchange Capacity (cmol$_c$kg$^{-1}$)</td>
<td>7.1</td>
<td>18.7</td>
<td>19.5</td>
</tr>
<tr>
<td>Exchangeable bases (cmol$_c$kg$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>2.9</td>
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<td>1.47</td>
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<tr>
<td>K</td>
<td>0.3</td>
<td>0.7</td>
<td>0.61</td>
</tr>
<tr>
<td>Na</td>
<td>0.11</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>Exchangeable Al$^{3+}$ (cmol$_c$kg$^{-1}$)</td>
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<td>0.2</td>
<td>0.13</td>
</tr>
<tr>
<td>Exchangeable H$^+$ (cmol$_c$kg$^{-1}$)</td>
<td>0.34</td>
<td>0.24</td>
<td>0.44</td>
</tr>
<tr>
<td>Effective CEC (cmol$_c$kg$^{-1}$)</td>
<td>5.25</td>
<td>6.91</td>
<td>7.47</td>
</tr>
</tbody>
</table>

SCL: Sandy Clay Loam    CEC: Cation Exchange Capacity
4.2 Populations of bradyrhizobia nodulating soybean and cowpea in Adenta, Bekwai and Nzima series.

The results from the population study using the Most Probable Number (MPN) plant infection technique revealed the presence of bradyrhizobia in all the soils studied (Table 2). The population sizes of indigenous bradyrhizobia nodulating the various legumes in the different soils varied widely ranging from $0.7 \times 10^1$ to $7.8 \times 10^3$ cells/g soil. The highest population of soybean nodulating *Bradyrhizobium* was recorded in Nzima series ($4.5 \times 10^2$ cells/g soil) and the lowest in Bekwai series ($0.7 \times 10^1$ cells/g soil). Ninety two cells of soybean nodulating *Bradyrhizobium* were present in each gram of the Adenta soil (Table 2). Cowpea was used as a control and nodulated in all the soils. The size of *Bradyrhizobium* nodulating cowpea in Bekwai series was the highest. The population of cowpea nodulating *Bradyrhizobium* present in the soils is ranked as follows Bekwai ($7.8 \times 10^3$ cells/g soil) > Nzima ($9.2 \times 10^2$ cells/g soil) > Adenta ($9.2 \times 10^2$ cells/g soil). In all the soils, the population of the *Bradyrhizobium* nodulating cowpea was higher than that of the *Bradyrhizobium* nodulating soybean.

Table 2. Populations of indigenous soybean and cowpea bradyrhizobia estimated by the Most Probable Number (MPN) technique.

<table>
<thead>
<tr>
<th>Legume Host</th>
<th>Most Probable Number technique (cells/g soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil series</td>
</tr>
<tr>
<td>Soybean</td>
<td>Adenta $9.2 \times 10^1$ Bekwai $0.7 \times 10^1$ Nzima $4.5 \times 10^2$</td>
</tr>
<tr>
<td>Cowpea</td>
<td>Adenta $6.8 \times 10^2$ Bekwai $7.8 \times 10^3$ Nzima $9.2 \times 10^2$</td>
</tr>
</tbody>
</table>
4.3 Responses of soybean and pigeonpea to nitrogen and phosphorus fertilizer application in Adenta and Bekwai series.

4.3.1 Phosphorus fertilizer application and nodulation of soybean and pigeonpea.

Although the application of P resulted in higher nodulation in pigeonpea grown in the Adenta soil beyond the 60 nodules/plant observed for the control, the differences were not significant (P > 0.05) up to the 120 kg P/ha rate. Further application of P to 160 kg P/ha, however, resulted in a non-significant (P > 0.05) decrease in nodulation whereas application of 200 kg P/ha resulted in a significant (P < 0.05) decrease in nodulation when compared to the control. In the case of soybean, application of P up to 120 kg P/ha gave no significant (P > 0.05) increases in number of nodules formed compared to the control (Fig. 1). Significant (P < 0.05) increases in the nodules formed on soybean were, however, observed with phosphorus application at rates of 160 kg P/ha and 200 kg P/ha in the Adenta soil over the control. About 4-fold and 6-fold increases in nodule numbers compared to the control were recorded when soybean was fertilized with 160 kg P/ha and 200 kg P/ha, respectively in the Adenta soil. The number of nodules recorded for soybean increased from 6 in the control to 40 when 200 kg P/ha was applied and this was the highest. Except for the number of nodules recorded for pigeonpea (19) at a rate of 200 kg P/ha which was lower than the number of nodules formed on soybean (40) at the same rate of phosphorus application, nodule numbers recorded for pigeonpea were higher than those for soybean at the other rates of phosphorus applied.

In the Bekwai soil, the application of 40 kg P/ha and 80 kg P/ha gave no significant increases in nodule numbers for pigeonpea over the control (Fig. 1). Nodule numbers recorded at rates 160 and 200 kg P/ha were significantly (P < 0.05) higher than that for the control (equivalent to about 5-fold and 8-fold increases at 160 and 200 kg P/ha, respectively). None of the rates of
phosphorus applied on the other hand was able to reverse the inability of soybean to form nodules in the Bekwai soil (Fig. 1).

![Fig. 1. Effect of phosphorus fertilizer application on the number of nodules formed on soybean and pigeonpea grown in the Adenta and Bekwai series](image)

**4.3.2 Effect of phosphorus fertilizer application on dry weight of nodules formed on soybean and pigeonpea.**

In the Adenta soil the dry weights of nodules formed on pigeonpea and soybean in the control treatments were, 63.30 mg and 3.30 mg, respectively. When 40 kg P/ha was applied to the Adenta soil, no increases over the controls in the nodule dry weights were recorded for pigeonpea and soybean. Phosphorus application up to 120 kg P/ha in the Adenta soil resulted in no significant (P > 0.05) increase in the dry weights of nodules formed on pigeonpea (Fig. 2). Above 120 kg P/ha, any increase in the rate of phosphorus applied resulted in decreases in the dry weight of nodules formed (Fig. 2). Pigeonpea receiving 200 kg P/ha recorded a 52.6% decrease in nodule dry weight compared to the control.

Nodulation in soybean was increased to 6.70 mg and 10.00 mg when 80 kg P/ha and 120 kg P/ha, respectively, were applied. Increased application to 160 kg P/ha and 200 kg P/ha resulted
in 40.00 mg and 80.00 mg nodule dry weights, respectively, which translate into 11-fold and 23-fold increases (significant at P < 0.05), respectively. The highest dry weight of nodules was recorded at 200 kg P/ha.

Application of phosphorus to pigeonpea in the Bekwai soil resulted in a significant increase in nodule dry weight from 10.00 mg at the 0 kg P/ha rate to 116.7 mg at the 200 kg P/ha rate. No significant increases in nodule dry weights were recorded among treatments 0, 40, 80, 120 and 160 kg P/ha. The increase recorded when 200 kg P/ha was applied translates into 10-fold that of the control and was the highest.

4.3.3 Nitrogen fertilizer application and nodulation of soybean and pigeonpea

In contrast to pigeonpea, with or without applied N, soybean did not nodulate in the Bekwai soil (Fig. 3). The number of nodules recorded for pigeonpea and soybean in the control treatments (when no nitrogen was applied) were 6 and 0, respectively. For both legumes, nitrogen fertilizer application was detrimental to nodule formation in both soils. In the Adenta
soil the highest numbers of nodules were formed on both legumes at the 0 kg N/ha rate (Fig. 3). The numbers of nodules recorded for pigeonpea and soybean in the control treatments were, 65 and 6, respectively. When 40 kg N/ha was applied to the Adenta soil, the number of nodules recorded for pigeonpea was 48 and this was 26.2 % lower than that recorded for the control. Further decreases in nodule numbers were recorded on pigeonpea upon increasing the rate of nitrogen applied up to 200 kg N/ha (Fig. 3). However, nodulation among the treatments receiving 120, 160 and 200 kg N/ha was not significant (P > 0.05). In the case of soybean, application of 40 kg N/ha decreased the number of nodules formed in the Adenta soil by 33.3 % and the application of 200 kg N/ha decreased the number of nodules formed from six (6) in the control to zero (0), indicating a complete inhibition of nodulation at the 200 kg N/ha rate. The number of nodules recorded for pigeonpea were significantly higher than those recorded for soybean at all the rates of nitrogen fertilizer applied with the mean number of nodules recorded for pigeonpea being significantly higher than that recorded for soybean.

![Fig. 3. Effect of nitrogen fertilizer application on the number of nodules formed on soybean and pigeonpea grown in the Adenta and Bekwai series.](http://ugspace.ug.edu.gh)
4.3.4 Effect of nitrogen fertilizer application on the dry weight of nodules formed on soybean and pigeonpea.

In the control treatments, the dry weights of nodules formed were, 63.30 mg and 3.30 mg for pigeonpea and soybean, respectively in the Adenta soil and 10.00 mg for pigeonpea in the Bekwai soil. In the Adenta soil, application of nitrogen at 40 kg N/ha resulted in 26.2 % decrease in the dry weight of nodules formed on pigeonpea. The dry weights of nodules recorded at rates 120 kg N/ha, 160 kg N/ha and 200 kg N/ha were all significantly lower (P < 0.05) than that for the control (Fig. 4). When 40 kg N/ha was applied to the Adenta soil, the measuring scale could not weigh the dry weight of soybean nodules obtained.

In the Bekwai soil, application of 40 kg N/ha decreased the dry weight of nodules formed on pigeonpea to 3.00 mg. The measuring scale could not weigh the dry weight of pigeonpea nodules formed at 80 Kg N/ha. No nodules were formed beyond 80 kg N/ha.

![Fig. 4. Effect of nitrogen fertilizer application on dry weight (g) of nodules formed on soybean and pigeonpea grown in the Adenta and Bekwai series.](http://ugspace.ug.edu.gh)
4.3.5 Combined application of phosphorus and nitrogen fertilizers on nodulation of soybean and pigeonpea.

In the Adenta soil, with no N and P application, numbers of nodules formed on pigeonpea and soybean were 65 and 6, respectively. With 40 kg P/ha applied there was no effect on the number of nodules formed on soybean whereas the nodules formed on pigeonpea were increased to 72. Nodulation was enhanced (26 nodules) when 100 kg N/ha was combined with 120 kg P/ha. A combination of 160 kg P/ha and 100 kg N/ha increased the nodule number over the control. The combined application of 100 kg N/ha and 200 kg P/ha, however, decreased nodulation compared to the control (Fig. 5). Application of combined 100 kg N/ha and 40 kg P/ha revived nodulation (3 nodules) in soybean. When 80 kg P/ha was applied together with 100 kg N/ha, the number of nodules formed on soybean in the Adenta soil was increased slightly to seven (7) and this was similar to the control. Soybean in the Adenta soil receiving P at the rates of 120 kg P/ha and 160 kg P/ha each produced more than 4 times the number of nodules in the control treatment with the value for the 120 kg P/ha rate being the highest (Fig. 5). Increasing the P rate to 200 kg P/ha reduced the number of nodules compared to the control treatment (N0P0 kg/ha) but not significantly.

In the Bekwai soil, six nodules were recorded for pigeonpea in the control treatment. Application of P at rates 40 kg P/ha and 80 kg P/ha in combination with the inhibitory rate of 100 kg N/ha resulted in the formation of 1 nodule in each case. Nodulation was restored to the level of the control (Fig. 5) when 100 kg N/ha was combined with 120 kg P/ha. Further increase in the P rates beyond 120 kg P/ha up to 200 kg P/ha in combination with 100 kg N/ha increased the number of nodules to 21 and 32 for the 160 kg P/ha and 200 kg P/ha rates, respectively. The increases observed translate into 3-folds and 5-folds for the 160 kg P/ha and 200 kg P/ha rates, respectively, compared to the control. Soybean on the other hand did not nodulate at all in all the treatments.
4.3.6 The combined application of nitrogen and phosphorus fertilizers on the dry weight of nodules formed on soybean and pigeonpea.

In the Adenta soil, with no N and P applied, dry weight of nodules formed on pigeonpea and soybean were 63.30 mg and 3.30 mg, respectively. Combined application of 100 kg N/ha and 40 kg P/ha did not give any significant increase in dry weight of nodules above the control in both legumes. Nodulation in pigeonpea was, however, boosted when 100 kg N/ha was combined with 200 kg P/ha resulting in the formation of nodules weighing 60.00 mg. With the combined application of 100 kg N/ha and 80 kg P/ha, the dry weight of nodules formed on soybean increased greatly to 13.30 mg. The highest dry weight of nodules formed on soybean (73.30 mg) was recorded when 100 kg N/ha was applied in combination with 120 kg P/ha. The dry weight of nodules decreased with combined application of 100 kg N/ha and 160 kg P/ha although the value recorded (60 mg) was still high compared to the control (3.30 mg). The dry weight of nodules formed on soybean was further decreased when 100 kg N/ha was applied
in combination with 200 kg P/ha and the value recorded was same as that for the control (Fig. 6).

In the Bekwai soil, with no N and P application, dry weight of nodules formed on pigeonpea was 10.00 mg. Nodulation was revived when 100 kg N/ha was combined with 120 kg P/ha resulting in the formation of nodules weighing 33.30 mg. Increasing the rate of phosphorus applied in the combined fertilizer treatment to 160 kg P/ha resulted in a dry weight of 166.7 mg for the nodules formed and this was the highest. The increases observed at rates 120 kg P/ha and 160 kg P/ha applied were significant (P < 0.05) and translate into 9-fold and 50-fold, respectively. A decrease in nodule dry weight relative to the 160 kg P/ha rate was observed when phosphorus was applied at 200 kg P/ha. Thus, treatments receiving 120, 160 and 200 kg P/ha reversed the N inhibition effect on the dry weight of nodules formed.

Fig. 6. The combined application of nitrogen and phosphorus fertilizers on dry weight (g) of nodules formed on soybean and pigeonpea.
4.3.7 Effect of phosphorus fertilizer application on shoot dry weight production by soybean and pigeonpea.

The shoot dry weight values for pigeonpea and soybean in the phosphorus control treatments in the Adenta soil were 2.31 g and 5.99 g, respectively (Fig. 7). Application of 40 kg P/ha resulted in about 90.9 % and 3.3 % increases in shoot dry weight for pigeonpea and soybean, respectively. When the rate of phosphorus applied was increased to 80 kg/ha, pigeonpea recorded an even higher (138 %) shoot dry weight over the control than soybean (5 %). Pigeonpea recorded the highest response at rate 160 kg P/ha which was more than 2-folds compared to the control. Soybean on the other hand recorded the highest response at rate 200 kg P/ha though this was very low (0.3 fold) when compared to that for pigeonpea. Pigeonpea recorded a decrease in shoot dry weight when 200 kg P/ha was applied. Soybean recorded, higher shoot dry weights than pigeonpea at all levels of phosphorus applications.

The shoot dry weights of pigeonpea and soybean grown in unfertilized Bekwai soil were 2.37 g and 1.77 g, respectively. Application of 40 kg P/ha resulted in about 22 % and 183 % increases in shoot dry weights of pigeonpea and soybean, respectively. Whereas an increase in the rate of phosphorus fertilizer to 80 kg P/ha gave a 0.7-fold increase in shoot dry weight over the control for pigeonpea, the increase observed for soybean receiving the same level of phosphorus application was about 2-folds more than the control. When the P rate was further increased from 80 kg P/ha up to 200 kg P/ha, both legumes gave significant increases in shoot dry weight and both legumes recorded highest weight of dried shoots at 200 kg P/ha.
4.3.8 Effect of nitrogen fertilizer application on shoot dry weight of soybean and pigeonpea.

Except for soybean growing in the Bekwai soil which recorded varying responses with nitrogen application, the control (0 kg N/ha) in all cases recorded the lowest dry weight of shoots (Fig. 8). In the Adenta soil, without N fertilizer, the shoot dry weights were 2.31 g and 5.99 g for pigeonpea and soybean respectively, and when nitrogen was applied at the rate of 40 kg N/ha, 64% and 15% increases in the dry weight of shoots were obtained for pigeonpea and soybean, respectively. Doubling the nitrogen rate at 80 kg N/ha increased the weight of dried shoots to 5.61 g and 9.21 g for pigeonpea and soybean, respectively. The increases in shoot dry weight were 142% and 54% more than those for the controls for pigeonpea and soybean, respectively. Increasing the nitrogen rates to 120, 160 and 200 kg N/ha resulted in lower shoot dry weights than the values recorded for the treatments at the 80 kg N/ha. There was, however, no significant difference between shoot dry weights recorded for pigeonpea at rates of 80 kg N/ha and 120 kg N/ha. The shoot dry weights for soybean were higher than those for pigeonpea at all levels of
nitrogen application in the Adenta soil. In the Bekwai soil the shoot dry weight values recorded for pigeonpea and soybean in the nitrogen control treatments were 2.37 g and 1.77 g, respectively (Fig. 8). Application of 40 kg N/ha resulted in only 4% and 7% increases in shoot dry weight for pigeonpea and soybean, respectively. Whereas an increase in the rate of nitrogen fertilizer to 80 kg N/ha gave a 40% increase in shoot dry weight of pigeonpea over the control, a negative response was recorded for soybean indicating a decrease. When the rate was further increased to 160 kg N/ha soybean still recorded decreases in shoot dry weights but with a dramatic increase of 46% over the control when 200 kg N/ha was applied. Both legumes recorded highest shoot dry weights in the treatments receiving 200 kg N/ha.

![Fig. 8. Effect of nitrogen fertilizer application on shoot dry weight (g) of soybean and pigeonpea grown in the Adenta and Bekwai series.](image)

4.3.9 The effect of the combined application phosphorus and nitrogen fertilizers on shoot dry weight of soybean and pigeonpea.

In the Adenta soil with no N or P applied shoot dry weights were 5.99 g and 2.31 g for soybean and pigeonpea, respectively. When only phosphorus was applied, at a rate of 40 kg P/ha, the shoot dry weights for soybean and pigeonpea, increased to 6.19 g and 4.41 g, respectively.
These values, although higher than those for the controls in the separate applications of the fertilizers, were further increased when either N or P rates were increased. (Fig. 7 and Fig. 8). Addition of 100 kg N/ha to the 40 kg P/ha led to a 25.8 % increase in shoot dry weight of soybean. When the rate of phosphorus was increased to 80 kg P/ha while maintaining the rate of nitrogen at 100 kg N/ha, increases in shoot dry weights were recorded for both pigeonpea and soybean (Fig. 9). The increases recorded for both pigeonpea and soybean were comparable to the highest shoot dry weight recorded in the treatments with separate fertilizer applications (Tables 3 and 4). Soybean recorded the highest dry weight of shoot when 80 kg P/ha was applied together with 100 kg N/ha. When 160 kg P/ha was combined with 100 kg N/ha in the Adenta soil, the dry weight of soybean shoots increased but not significantly higher than the shoot dry weight recorded when 80 kg P/ha was combined with 100 kg N/ha. Pigeonpea recorded the highest shoot dry weight when 160 kg P/ha was applied. Application of 200 kg P/ha combined with 100 kg N/ha decreased the shoot dry weight (Fig. 9). Soybean recorded significant decreases when phosphorus was applied above 80 kg P/ha. In the Bekwai soil the control without N or P produced 1.77 g and 2.37 g of dried shoot for soybean and pigeonpea, respectively. Application of 40 kg P/ha resulted in significant increases to 2.89 g and 5.01 g of shoot dry weight for pigeonpea and soybean, respectively. Application of 100 kg N/ha together with 40 kg P/ha gave 3.07 g and 4.86 g shoot dry weight for pigeonpea and soybean, respectively. The highest shoot dry weight produced by soybean (7.17g) was recorded when 100 kg N/ha was applied in combination with 200 kg P/ha. There were progressive increases in shoot dry weight of pigeonpea up to P120 kg/ha (5.37 g), after which a decrease occurred (Fig. 9).
Fig. 9. Effect of combined application of phosphorus and nitrogen on shoot dry weight (g) of soybean and pigeonpea.

4.4 Effect of phosphorus application on nodulation and nitrogen fixation in soybean in Adenta series

Number of nodules formed

Nodule formation was enhanced by P application (Table 3). Two nodules were formed on soybean in the treatment receiving no phosphorus application and this was the lowest. Phosphorus application at 40 kg P/ha resulted in increased number of nodules equivalent to about 10 times the number of nodules recorded in the treatment receiving no phosphorus. Increasing the rate of phosphorus applied to 80 kg P/ha resulted in the formation of 40 nodules which was 20-folds the number of nodules recorded for the control indicating that doubling the rate of phosphorus application gave twice as much the number of nodules formed. A further increase in the rate of phosphorus applied to 120 kg P/ha resulted in the formation of 44 nodules on soybean which also gave 22-folds the number of nodules formed on the control plants. However, the increase in nodule number was not significantly different from the nodules produced by the application of 40 kg P/ha.
Dry weight of nodules formed

The results for the dry weights of nodules revealed a trend similar to that observed for nodule numbers. The lowest dry weight of nodules (2.99 mg) was recorded in the control (Table 3). Application of phosphorus resulted in increased dry weight of nodules up to the 120 kg P/ha rate (Table 3). The highest dry weight of nodules recorded was 65.78 mg, was obtained when 120 kg P/ha was applied. Significant increases in dry weight of nodules over the control were recorded at all levels of P applied (Table 3). The dry weight produced per nodule was found not to respond to phosphorus application (Table 3).

Total nitrogen in shoot

The values for total nitrogen in shoot was found to increase with P application, and ranged from the lowest (65.73 mg) in the control treatment to the highest (93.86 mg) when 120 kg P/ha was applied. Application of P above 40 kg P/ha gave significant (P < 0.05) increases over the control (Table 3) and also over the preceding rates.

Percent nitrogen derived from fixation

When no P was applied, 23.1 % of the total nitrogen in shoot was derived from atmospheric N₂ fixation. The percentage of N derived from the atmosphere (% Ndfa) increased with increased P application with the 120 kg P/ha rate giving the highest (54.7 %). However, the most efficient response in % Ndfa occurred when 40 kg P/ha was applied increasing % Ndfa from 23.1 % in the control to 44.80 %, almost double.

Total nitrogen derived from fixation

The total nitrogen derived from fixation for the control was 15.6 mg and this was the lowest recorded. Application of phosphorus at a rate of 40 kg P/ha increased the amount of nitrogen fixed to 32.27 mg, which was more than two times that of the control. With a further increase
in P application to 80 kg P/ha increase in total nitrogen fixed was not significant. The highest and significant increase in total nitrogen fixed (51.50 mg) occurred when 120 kg P/ha was applied (Table 3).

**Nitrogen fixation efficiency (N$_2$-fixed/mg nodule)**

The total nitrogen fixed per mg nodule decreased with P application (Table 3). With no P applied, each mg dry weight of nodule fixed 5.21 mg N in soybean and this was the highest for all treatments. The application of P at a rate of 40 kg P/ha resulted in a substantial (23.3 \%) decrease in N$_2$ fixed. Further increases in P application gave non-significant (P > 0.05) decreases in N$_2$ fixed and the values were 1.11 mg, 0.79 mg and 0.78 mg for the rates 80 kg P/ha, 120 kg P/ha and 160 kg P/ha, respectively.
Table 3. Effect of phosphorus application on nodulation and nitrogen fixation in soybean grown in Adenta soil.

<table>
<thead>
<tr>
<th>Phosphorus (kg/ha)</th>
<th>NN</th>
<th>NDW (mg)</th>
<th>DW/N (mg)</th>
<th>SDWT (g)</th>
<th>TNS (mg)</th>
<th>% Ndfa</th>
<th>TNF (mg)</th>
<th>TNF (mg)/ mg NDW</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>2c</td>
<td>2.99d</td>
<td>1.50a</td>
<td>1.0d</td>
<td>67.53c</td>
<td>23.1c</td>
<td>15.57c</td>
<td>5.21a</td>
</tr>
<tr>
<td>P40</td>
<td>19b</td>
<td>28.99c</td>
<td>1.53a</td>
<td>2.5c</td>
<td>71.67c</td>
<td>44.8b</td>
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</tr>
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<td>P80</td>
<td>40a</td>
<td>51.17b</td>
<td>1.28a</td>
<td>4.4b</td>
<td>84.15b</td>
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<tr>
<td>P120</td>
<td>44a</td>
<td>65.78a</td>
<td>1.50a</td>
<td>5.3a</td>
<td>93.86a</td>
<td>54.7a</td>
<td>51.50a</td>
<td>0.78b</td>
</tr>
<tr>
<td>Mean (SE)</td>
<td>26 (± 9.77)</td>
<td>37.23 (± 13.69)</td>
<td>1.45 (± 0.06)</td>
<td>3.3 (± 0.96)</td>
<td>79.3 (± 6.00)</td>
<td>42.6 (± 6.82)</td>
<td>34.94 (± 7.56)</td>
<td>1.97 (± 1.08)</td>
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<td>LSD (0.05)</td>
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<td>8.12</td>
<td>0.76</td>
<td>0.3</td>
<td>9.59</td>
<td>5.1</td>
<td>8.60</td>
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<tr>
<td>CV (%)</td>
<td>24.8</td>
<td>14.2</td>
<td>3.09</td>
<td>21.6</td>
<td>7.9</td>
<td>7.8</td>
<td>16.0</td>
<td>17.9</td>
</tr>
</tbody>
</table>

NB: Means having the same letters under the same column are not significantly different from one another.

SDW: Shoot dry weight; NDW: Nodule dry weight; NN: Nodule number; DW/N: Dry weight per nodule

% NS: Percent nitrogen in shoot; TNS: Total nitrogen in shoot; % Ndfa: Percent nitrogen derived from atmosphere (fixation)

TNF: Total nitrogen fixed.
4.4.1 Effect of phosphorus application on shoot dry weight of soybean grown in the Adenta and Nzima soils.

With no phosphorus application, shoot dry weight of soybean was higher in the Adenta series (2.16 g) than in the Nzima series (1.00 g). Application of 40 kg P/ha more than doubled the dry weight of shoots in both soils (Fig. 10). The dry weight of shoot recorded for the treatment receiving 40 kg P/ha in the Adenta soil was significantly higher than that in the Nzima soil (Fig.10). When the fertilizer rate was increased to 80 kg P/ha, shoot dry weight produced by soybean in the Nzima soil was about hundred percent more than that for the preceding rate (40 kg P/ha). The case was different in the Adenta soil where the corresponding increase in shoot dry weight recorded was less than 10 percent. A further increase in the fertilizer rate resulted in an increase in the dry weight of shoots from both soils. The higher responses observed in the Nzima soil with P application resulted in the shoot dry weight of soybean becoming higher in the Nzima than in the Adenta soil at the 120 kg P/ha rate (Fig 10).

![Fig. 10. Effect of phosphorus application on shoot dry weight production by soybean grown in the Adenta and Nzima soils.](image)

<table>
<thead>
<tr>
<th>Fertilizer rates applied (kg P/ha)</th>
<th>P0</th>
<th>P40</th>
<th>P80</th>
<th>P120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenta</td>
<td>2.16</td>
<td>4.08</td>
<td>4.4</td>
<td>4.69</td>
</tr>
<tr>
<td>Nzima</td>
<td>1</td>
<td>2.47</td>
<td>4.44</td>
<td>5.25</td>
</tr>
</tbody>
</table>

Lsd (5 %): Phosphorus = 0.34, Soil = 0.24, Soil*Phosphorus interaction = 0.48
4.5 Genetic diversity of indigenous soybean, pigeonpea and cowpea rhizobial strains in Adenta, Bekwai and Nzima soils.

The diversity of *Bradyrhizobium* isolates that nodulated soybean and pigeonpea in the Adenta, Bekwai and Nzima soils was evaluated with or without phosphorus fertilization. The inclusion of cowpea was to examine how related the *Bradyrhizobium* isolates that nodulated soybean and pigeonpea were to those that nodulated cowpea. Neither of the locations from which the soils were sampled had a known history of inoculation with rhizobia. As such the isolates trapped by these legumes were assumed to be indigenous to the soils.

4.5.1 Electrophoresed gel images of the PCR amplified products by the different Primers.

4.5.1.1 PCR amplification of the 16S rDNA region of the isolates

The amplification profiles for the 16S PCR revealed a 1500 bp DNA fragment (Fig. 11) that was consistent for all the *Bradyrhizobium* isolates. A non-variable 600 bp fragment that persisted in the majority of isolates was also observed from this PCR. From Fig. 11, this fragment was amplified for pigeonpea isolates PBF-3, PBF-13, PBF-14, PBF-15, PBF-16, PBF-17, PBF-18 and PBF-19, PNF-7 and PNF-8.

![Fig. 11. Sample of PCR amplification of *Bradyrhizobium* DNA using primer targeting the 16S conserved region.](http://ugspace.ug.edu.gh)
The letter P stands for pigeonpea. The letters B and N stand for Bekwai and Nzima soils, respectively. The letters M and V represent ladder lane and negative control, respectively. The letter F stands for P fertilised treatments.

**4.5.1.2 PCR amplification of the 23S rDNA region of the isolates**

The 23S PCR amplifications produced a DNA fragment of size 2900 bp (Fig. 12) which was consistent for all the *Bradyrhizobium* isolates including the reference strain USDA 110. No additional fragment was observed from this PCR amplification. Some amplicons recorded no bands (Fig. 12) because of technical problems. As such PCR amplification of the DNAs corresponding to these regions was repeated and the 2900 bp DNA fragment was successfully amplified for the isolates.

![Sample of PCR amplification of *Bradyrhizobium* DNA using primer targeting the 23S conserved region.](image)

The letters C and P stand for cowpea and pigeonpea, respectively, while A, B and N stand for Adenta, Bekwai and Nzima soils, respectively, and M represents ladder lane. The letters F and U stand for P fertilised and unfertilised treatments, respectively.
4.5.1.3 PCR amplification of the ITS region of the isolates

From Fig. 13, the ITS primer targeted PCR for the bradyrhizobial isolates was able to amplify the ITS conserved region for all the DNA samples and discriminated between isolates by producing fragments with band size ranging from 300 bp to 700 bp.

![PCR amplification of Bradyrhizobium DNA using primer targeting the 16S-23S ITS conserved region.](image)

The letter C stands for cowpea, A and N represent Adenta and Nzima soils, respectively and M represents ladder lane. The letters F and U stand for P fertilised and unfertilised treatments, respectively.

4.5.1.4. PCR analysis of the total genomic DNA of the isolates using primer RPO1

Figure 14 shows RPO1 PCR amplified DNA from the isolates. This produced fragments with band sizes ranging from as short as 117 bp to as long as 1800 bp. Figure 14 further showed the discriminating ability of the RPO1 primer as it differentiated among the isolates. The DNA pattern of isolate SNF-2 was similar to the banding patterns of the soybean isolates from the Adenta soil with P-fertilization and different from the other soybean isolate SNF-1 from Nzima soil with P-fertilization. The banding patterns of the cowpea isolates CNF-12 and CNF-16 were similar and different from those of the other cowpea isolates CNF-1, 2, 17 and CNF-18 (Fig. 14).
Amplification of a 600 bp DNA fragment was observed from this PCR and was consistent for
the majority of isolates (Fig. 14). No two isolates produced the same banding patterns from this
PCR to infer 100 % similarity.

![Fig. 14. Sample of PCR amplification of Bradyrhizobium DNA using primer RPO1.](image)

The letters S and C represent soybean and cowpea, respectively, N and A represent Nzima and
Adenta soils, respectively and M represents ladder lane. The letter F stands for P fertilised
treatments.

4.5.1.5 PCR analysis of the total genomic DNA of the isolates using primer RPO4

From Fig. 15, PCR amplification of DNA from the isolates using the arbitrary oligonucleotide
primer RPO4 produced fragments with band sizes ranging from as short as 157 bp to as long as
3000 bp. The banding patterns of the DNA fragments obtained by this PCR could also
differentiate among the isolates and the reference strain as well and established groups among
the isolates. From Fig. 15 the PCR differentiated between isolates that nodulated cowpea in the
Nzima soil with P and those that nodulated cowpea in the Adenta soil with or without P and
further differentiated within isolates from the Adenta soil. As shown in Fig. 15, cowpea isolates
from the Adenta soil with P, had two groups (five isolates in each group) with isolates in each
group having similar banding patterns
Fig. 15. Sample of PCR amplification of *Bradyrhizobium* DNA using primer RPO4.

The letter C represents cowpea, A stands for Adenta soil and M represents ladder lane. The letters F and U stand for P fertilised and unfertilised treatments, respectively.

4.5.2 Clustering on the basis of PCR amplification with RPO1 and RPO4.

This section presents the results of hierarchical clustering of the data generated from the PCR gel images using the Unweighted Paired Group Arithmetic Method (UPGMA). Indices of diversity were calculated based on clusters that were formed at 80 % similarity.

4.5.2.1 Phylogenetic relationships among cowpea *Bradyrhizobium* isolates in Adenta soil as determined by combined matrices for RPO1 and RPO4 PCR.

All the 21 isolates (eleven from unfertilised treatments and ten from fertilised treatments) that nodulated cowpea in the Adenta soil were 66 % similar (Fig. 16). Above the mean similarity of 66 %, two main clusters A and B were observed. One isolate, CAU-11 being the most similar (72 %) to the reference strain, USDA110 grouped together with the reference strain in cluster A. The remaining 20 cowpea isolates were differentiated into sub-clusters under cluster B above a mean 74 % similarity level. At 80 % similarity, the isolates within cluster A were differentiated into two sub-clusters, I and II. The isolates within cluster B were further differentiated into three sub-clusters which were designated I, II and III at the same 80 % similarity level. About 33.3 %
of the cowpea isolates were grouped into sub-cluster I with a mean similarity of 84 %. The isolates that grouped under sub-cluster I were those that nodulated cowpea in either P-fertilized or P-unfertilized Adenta soil (Fig. 16). The isolates within sub-clusters II and III were 88 % and 82 % similar, respectively. All the five isolates within sub-cluster II (CAF-6-10) were from treatments receiving P. Sub-cluster III on the other hand contained eight isolates all of which were from treatments without P. The isolates in sub-cluster III were placed farther apart from the other cowpea isolates (CAU-11 and CAU-9, 10) from Adenta soil without P. The isolates CAU-11 and CAU-9, 10 were also clustered farther apart from each other (Fig. 16). The following groups of isolates; CAU-2-3, CAU-6-7, CAF-6-9 and CAF-1, 4 were similar, scoring 95 % similarity. The most similar isolates were CAF-6-8 with a similarity of 96 %. All the isolates were dissimilar above 96 % similarity level. The Shannon-Weaver diversity and Pielou Evenness indices were 1.22 and 0.88, respectively for the clustering among the 21 cowpea isolates.

Fig. 16. Dendogram indicating relationships among cowpea isolates in Adenta soil based on combined RPO1 and RPO4 PCRs
4.5.2.2 Phylogenetic relationships among cowpea <i>Bradyrhizobium</i> isolates in Bekwai soil as determined by combined matrices from RPO1 and RPO4 PCR.

All the 12 cowpea isolates in the Bekwai soil were from treatments receiving P. At a similarity of 61 %, all the isolates together with the reference strain were similar (Fig. 17). Beyond the mean similarity of 61 %, two distinct clusters, A and B were formed. The reference strain was separated into Cluster A while all the 12 cowpea isolates were grouped under Cluster B at 69 % similarity. At 80 % similarity, seven sub-clusters were formed under cluster B (Fig. 17). The isolates CBF-6, CBF-12, CBF-5, CBF-3 and CBF-11 were separately clustered into BI, BII, BIV, BV and BVII, respectively. Sub-clusters BIII and BVI contained three isolates each equivalent to 25 % of the total isolates. The isolates within sub-cluster BIII were 80 % similar whereas those in BVI were 83 % similar. The isolates CBF-7 and CBF-8 were the most similar at 93 % level above which all the isolates were dissimilar. The Shannon-Weaver diversity and the Pielou Evenness indices were calculated as 1.74 and 0.90, respectively for the clusters formed in Fig. 17.
4.5.2.3 Phylogenetic relationships among cowpea *Bradyrhizobium* isolates in Nzima soil as determined by combined matrices from RPO1 and RPO4 PCR.

All the 19 isolates were from nodules on cowpea grown in Nzima soil with P fertilization and were similar at 67 % level (Fig. 18). At 64 % level, all the cowpea isolates were similar to the reference. Above the mean similarity of 64 %, two main clusters A and B were formed. Cluster A contained the reference strain whereas cluster B contained the 19 cowpea isolates that were further clustered into two distinct groups above a mean similarity of 67 %. Nine out of the 19 isolates were differentiated from the other cowpea isolates into one group at 70 % similarity level with the remaining isolates forming another group with a mean similarity of 72 %. At 80 % similarity level, the cowpea isolates were grouped into ten new sub-clusters, I-X. Isolates CNF-
15 and CNF-16 were the most similar at 98% level above which all the isolates were dissimilar. The Shannon-Weaver diversity and the Pielou evenness indices for this dendogram were 2.11 and 0.91, respectively.

**Fig. 18.** Dendogram indicating relationships among cowpea isolates in Nzima soil based on combined RPO1 and RPO4 PCRs
4.5.2.4 Phylogenetic relationships among cowpea *Bradyrhizobium* isolates from all the soils as determined by combined matrices from RPO1 and RPO4 PCR.

From Fig. 19, all the 52 cowpea isolates from the study were 65 % similar to the reference strain. Above the mean similarity of 65 %, the isolates were grouped into two main clusters, A and B. Isolate CAU-11 was clustered together with the reference strain, USDA110 in A at 70 % similarity. The remaining 51 cowpea isolates from this PCR were clustered into B at about 67 % similarity level. The isolates in cluster B were further grouped into BI and BII at about 73 % similarity. Sub-cluster BI contained only isolates from the Nzima soil which were 76 % similar. Cluster BII contained isolates that nodulated cowpea grown in the three soils studied. At about 73 % similarity, the isolates in cluster BII were grouped into five sub-clusters a, b, c, d and e. Sub-cluster IIa contained four isolates that were differentiated into separate clusters at 80 % similarity (Fig. 19). Sub-clusters IIb and IIc contained only isolates that nodulated cowpea in the Bekwai soil. Sub-cluster IIId contained two cowpea isolates from the Nzima soil with P grouped together with two cowpea isolates from the Adenta soils without P and five other isolates from the Adenta soil with P application. Cluster IIe contained isolates obtained from all the soils used with or without P application at 73 % similarity. Isolates CNF-15 and CNF-16 were the most similar at 98 % similarity above which all the isolates were dissimilar. The sub-clusters formed at 80 % similarity are presented in Table 4 for simplicity. The Shannon-Weaver diversity and the Pielou evenness indices were 2.75 and 0.90, respectively.
Fig. 19. Dendogram indicating relationships among Cowpea isolates in Adenta, Bekwai and Nzima soil based on combined RPO1 and RPO4 PCRs
Table 4. Clustering of isolates obtained from cowpea nodules from all the soils at 80 % similarity

<table>
<thead>
<tr>
<th>Similarity level</th>
<th>66 %</th>
<th>67 %</th>
<th>70 %</th>
<th>80 %</th>
</tr>
</thead>
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<td>Cluster</td>
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<td></td>
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<tr>
<td>A</td>
<td>CAU-11</td>
<td>USDA110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>I a 1</td>
<td>CNF-12 to 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CNF-1,2,17,18</td>
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<td></td>
</tr>
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<td>II a 1</td>
<td>CNF-3</td>
<td></td>
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<tr>
<td></td>
<td>2</td>
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<td>4</td>
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<td>CBF-10</td>
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<td>7</td>
<td>CAU-1 to 3, CNF-9</td>
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</table>

4.5.2.5 Phylogenetic relationships among pigeonpea *Bradyrhizobium* isolates in Adenta soil as determined by combined matrices from RPO1 and RPO4 PCR.

All the 13 pigeonpea isolates (three from unfertilised treatments and ten from fertilised treatments) from the Adenta soil together with the reference strain USDA110 were 66 % similar (Fig. 20). Two main clusters, A and B, were formed beyond the mean similarity of 66 %. Cluster A contained the reference strain USDA110 grouped together at 72 % similarity level with four pigeonpea isolates from P fertilized treatments in the Adenta soil. In Cluster B, the three isolates
from nodules of pigeonpea grown in the Adenta soil without P application were clustered together at 71 % similarity with the remaining pigeonpea isolates from the Adenta soil with P application. At 80 % similarity, nine sub-clusters were formed under clusters A and B. The isolates in cluster A were differentiated into three sub-clusters (I, II and III). Isolate PAF-1 and the reference strain were clustered separately into sub-clusters III and I, respectively. All the three isolates within sub-cluster II were from nodules of pigeonpea grown in the Adenta soil receiving P and were found to be 80 % similar. Cluster B consisted of six sub-clusters I, II, III, IV, V and VI. Sub-cluster II contained isolates PAF-7 and PAF-9. Isolates PAF-10, PAF-6, PAF-4 and PAF-3 were differentiated into separate clusters I, III, IV and V, respectively. The isolates from nodules of pigeonpea that was grown in the P-unfertilised Adenta soil were clustered into VI (Fig. 20). Isolates PAU-1 and PAU-2 were the most similar at 93 % level above which all the isolates were dissimilar. The Shannon-Weaver diversity and the Pielou evenness indices were 1.95 and 0.94, respectively.
Fig. 20. Dendogram indicating relationships among Pigeonpea isolates in the Adenta soil based on combined RPO1 and RPO4 PCRs

4.5.2.6 Phylogenetic relationships among pigeonpea *Bradyrhizobium* isolates in Bekwai soil as determined by combined matrices from RPO1 and RPO4 PCR.

All the 20 isolates that nodulated pigeonpea in P-fertilised Bekwai soil were similar at 68 % level (Fig. 21). The pigeonpea isolates were similar to the reference strain at 61 % similarity. Grouping of all the isolates into Clusters A and B occurred beyond the mean similarity level of 61 %. All the 20 isolates within cluster B were 68 % similar. At about 70 % similarity level, isolate PBF-
20 was differentiated from the remaining isolates which were extensively differentiated beyond 73 % similarity level. At 80 % similarity, there was further differentiation within Cluster B resulting in the formation of 11 sub-clusters (Fig. 21). Sub-cluster IV contained 25 % of the isolates whereas 15 % of the isolates grouped under sub-cluster III. Isolates PBF-15 and PBF-18 were the most similar among the isolates at 89 % level. Beyond the 89 % similarity level, all the isolates were dissimilar. Three pairs of isolates (PBF-1 and PBF-12, PBF-1 and PBF-5 and PBF-4 and PBF-8) were similar at about 88 % level beyond which complete differentiated among all the isolates occurred. The Shannon-Weaver diversity and the Pielou evenness indices were 2.20 and 0.92, respectively.
4.5.2.7 Phylogenetic relationships among pigeonpea *Bradyrhizobium* isolates in Nzima soil as determined by combined matrices from RPO1 and RPO4 PCR.

All the 20 pigeonpea isolates (10 each from pigeonpea nodules from fertilised and unfertilised Nzima soils) together with the reference strain USDA110 were 63 % similar (Fig. 22). Above the mean similarity of 63 %, two main clusters, A and B were formed. Isolate PNF-7 was clustered together with the reference strain USDA110 into A at a similarity of 68 %. The remaining 19 isolates were clustered into B. All the isolates in cluster B were similar at 66 %
level. At 80 % similarity, 12 sub-clusters were formed two of which were grouped under A with the remaining ten sub-clusters grouped under B (Fig. 22). There was complete differentiation between the reference strain and PNF-7 at 80 % similarity into separate clusters I and II, respectively. Twenty five percent of the isolates were differentiated from the rest of the isolates into separate clusters (Fig. 22). Within each of sub-cluster VIII and X an isolate from nodules of P-fertilised pigeonpea plants was clustered together with two isolates from P-unfertilised plants. Clusters IV and V contained isolates from nodules of P-fertilised pigeonpea plants. Isolates PNU-3 and PNU-4 were the most similar at 91 % level beyond which all the isolates were dissimilar. Isolates from the Nzima soil with P application were represented in five out of the six clusters formed at 80 % similarity whereas isolates from the same soil without P application were represented in only two out of the six clusters. The Shannon-Weaver diversity and the Pielou evenness indices were 2.27 and 0.94, respectively.
Fig. 22. Dendogram indicating relationships among pigeonpea isolates in the Nzima soil based on combined RPO1 and RPO4 PCRs
4.5.2.8 Phylogenetic relationships among pigeon pea *Bradyrhizobium* isolates from all the soils as determined by combined matrices from RPO1 and RPO4 PCR.

From Fig. 23, the mean similarity of the 53 pigeon pea isolates from all the soils was 64 %. Above the 64 % mean similarity level, the isolates were clustered into A and B. Isolate PNF-7 was clustered together with the reference strain and isolate PAF-1 under A. Within cluster B, the isolates were differentiated into three clusters, I< II and III at 70 % similarity level. Cluster B was further clustered at 80 % similarity level into 28 sub-clusters with further clustering beyond 80 % similarity level. At the same 80 % similarity the two pigeon pea isolates in cluster A were differentiated into two separate groups. The sub-clusters formed at 80 % similarity have been summarised and presented in Table 5. From Table 5, it is evident that Cluster BI contained only isolates from the Bekwai soil with P application. Within Cluster BIIa, isolate PNU-2 from the unfertilized Nzima soil was grouped together with four isolates from the fertilized Adenta soil. Cluster BIIb on the other hand, contained two isolates one each from the Nzima and the Adenta soils with P. The groupings in III occurred at 74 % similarity and were further differentiated at 80 % similarity level. The cluster BIIId contained only pigeon pea isolates from fertilized the Nzima soil whereas BIIIc contained only isolates from fertilised Bekwai soil. Isolates from all the soils with or without P were grouped under BIIia and BIIle. Within BIIId, isolate PNU-7 from unfertilised Nzima soil grouped together with isolate PBF-6 from fertilized Bekwai soil. The isolates PAU-1 and PAU-2 were the most similar at 93 % similarity level. Beyond 93 % similarity, all the isolates were dissimilar. The Shannon-Weaver diversity and the Pielou evenness indices were 3.29 and 0.97, respectively.
Table 5. Clustering isolates obtained from nodules on pigeonpea grown in the soils at 80% similarity

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</table>
Fig. 23. Dendogram indicating relationships among Pigeonpea isolates in the Adenta, Bekwai and Nzima soils based on combined RPO1 and RPO4 PCRs
4.5.2.9 Phylogenetic relationships among soybean *Bradyrhizobium* isolates in Adenta, and Nzima soils as determined by combined matrices from RPO1 and RPO4 PCR.

The dendogram (Fig. 24) shows the level of similarity among 15 soybean isolates from Adenta (13, all from fertilized treatments) and Nzima (2, all from fertilized treatments) soils and their genetic relatedness to the reference strain USDA 110. All the soybean isolates were 62 % similar to the reference strain. Beyond the mean similarity of 62 %, the isolates were grouped into two main clusters, A and B. Isolate SAF-10 was the most similar to the reference strain, and was grouped together with the reference strain in Cluster A at 66 % similarity level. The two isolates from Nzima series were 64 % similar. All the isolates in cluster B were 73 % similar. At 80 % similarity, the isolates were grouped into eight clusters all of which were dissimilar to the reference strain, USDA 110. Isolates within Cluster B were differentiated into 7 sub-clusters (I, II, III, IV, V, VI and VII) at the 80 % similarity level. Isolates SNF-2, SAF-12 and SAF-11 were separately clustered into I, V, and VII, respectively. Isolates SAF-1 and SAF-13 with 80 % similarity were clustered into VI. In sub-cluster IV, isolate SNF-1 from nodules of soybean grown in the Nzima soil was grouped together with other isolates from the Adenta soil. The isolates from the Nzima series were dissimilar at 80 % level and were genetically distant apart (Fig. 24). Isolates SAF-6 and SAF-8 were the most similar at 98 % level. All the isolates were dissimilar above 98 % similarity level. The Shannon-Weaver diversity and Pielou evenness indices were 1.90 and 0.92, respectively for the clustering of all the soybean isolates. The Shannon-Weaver diversity and Pielou evenness indices for isolates from only the Adenta and the Nzima series were 1.58, 0.88 and 0.69, 1.00, respectively. In all, seven clusters were formed for the soybean isolates from the Adenta Series.
Fig. 24. Dendogram indicating relationships among soybean isolates in Adenta and Nzima soils based on combined RPO1 and RPO4 PCRs
4.5.2.10 Phylogenetic relationships among *Bradyrhizobium* isolates from soybean, pigeonpea and cowpea grown in Adenta soil as determined by combined matrices from RPO1 and RPO4 PCR.

The mean similarity for the 57 isolates from Adenta series was 64% (Fig. 25). Above the mean similarity level, the isolates were grouped into two main clusters A and B. The isolates in A were 75% similar whereas the isolates in B were 67% similar. At 80% similarity, eighteen sub-clusters were formed among the isolates obtained from the Adenta soil. The three isolates in cluster A were those that nodulated soybean in P-fertilised treatments. About 95% of the isolates were grouped in cluster B and were differentiated into 16 sub-clusters (Fig 25 and Table 6). Cluster BI contained three isolates from soybean, pigeonpea and cowpea grouped together with the reference strain USDA 110. Within BIIa, one soybean isolate SAF-11 was clustered together with one pigeonpea isolate PAF-10 all from fertilised treatments. The clusters BIIb, BIIId and BIIe contained four, one and four pigeonpea isolates, respectively from fertilised treatments. The eight soybean isolates within cluster BIIc were those obtained from fertilised treatments. Within BIIf, two unfertilised pigeonpea isolates were clustered together with cowpea isolates from either P-fertilised or P-unfertilised treatments. At 80% similarity, none of the isolates were similar to the reference strain. Isolates SAF-6 and SAF-8 were the most similar with 98% similarity. Beyond 98% similarity level, all the isolates were dissimilar. The sub-clusters formed at 80% similarity are presented in Table 6 for simplicity. The Shannon-Weaver diversity and the Pielou evenness indices were 2.51 and 0.86, respectively.
Table 6. Clustering isolates obtained from nodules on legumes grown in Adenta soil at 80% similarity

<table>
<thead>
<tr>
<th>Similarity level</th>
<th>65%</th>
<th>67%</th>
<th>74%</th>
<th>80%</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>A</td>
<td>SAF-13, SAF-1</td>
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<tr>
<td></td>
<td>SAF-12</td>
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<td></td>
<td></td>
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<tr>
<td>B</td>
<td>I</td>
<td></td>
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<tr>
<td></td>
<td>USDA 110</td>
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<tr>
<td></td>
<td>SAF-10</td>
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<tr>
<td></td>
<td>PAU-3, CAU-11</td>
<td></td>
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</tr>
<tr>
<td>II</td>
<td>a</td>
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<td></td>
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<tr>
<td></td>
<td>SAF-11</td>
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<td></td>
<td>PAF-10</td>
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<td></td>
<td>b</td>
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<td></td>
<td>PAF-1</td>
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<td></td>
<td>c</td>
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<tr>
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<td>SAF-6,7,8,9</td>
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<td>d</td>
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<td></td>
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<td>CAU-1,2,3,4, 6,78</td>
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</table>
Fig. 25. Dendogram indicating relationships among isolates from Adenta soil based on combined RPO1 and RPO4 PCRs
4.5.2.11 Phylogenetic relationships among *Bradyrhizobium* isolates from Bekwai soil as determined by combined matrices from RPO1 and RPO4 PCR.

The mean similarity of the 32 isolates from Bekwai series was 61 % (Fig. 26). Two main clusters A and B were formed above the mean similarity level of 61 %. The reference strain was separated into cluster A. Cluster B consisted of cowpea and pigeonpea isolates from the Bekwai series with a mean similarity of 66 %. At 80 % similarity, 16 sub-clusters were formed under cluster B. Cluster BI contained only isolates from pigeonpea grown in fertilised soils. Cluster BIIa also contained only isolates from pigeonpea whereas two isolates from fertilised pigeonpea and fertilised cowpea were grouped in BIIb. Within BIIb, isolate CBF-3 and from fertilised cowpea was clustered grouped together with 11 isolates from fertilised pigeon pea. Clusters BIIe, BIIf and BIIg contained isolates from cowpea in fertilised soils. At the highest similarity of 93 %, the isolates CBF-7 and CBF-8 were similar. All the isolates were dissimilar beyond 93 % similarity. The sub-clusters formed at 80 % similarity are presented in Table 7 for simplicity. The Shannon-Weaver diversity and the Pielou evenness indices were 2.62 and 0.97, respectively.
Table 7. Clustering isolates obtained from nodules on legumes grown in Bekwai soil at 80% similarity

<table>
<thead>
<tr>
<th>Similarity level</th>
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<th>68 %</th>
<th>75 %</th>
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<tr>
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<td>USDA 110</td>
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<td></td>
</tr>
<tr>
<td>B</td>
<td>I</td>
<td>PBF-9,10, PBF-20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>a</td>
<td>PBF-16</td>
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<td></td>
<td></td>
<td>PBF-11,12,</td>
<td></td>
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<tr>
<td></td>
<td>b</td>
<td>PBF-17, CBF-6</td>
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<tr>
<td></td>
<td>c</td>
<td>PBF-6,14</td>
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<tr>
<td></td>
<td>d</td>
<td>PBF-3,4,8 PBF-2</td>
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<td>e</td>
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<td></td>
<td>f</td>
<td>CBF-7-10</td>
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<tr>
<td></td>
<td>g</td>
<td>CBF-5 CBF-1,2,4, CBF-11</td>
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</table>
Fig. 26. Dendogram indicating relationships among isolates from Bekwai series based on combined RPO1 and RPO4 PCRs
4.5.2.12 Phylogenetic relationships among *Bradyrhizobium* isolates from Nzima series as determined by combined matrices from RPO1 and RPO4 PCR.

Figure 27 showed that all the 41 isolates in the Nzima soil were 62 % similar. The isolates from Nzima series were differentiated into clusters A and B beyond the mean similarity of 62 %. Isolate PNF-7 was clustered together with the reference strain USDA110 in cluster A at 68 % similarity. The isolates within cluster B were grouped into twenty new clusters (Fig. 27) at 65 % similarity level. At 71 % similarity level, the isolates within cluster BII were further differentiated into sub-clusters a, b, c, d and e. At 80 % similarity, the isolates from Nzima series were differentiated into 21 clusters. Isolate PNU-2 and PNF-1 were the only isolates in Clusters BI and BIIb, respectively. Cluster BIIa consisted of the isolates from soybean clustered together with some cowpea isolates. Three isolates from fertilised pigeonpea and two isolates from fertilised cowpea were grouped under BIIc. Within BIIId cowpea isolates CNF-7 and CNF-8 were grouped together with pigeonpea isolates from either fertilised or unfertilised treatments. Four fertilised cowpea isolates formed one group BIIe together with two isolates from unfertilised pigeonpea and five isolates from fertilised soils. The isolates CNF-15 and CNF-16 were the most similar at 98 % similarity. The isolates were completely differentiated into clusters containing single isolates beyond 98 % similarity. For simplicity, the main clusters formed at 80 % similarity and their sub-clusters at higher similarity levels are presented in Table 8. The Shannon-Weaver diversity and the Pielou evenness indices were 2.81 and 0.92, respectively.
### Table 8. Clustering of isolates obtained from nodules on legumes grown in Nzima soil at 80% similarity

<table>
<thead>
<tr>
<th>Similarity level</th>
<th>63 %</th>
<th>65 %</th>
<th>71 %</th>
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</tr>
<tr>
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<td>PNF-7</td>
<td></td>
<td></td>
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<tr>
<td>B</td>
<td>I</td>
<td>PNU-2</td>
<td></td>
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<tr>
<td></td>
<td>II a</td>
<td>SNF-1</td>
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<td></td>
<td>b</td>
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<td></td>
<td>c</td>
<td>PNF-8,9,10</td>
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<td>CNF-3</td>
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<td>CNF-19</td>
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<td>PNF-1,2</td>
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<td></td>
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<td>PNU-5</td>
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<td>CNF-4,9, PNU-10</td>
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Fig. 27. Dendogram indicating relationships among isolates from Nzima series based on combined RPO1 and RPO4 PCRs
CHAPTER FIVE

5.0 DISCUSSION

5.1 Physico-chemical properties of the Adenta, Bekwai and Nzima series.

The particle size distribution and the dry bulk density for the soil from the coastal savannah Haplic Acrisol agrees with the findings of Dowuonna et al. (2012) who classified the coastal savannah Haplic Acrisol as sandy clay loam. The particle size distribution and the dry bulk densities reported for the Ferric Acrisol and the semi-deciduous Haplic Acrisol from this study are the same as reported by Adu (1992) and Owusu-Bennoah et al. (2000) who classified these soils from the semi-deciduous forest as clay soils. The bulk densities for all the soils (1.35-1.49 Mg/m$^3$) were medium and as such should not be growth limiting, using the growth limiting bulk density (GLBD) values for the different textural classes of soils (Appendix 1) reported by Daddow and Warrington (1983). However, based on the GLBD value of 1.4 Mg/m$^3$ (Morris and Lowery, 1988; Coder, 1995; Coder, 1996), the bulk densities of these clay textured soils from the semi-deciduous forest could have the potential to limit plant growth. The effect of GLBD on plant growth has been reported by Jaramillo-C et al. (1992) who stated that soils with dry bulk densities found to be growth limiting (compacted soils), often limit the root length of crops growing in those soils thus preventing plants from exploring nutrients within the larger soil volume for growth.

The Adenta soil from the coastal savannah agro-ecological zone had higher available P than the soils from the semi-deciduous agro-ecological zone. The low levels of available P in the soils from the semi-deciduous forest may be due to low levels of mineral apatite in the parent material (Acquaye and Oteng, 1972; Adu, 1992) and or may also be due to P-fixation in low pH soils through reactions with Fe and Al hydroxides (Sanchez and Salinas, 1981; Mokwunye et al., 1986; Warren, 1992; Juo and Fox, 1997; Abekoe and Sahrawat, 2001).
The Bekwai and Nzima soils have high organic carbon content and total nitrogen partly due to the high litter deposition from the canopies as well as low organic matter turnover in the semi-deciduous soils. Similar findings have been reported by Jones et al. (2006).

The higher CEC values for Bekwai and Nzima may be due to the high proportions of clay in these soils and the presence of considerably high organic carbon compared to the Adenta soil. Similar findings have been reported by Dwomo and Dzedzoe (2010).

5.2 Populations of indigenous Bradyrhizobia nodulating soybean and cowpea in the Adenta, Bekwai and Nzima series.

The presence of cowpea and soybean nodulating Bradyrhizobium spp. in the three Ghanaian soils examined suggests that these soils do provide favourable conditions for the Bradyrhizobium spp. to live saprophytically in the absence of their symbiotic host legumes. Similar findings have been reported by Duodu et al., (2005).

The wide range of Bradyrhizobium spp. that nodulated cowpea and soybean in the studied soils further suggests that growth conditions were more favourable in some soils and that some of the soils may have an unknown history of legume cultivation. This finding agrees with that of Abaidoo et al. (2007). The population of Bradyrhizobium cells that nodulated soybean were low in all the soils compared to the population of Bradyrhizobium spp. nodulated cowpea suggesting that soybean is more specific compared to cowpea in rhizobia strain selection and that cowpea as a promiscuous legume is nodulated by a larger pool of Bradyrhizobia. This may be due to the fact that legumes within the cowpea cross-inoculation group nodulate only with subgroups of the indigenous populations of Bradyrhizobium spp. (Burton, 1979; Thies et al., 1991; Abaidoo et al., 2007; Jaramillo et al., 2013).
5.3 Effect of P application on nodulation of soybean and pigeonpea grown in the Adenta and Bekwai series.

In both the Adenta and the Bekwai soils, the addition of soluble P fertilizer generally had highly positive effects on nodulation and nodule growth. Phosphorus is known to be the main energy source for powering nodulation of legumes (Gentili and Huss-Danell, 2003). Nodule P requirements forms a greater portion of a legume’s total P requirement (Bonetti et al., 1984; Qin et al., 2012). Hence, the increased nodulation with P addition which is similar to earlier reports (Singh et al., 1981; Rao and Reddy, 1997; Olivera et al., 2004), is expected.

While these responses applied to both legumes used in this study, the magnitude was often influenced by the soil type and legume genotype. For example, while in the Adenta soil, pigeonpea gave higher responses than soybean with P application up to 120 kg P/ha, the application of P above 120 kg P/ha decreased the number of nodules formed on pigeonpea but not on soybean. Plant species differ in their relation with mycorrhizal fungi as well as the response of arbuscular mycorrhizal association to varying levels of P in soil solution (Kahluoto et al., 2000). Thus, plants with extensive fibrous roots are often less dependent on mycorrhizae compared to plants having less extensive root systems (Plenchette et al., 1983). Pigeonpea is known to release bond sources of P in soil by its root exudates (Ae et al., 1990) and mycorrhizal association (Dighton et al., 1993). This together with the high phosphorus utilisation efficiency of pigeonpea which makes the legume adapt to soils with low P status (Ascencio and Lazo, 2001) may have contributed to the higher response of pigeonpea to P application than soybean in this study. Similar findings on differences in legumes’ P requirements for nodulation and nitrogen fixation in different soils have been reported (Shu jie et al., 2007). Phosphorus application that usually exceeds that required by plants have been reported to result in decreased mycorrhizal development (Chulan and Ragu, 1986). When the release of P from fertilizers is not in balance with the plant demand, it results in increased P concentration in the soil’s solution near the
rhizosphere. The excess P in solution is bound to Ca, Mg or to Fe and Al oxides through fixation reactions (Mitchell, 1957; Sample et al., 1980). The nutrients Ca, Mg and Fe therefore become unavailable to the growing plant. At this stage growth ceases and further developments are retarded and may be the reasons why higher rate of P application decreased nodulation in pigeonpea. Shekhar and Sharma (1991) reported similar decreases in nodulation parameters in *Pisum sativum* when phosphorus was applied above a threshold rate.

5.4 Effect of N application on nodulation of soybean and pigeonpea grown in the Adenta and Bekwai series.

Application of nitrogen generally decreased the number of nodules and also the dry weight of nodules formed. The N$_2$ fixation process together with the assimilation of the end product is high and costly in terms of energy (Evans et al., 1980; Al-Neimi et al., 1997) compared to the energy cost of assimilating supplied N (Salsac et al., 1987; Bloom et al., 1992). As such, leguminous plants will prefer mineral N to fixed N$_2$ when presented with these two options. Such conditions do not favour the nodulation of the growing legume and the process is inhibited (Danso et al., 1990; Abdel-Wahab et al., 1996). Higher levels of mineral N in soils have been reported to depress nodulation (Davidson and Robson, 1986; Eaglesham, 1989; Gentilli and Huss-Dannel, 2002) and nitrogen fixation in actively growing legumes (Herdina and Silsbury, 1989; Sun et al., 2008).

That nodules were formed and their weights could not be measured by the scale used suggests that nodule growth may have been more severely affected than nodule initiation. In the presence of high soil mineral N, there is reduced supply of carbohydrates to nodules for nitrogenase activity (Streeter, 1985). Nitrogenase activity is therefore inhibited by this process (Purcell and Sinclair, 1990; Sanginga et al., 1996; Arreseigor et al., 1997). This together with decreased diffusion of oxygen into nodules causes restricted respiration of bacteriods (Gordon et al., 2002)
which may have inhibited the development of the nodules formed (Atkins et al., 1984; Imsande, 1986; Sodek and Silva, 1996) and consequently decreased the weight of nodules formed at higher N rates.

5.5 The counteracting effect of P on nodulation inhibition by soil inorganic N by soybean and pigeonpea grown in the Adenta and Bekwai series.

Where high soil mineral N had completely “knocked-off” nodulation and nodule growth, application of phosphorus revived nodulation and nodule growth in all cases. However, the levels of phosphorus required differed for the different soils and for the different legumes. Due to its key role in the energy metabolism of all plant cells and particularly in nitrogen fixation (Dilworth, 1974), P application has been reported to increase nitrogenase activity, photosynthetic rate and legume biomass production (Geneva et al., 2006). With increased photosynthetic rate and biomass production the plant grows bigger with a higher N requirement. The increased N requirement for growth leads to increased N uptake from the soil thereby reducing the N in the soil (Corti et al., 2005) to a low level where N inhibition is “knocked-off”. This finding is supported by earlier reports that where nodule numbers and nodule dry weights were inhibited by N application, there was a counteracting effect by high P (Gentili and Huss-Danell, 2002).

Soybean grown in the Bekwai soil was not nodulated at all. The inability of soybean to nodulate in the Bekwai soil may be due to factors other than P. Similar findings on the inability of soybean to nodulate in the Bekwai soil have been reported by Klogo (2006).

5.6 Effect of N and P application on shoot dry weight of soybean and pigeonpea grown in the Adenta and Bekwai series.

Application of P increased the dry weight of shoots produced by the legumes in both soils. However, in the Adenta soil, P application above 160 kg/ha decreased pigeonpea shoot
production. The importance of P in promoting vegetative growth and nodulation as well as N\textsubscript{2} fixation as stated earlier (Dilworth, 1974; Gentili and Huss-Danell, 2002; Geneva \textit{et al.}, 2006) may have been the reasons for the increased shoot biomass observed. Similar findings on P application on increased biomass production have been reported (Gate and Wilson, 1974; Olofintoye, 1986; Pereira and Bliss, 1987). Also, the decreased dry weight of pigeonpea shoots observed with high P application could be due to the problems as associated high P in soils stated earlier (Mitchell, 1957; Sample \textit{et al.}, 1980; Chulan and Ragu, 1986; Shekhar and Sharma, 1991). This observation is similar to the findings of Tsvetkova \textit{et al.}, (2003).

The study again showed that nitrogen application increased shoot production by the legumes in both soils. In the Bekwai soil series, increased dry weight of pigeonpea shoots were observed at all rates of N applied. The increase in the dry weight of shoots with nitrogen application gives an indication of the N requirements of these legumes for adequate growth and development (Walker \textit{et al.}, 2001) and that the soils’ solution do not contain enough N to satisfy all the N requirements of legumes, neither was N\textsubscript{2} fixation high enough to provide all the additional N required. This finding is in agreement with work done by Singleton \textit{et al.} (1985) who reported that N application enhances shoot dry weight production.

In the Adenta series, however, N application above 80 kg N/ha decreased dry shoot production by both legumes. This may be due to toxicity induced by oversupply of N. Similar findings of decreased shoot production due to N toxicity have been reported for soybean varieties grown in some Ghanaian soils (Fening, 1999).

The combined application of N and P in this study enhanced shoot dry weight production by both legumes. Because of its importance in energy metabolism (Dilworth, 1974) and the ability to increase nitrogenase activity, photosynthetic rate and legume biomass production (Jia \textit{et al.}, 2004: Geneva \textit{et al.}, 2006), P application results in increased plant N uptake from soils thereby
reducing the N in the soil (Corti et al., 2005). In this way, any toxicity effect induced by the high N in the soil (Fening, 1999) is also reduced.

A similar finding was reported by Gentilli and Huss-Danell (2002) who concluded that in low P soils the dry weight of shoots produced is low compared to the high dry weight of shoots (10-fold increase) at high P levels under toxic levels of N.

Although P effect was general on growth of the legumes, its effect was more drastic on nodulation. This may be due to the more P requirement for nodule initiation and development which often sinks more of the supplied P (Eaglesham and Ayanaba, 1984), the excess of which is used for enhancing vegetative growth (Jia et al., 2004; Geneva et al., 2006). Similar finding has been reported by Wall et al. (2000).

5.7 Effect of phosphorus application on nitrogen fixation by soybean in Adenta series.

Phosphorus application though increased the nodulation of soybean by the indigenous \textit{Bradyrhizobium} populations, the tendency with increased P was for the dry weight per nodule formed and perhaps nodule size too to be the same as that of the control. Phosphorus application had no significant positive effect on the dry weights per nodule formed in this study. Without P application, the indigenous populations of \textit{Bradyrhizobium} that nodulated soybean fixed only 15.57 mg N/pot equivalent to 15.57 kg N/ha in the soil. The total N derived from atmospheric N$_2$ fixed constituted 23.1\% of the total N in shoot. With P application, nodulation in the soybean-\textit{Bradyrhizobium} symbiosis was enhanced, resulting in increased total N accumulation in the shoot up to the 120 kg P/ha rate where 51.50 mg N/pot equivalent to 51.50 kg N/ha was derived from fixation. This finding could be explained by the conclusions that phosphorus enhances photosynthetic rate and biomass production (Jia et al., 2004) as well as nitrogenase and acid phosphatase activities (Geneva et al., 2006; Olivera et al., 2004). Eventually the plant grows bigger with a higher N demand which leads to an increased N uptake from soil. With increased
N uptake, the soil’s N is depleted and mechanisms for nodulation and nitrogen fixation is initiated especially in soils with low N status. This finding is in line with results reported earlier for increased nodulation and also increased amount of nitrogen fixed with P addition (Herridge et al., 2008; Salvaglotti et al., 2008). In uninoculated soils, N₂ fixation induced by an indigenous bradyrhizobial community supplies less than optimal amounts of nitrogen (Sanginga et al., 2002). Most of the increase in N₂ fixation with P addition was in total N₂ fixed rather than in % Ndfa, with the greatest increase in % Ndfa occurring with the first addition of P, from 0 kg P/ha to 40 kg P/ha. This observation is similar to the trend reported by Sanginga et al. (1990).

5.8 Diversity of indigenous *Bradyrhizobium* strains nodulating soybean, pigeonpea and cowpea in Adenta and Bekwai series as determined by combined RPO1 and RPO4 PCRs.

The results from the present study have shown that the indigenous bradyrhizobial isolates that nodulated cowpea, soybean and pigeonpea grown in the soils studied differed genetically and were highly diverse. Polymerase chain reactions (PCR) using RPO1 and RPO4 primers were able to generate a high degree of polymorphic *Bradyrhizobium* DNA (Versalovic et al., 1994; Teaumroong and Boonkerd, 1998; El-Fiki, 2006). The resulting multiple DNA fragments had band sizes ranging between 117-1800 bp and 157-3000 bp for the RPO1 and RPO4 PCR respectively. These band sizes corresponded to the expected sizes reported previously (Richardson et al., 1995; Sikora and Redzepovic, 2003). The 1500 bp and 2900 bp DNA fragments for the 16S and 23S gene PCR were also expected (Pronk and Sanderson, 2001; Yasuda and Shiaris, 2005; Zhu et al., 2007). The use of combined matrices of RPO1-PCR and RPO4-PCR in the present study to discriminate among bradyrhizobial isolates was successful and the observation made agrees with earlier reports (Bostock et al., 1993; Wang et al., 1993; Sikora et al., 1997; Wolde-meskel et al., 2005; El-Fiki, 2006).
The high diversity in indigenous populations of Bradyrhizobia that nodulated legumes in this study is similar to work done elsewhere on rhizobia (Madrzak, et al., 1995; Niemann et al., 1997; Ando, et al., 1999).

Several researchers have reported on the effect of environmental variables and management practices on population structure and diversity under field conditions where host legumes were previously absent (Bala et al., 2001; Andrade et al., 2002; Depret et al., 2004; Kaschuk et al., 2006). The diversity of rhizobia between sites (Mothapo et al., 2013) is usually due to prevailing site-specific environmental variables imposing general genetic adaptations on soil rhizobia (Bernal and Graham, 2001; Mutch et al., 2003; Tian et al., 2007; Yang et al., 2006; Farooq and Vessey, 2009). Compared to the semi-deciduous forest soils, the Adenta soil from the coastal savannah agro-ecological zone supported a low diversity of bradyrhizobia that were capable of nodulating pigeonpea and cowpea. Conversely, the diversity of Bradyrhizobium from the Adenta soil that nodulated soybean was higher than that for the soybean isolates from the Nzima soil. Earlier reports show that soils from humid regions have relatively high diversities of nitrogen-fixing bacteria than those from semi-arid regions (Wasike et al., 2009). Similar findings have also been reported by Danso and Owiredu (1988) and Rupela et al. (1982). The high diversity of the pigeonpea isolates from this study may be due to the low specificity of the legume in terms of nodulating with indigenous rhizobial communities as previously reported (Coutinho et al., 1999; Lombardi et al., 2009).

5.9 Effect of Phosphorus on the diversity of indigenous Bradyrhizobium strains nodulating soybean, pigeonpea and cowpea in Adenta, Bekwai and Nzima series.

Application of P in the Adenta soil decreased the diversity of Bradyrhizobium that nodulated cowpea. Different levels of phosphorus are stored by different strains of Bradyrhizobium japonicum (Cassman et al., 1981). The nitrogen fixing capability of superior strain-legume
symbiosis is usually at the highest level of P application (Erman et al., 2009). These conclusions together with the selectivity for effective strains (Perret et al., 2000) under N-limited conditions may have led to the selection of strains which were similar in this case and dominated the nodulation of this legume or that some of the different strains that nodulated the cowpea prior to P addition were suppressed in the presence of added P.

Contrary to the observation made for cowpea in the Adenta soil, the diversity of the isolates that nodulated pigeonpea in both the Adenta and Nzima soils was increased with P addition. The same explanation given for enhanced nodulation of some cowpea isolates from the Adenta soil with P application may be applicable to this observation except that the higher diversity observed with P application may be that pigeonpea in addition, was nodulated by the strains that nodulated when no P was applied or that the enhanced strains in this case were diverse.

It is suspected that *Bradyrhizobium japonicum* strains may be present in the Adenta and Nzima soils but absent in the Bekwai soil. Results on soybean not being nodulated by indigenous Bradyrhizobial populations in Bekwai soil have been reported by Attuah (2001). However, upon inoculation with *Bradyrhizobium japonicum* strain USDA 110 it was found that the legume was profusely nodulated (Attuah, 2001). These findings are in agreement with reports that where a high degree of host-specificity exists between legume hosts and rhizobial species (Thrall et al., 2000) loss of a single rhizobial species can result in loss of nodulation and N₂-fixation by that legume (Lowendorf et al., 1980).
CHAPTER SIX

6.0 SUMMARY CONCLUSION AND RECOMMENDATIONS

The present study aimed at studying the nodulation and nitrogen fixation of soybean and pigeonpea under phosphorus fertilization and the need for *Bradyrhizobium* inoculation in Ghanaian soils.

The presence of bradyrhizobia indigenous to Ghanaian soils that are capable of nodulating with soybean and cowpea has been reported. The populations of bradyrhizobia nodulating soybean and cowpea in Ghanaian soils estimated by the MPN plant infection assay vary widely ranging from $0.7 \times 10^1$ cells/g soil to $7.8 \times 10^3$ cells/g soil. The soils from the semi-deciduous forests were found to harbour more indigenous cowpea bradyrhizobial populations than the soil from the coastal savannah. The Ferric Acrisol contained the highest population ($7.8 \times 10^3$ cells/g soil) of cowpea nodulating *Bradyrhizobium*, however, the least population of Soybean nodulating *Bradyrhizobium* ($0.7 \times 10^1$ cells/g soil) was observed in this soil. The semi-deciduous Haplic Acrisol contained the highest population of soybean rhizobia ($4.5 \times 10^2$ cells/g soil). Soybean is probably nodulated by both bradyrhizobia belonging to the cowpea miscellany and those that do not belong to the cowpea miscellany.

Application of P generally increased nodulation and growth of nodules except for soybean grown in the Ferric Acrisol. Nitrogen application inhibited nodulation in the legumes up to levels where complete inhibition of nodulation and growth of nodules occurred. Application of P in combination with an inhibitory rate of N revived nodulation and nodule growth in all cases. Single applications of N and P generally increased shoot dry weight production except in some few cases where higher applications resulted in decreased shoot production. Combined application of N and P also increased shoot dry weight production by the legumes. However, at higher rates of combined N and P, toxicity was induced on shoot dry weight production.
The present study investigated the nitrogen fixing potential of the indigenous bradyrhizobia on soybean in the Coastal savannah Haplic Acrisol. Without P application, the soybean-\textit{Bradyrhizobium} symbiosis fixed 15.57 kg N/ha equivalent to 23.1 \% of the total N in shoot. Phosphorus application increased the amount of nitrogen fixed by the symbiosis, and by the 120 kg P/ha rate, the amount fixed was more than 3 times that of the control without P, and was equivalent to 54.7 \% of the total N in shoot. Thus, with 120 kg P/ha applied, the indigenous populations of bradyrhizobia through symbiosis were able to supply the soybean cultivar Anidaso with more than half of its total nitrogen requirement.

The combined DAPD and RAPD fingerprinting patterns have been able to successfully discriminate within and between isolates that nodulated cowpea, pigeonpea and soybean in the soils under study. The cluster analysis revealed that a high diversity existed within and between indigenous bradyrhizobial isolates that nodulated cowpea, pigeonpea and soybean grown in the soils from different agro-ecological zones in Ghana. The diversity of isolates from the different phosphorus fertilized soils that nodulated soybean, pigeonpea and cowpea varied considerably. For example, whereas P application reduced the diversity of cowpea isolates from the coastal savannah Haplic Acrisol, the diversity of pigeonpea isolates from the same soil were increased with P application.

The diversities identified especially for soybean considering the nitrogen fixing potentials of the symbiosis represents a valuable genetic resource potential for selecting more competitive and effective strains for inoculum production to improve BNF and increase the yields of the legumes at a low cost of production.

I would recommend that

- To achieve maximum benefits from the symbiosis, P fertilization is essential, especially for our soils that are generally low in available P
• Nitrogen fertilization generally is inhibitory to \( N_2 \) fixation, and is not recommended if the maximum benefits from BNF are expected.

• In soils where nodulation and \( N_2 \) fixation are inhibited by nitrogen availability, the application of \( P \) fertilizer is essential to counter the inhibitory effects of the high soil \( N \) on nodulation and \( N_2 \) fixation.

• The high diversity of bradyrhizobia observed although could ensure the nodulation of different tropical legumes by the indigenous strains without the need for artificial inoculation, there is the possibility that these legumes could be nodulating with the diverse array of these bradyrhizobia, of which a proportion might not be active in fixing \( N_2 \) on that particular legume because they are not specific to it. For this reason, more studies are necessary to assess actual \( N_2 \) fixed with or without inoculation by selected, highly effective strains, distinct from number of nodules formed, so as to assess the need for inoculation of many of these legumes.

• Based on the higher yield of the \( N \)-fertilized legumes than the unfertilized controls, it appears that the indigenous bradyrhizobia were not highly efficient and could therefore not fix the optimum amounts of \( N \) required for the growth of these legumes. Such results call for the use of rhizobial inoculation with strains more effective than the indigenous ones.
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APPENDICES

Appendix 1

USDA soil texture triangle

Source: Soilsensor.com (http://www.soilsensor.com/soiltypes.aspx)
Appendix 2

Growth- Limiting bulk density textural triangle

Source: Warrington and Dadow (1983)
Appendix 3

Effect of Phosphorus application on the diversity of *Bradyrhizobium* nodulating soybean, pigeonpea and cowpea growing in Adenta, Bekwai and Nzima series.

<table>
<thead>
<tr>
<th>Indices</th>
<th>Legume</th>
<th>NI</th>
<th>Soil</th>
<th>Diversity</th>
<th>Evenness</th>
<th>Effect of P on diversity</th>
<th>New strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cowpea</td>
<td>21</td>
<td>Adenta</td>
<td>1.22</td>
<td>0.88</td>
<td>-</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Bekwai</td>
<td>1.74</td>
<td>0.90</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>Nzima</td>
<td>2.11</td>
<td>0.91</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>All</td>
<td>2.75</td>
<td>0.90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigeonpea</td>
<td>13</td>
<td>Adenta</td>
<td>1.95</td>
<td>0.94</td>
<td>+</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Bekwai</td>
<td>2.20</td>
<td>0.92</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Nzima</td>
<td>2.27</td>
<td>0.94</td>
<td>+</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>All</td>
<td>3.29</td>
<td>0.97</td>
<td></td>
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</tr>
<tr>
<td>Soybean</td>
<td>13</td>
<td>Adenta</td>
<td>1.58</td>
<td>0.88</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Nzima</td>
<td>0.69</td>
<td>1.00</td>
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<td>NA</td>
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</tr>
<tr>
<td></td>
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<td>0.92</td>
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<td></td>
</tr>
<tr>
<td>All</td>
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<td></td>
<td>32</td>
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<td>Nzima</td>
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<td>0.92</td>
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</tr>
</tbody>
</table>

NB:  
- = P application increased diversity  
+ = P application decreased diversity  
0 = P application had no effect on diversity.  
NI= Number of isolates  
NA = Not applicable Indicating that there were no nodules formed without P application