UNIVERSITY OF GHANA

THE CHARACTERISTICS AND DIVERSITY OF INDIGENOUS RHIZOBIA THAT NODULATE SELECTED INDIGENOUS MULTIPURPOSE LEGUMINOUS TREES AND SHRUBS IN THREE SOILS OF GHANA

BY

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DECLARATION

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

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I dedicate this work solely to my god father Apostle (Dr.) Kwadwo Safo, the Founder and Leader of Kristo Asafo.

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TABLE OF CONTENTS

TITLE **PAGE**

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Trees in general and leguminous trees in particular form an integral part of the traditional farming systems in Ghana. Compared to other plants, leguminous trees have the advantage that, they are generally capable of growing better on N-deficient soils due to their ability to convert unavailable atmospheric N_2 into plant utilizable N. However, several factors including the abundance and effectiveness of the specific rhizobial partner, the available N and P in soil, are among the important factors that severely affect how much N_2 can be fixed in these trees. This study was thus conducted to assess the abundance and characteristics of the rhizobia that nodulate 18 selected indigenous tree legumes grown in three representative soils of Ghana and to ascertain important soil nutrient constraints that affect their nodulation, nitrogen fixation and growth. The three soils belonged to the Hatso, Toje and Alajo local series (equivalent to Haplic lixisol, Rhodic lixisol and Calcic vertisol, respectively). The 200 *Rhizobium* isolates obtained from nodules of these tree legumes were found to be highly diverse and varied in their abilities to nodulate legumes other than the host plants from which they were isolated. The isolates were further characterized culturally, metabolically, phenotypically and for their effectiveness in fixing atmospheric nitrogen. Of the 10 multi-purpose shrubs and tree species belonging to the sub-family Mimosoideae examined as much as 70% of them formed nodules in the three soils, whiles only 20% and 10% of the tree legumes that formed nodules belong to Papilionoideae and Caesalpinoideae sub-families respectively. As to the *Rhizobium* isolates from these shrubs and tree species, those obtained from *Pithecelobium spp* and *Melletia thonningi* appeared to be highly specific, nodulating only their respective homologous hosts, while those from *Acacia mangium, Albizia lebbek and Acacia auricloformis* appeared to be slightly promiscuous, and moderately promiscuous for those from *Leucaena leucocephela*, *Crotalaria ochloleuca, Cajanus cajan* and

Vigna unguiculata;, the NGR 234 strain, included as a standard was the most promiscuous, forming nodules on almost 67% of the eight host legumes on which it was tested. With regards to the occurrence of rhizobia in each soil, the three soils were found to harbour variable populations of indigenous rhizobia, ranging from 22 cells/g of soil for *A. mangium* and *C. ochloleuca* in Alajo soil, to 5,200 cells/g for compatible strains of *Pithecelobium sp*. (in each of the three soils), *M. thonningi* (in Toje and Hatso soils) and *A. lebbek* (in only the Hatso soil). Further examination of the characteristics of the *Rhizobium* isolates revealed that each shrub and tree legume species was nodulated by strains belonging to both the fast-growing (*Rhizobium*) and slow-growing (*Bradyrhizobium*) types. In terms of abilities to fix N_2, the greatest majority (63%) of the isolates were found to be ineffective on their homologous host plants, with ineffectiveness ranging from 43% of the *Acacia auricloformis* strains to 88% of isolates from *Acacia mangium*. The proportion classified as being effective averaged 15% and ranged from 5% in the case of *Acacia mangium* to 28% of the *Albizia lebbek* isolates. The polymerase chain reaction (PCR) of the 16S rRNA gene of almost all the 60 selected *Rhizobium* isolates from the tree legumes gave a single band of size 1.5 kb, corresponding to the expected band size reported for rhizobia, while the combined restrictions of the 16S rRNA genes of the 60 rhizobia isolates with five enzymes (Hae 111, Rsa 1, Hpa 1, Hpa 11 and Alu 1) distinguished clearly 60 different combinations of patterns or fingerprints, representing 60 distinct 16S rRNA genotypes among the isolates. Characterization of the test rhizobia isolates based on simple PCR of the intergenic spacer (ITS) gene was most resolute as it provided several distinct band sizes indicating great variation among the rhizobia isolates' ITS. However, restriction of the ITS with three restriction enzymes (Hae 111, Hpa 11 and Alu 1) did not give much further distinctions among the test isolates. Experiments conducted in pots in the screenhouse using the Hatso and Toje soils indicated significant increases in both

number of nodules formed, N_2 fixation and total biomass of *A. lebbek*, *A.mangium* and *A. auricloformis* with P addition to soils. However, the application of phosphorus beyond 90 kg P/ha resulted in significant decrease in all these parameters in Hatso and Toje soils. With nitrogen fertilizer application, even the minimum level (40 kg N/ha) resulted in significant reduction in numbers of nodules formed on the tree legumes, even though it resulted in significant increase in plant biomass. However, phosphorus application mitigated the negative effect of nitrogen addition. *Rhizobium* inoculation also resulted in significant increase in both number of nodules formed and total biomass of the tree legumes over the uninoculated controls. Thus although the rhizobia that nodulate tree legumes in Ghanaian soils may be widespread, the rhizobia that occur in each soil appear to be heterogeneous or very diverse both phenotypically and genotypically and in their abilities to form effective nodules. To enhance nodulation, nitrogen fixation and growth of the tree legumes, consideration must be given to augmenting these soils with selected, highly effective strains together with phosphorus fertilizer.

CHAPTER ONE

1.0 INTRODUCTION

Trees in general are considered as integral part of the traditional farming systems in Ghana. Forest trees such as *Gliricidia sepium, Sammania sammia, Acacia spp, Albizia spp.* etc provide shade for crops like cocoa, coffee etc, act as wind brakes, control erosion, provide timber for wood industries, provide building materials for construction, fuel wood (which is the most important source of energy in rural areas), medicines, chewing sticks etc. They provide sanctuary for wildlife, protect and enrich the soils, maintain and regulate stream flow and preserve the genetic diversity of the flora and fauna.

In many areas, forest trees are used for stabilizing coastal sand dunes or for reclaiming degraded lands (Diem and Dommergues, 1990). In view of the numerous contributions of forest trees to the environment and mankind, there is the need to protect and use forests rationally to ensure continuous supply of its functions.

Unfortunately, forest trees in Ghana are being depleted at an alarming rate without corresponding replacement. The traditional shifting cultivation and bush fallow system allowed the soil to be rested for many years for trees and shrubs to develop to help restore the soil's fertility following few years of cropping. However, due to population pressure and greater demand of farming land, the fallow period has been so reduced that the trees are not allowed to grow to accomplish the desired objective.Continuous cultivation (in response to increasing population) without much fertilizer addition has drastically led to reduced soil fertility and increased soil erosion in the humid tropics and the semi-arid areas (Lal, 1987).

Reduction in the periods of rest in the shifting cultivation and bush fallow farming practices have led to increased deforestation. According to F.A.O. (1986), the tropics are losing more than 10 million hectares of forest cover annually and farming is responsible for almost 70% of deforestation in tropical Africa. The situation is alarming in tropical Africa where deforestation exceeds the projected rate of tree planting by a ratio of 1:10 (F.A.O.1986). In addition to food crop farming, in drier areas, overgrazing and harvesting of trees for fuel wood have been major factors in reduced productivity, increased soil erosion and desertification (F.A.O., 1986). In wet areas, farming and excessive harvesting of trees for timber and fuel wood are the main causes of increased deforestation and reduced soil productivity (F.A.O., 1986). In order to reverse the present trend, there is the need to reduce the rate of population growth, introduce settled agriculture, develop alternative sources of energy and embark on serious tree planting programmes.

Attempts to improve the productivity of traditional farming systems in Ghana by introducing inorganic fertilizers have proved futile, mainly, because of the inability of poor farmers to afford the prices of fertilizers, aggravated by the removal of price subsidies on fertilizers by the government. According to Kang *et al.* (1981), the effective management of biological nitrogen fixing trees and other leguminous plants in farming systems is a key to restoring and maintaining soil fertility thereby reducing the need for fertilizer N.

For more than 100 years, biological nitrogen fixation (BNF) has commanded the attention of scientists concerned with plant mineral nutrition, and it has been exploited extensively in agricultural practice (Dixon and Wheeler, 1986; Burris, 1994). However, its importance as a primary source of N for agriculture has diminished in recent decades as increasing amounts of inorganic fertilizer N have been used for the production of food and cash crops (Peoples *et al.,*

1995). However, it is the opinion of Peoples *et al*.(1995) that, because of the current global interest and emphasis on environmental sustainable development, greater attention may need to be focused on the potential role of BNF in supplying N for Agriculture. Attention has been drawn to the fact that BNF is ecologically benign and that its greater exploitation can reduce the use of fossil fuels and can be helpful in reforestation and in restoration of misused land for productivity (Burris, 1994; Sprent and Sprent, 1990).

Currently, the subject of BNF is of great practical importance because the use of inorganic nitrogenous fertilizers has resulted in unacceptable levels of water pollution (increasing concentrations of toxic nitrates in drinking water supplies) and in the eutrophication of lakes and rivers (Dixon and Wheeler, 1986; Sprent and Sprent, 1990).

Moreover, whilst BNF may be tailored to the needs of soil microorganisms, chemical fertilizer is usually applied in a few large doses, up to 50% of which may be leached (Sprent and Sprent, 1990). This not only waste energy and money but also leads to serious pollution problems, particularly in water bodies.

In recent years, the potential of leguminous and actinorrhizal trees for restoring and maintaining soil fertility has aroused considerable interest and many international agencies (e.g. IITA, IAEA, IDRC etc.) have recommended their increased use in agroforestry systems and for crop shade. The major advantages of nitrogen fixing trees are, their ability to establish in nitrogen deficient soils, and the benefits of the nitrogen fixed and extra organic matter to succeeding or associated crops. Sanginga *et al.*(1986), found that maize yields in soil in which inoculated *Leucaena leucocephela* had grown for 6 months, were increased from 1.5 t/ha to 2.5 t/ha with prunnings removed and from 2.2 to 4 t/ha with prunnings returned to the soil. Ladha *et al.* (1989) indicated

that, nitrogen fixed by *Sesbania rostrata* can significantly increase subsequent grain yield of lowland rice.

Although nodules are important for nitrogen fixation in legumes, till date, with the exception of few leguminous trees including *Leucaena leucocephela, Albizia lebbek, Gliricidia sepium, Acacia mangium,* and *Acacia auricloformis*, the need to inoculate with appropriate rhizobia for enhanced nodule formation has not been examined for others.

It has been known for a long time that not all leguminous trees are able to nodulate and fix nitrogen (Kang *et al.,* 1981). This therefore raises questions about the suitabillity of integrating these non-N_2-fixing legumes into agroforestry systems. For example, earlier work by Boakye (2001) reported lack of nodulation in *Parkia biglobosa* and *Tetrapleura tetraptera* which are two very important economic trees in Ghana. With the exception of some species such as *Leucaena leucocephela* which have been reported to fix large amounts of nitrogen in Nigeria (Kang *et al.,* 1981), our knowledge on nitrogen fixation in trees in the sub-region is still very limited, and there is therefore the urgent need for more studies on nitrogen fixing trees, in developing countries like Ghana.

In this study, 18 species of some common indigenous leguminous trees and shrubs were assessed for ways of enhancing their growth, nodulation and nitrogen fixing abilities on three different soil types that occur in the coastal savannah zone of Ghana.

Justification

Compared to their herbaceous counterparts the available literature indicates that little work has been done on biological nitrogen fixation in trees.and till date, little is known about the rhizobia that nodulate and fix nitrogen in many indigenous tree legumes. It is to fill this gap as well as

towards improving benefits to be gained in the use of nitrogen fixing trees in sustaining soil fertility and productivity for food sufficiency in Ghana that this study was proposed.

The Objectives of the Study are:

i. To investigate the growth, nodulation and nitrogen fixing potential of some common indigenous multipurpose trees and shrubs in Ghana.

ii. To assess the need for inoculation for improving nitrogen fixation in selected leguminous trees of potential use in agroforestry and forestry.

iii. To assess the role of factors of soil nutrients such as nitrogen and phosphorus in nodulation and growth of indigenous nitrogen fixing trees.

iv. To isolate and identify strains of rhizobia likely to be of use in the preparation of inoculants.

v. To characterize phenotypically and genotypically the rhizobia that nodulate the indigenous leguminous tree legumes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Role of Nitrogen in the Biosphere

Nitrogen, the most abundant element on earth also happens to be one of the mostt essential nutrients for life on earth, coming after Carbon (C), Hydrogen (H) and Oxygen (O), in relative importance The gaseous atmosphere consists about 78% dinitrogen (N_2) and each square foot of the earth's surface contains approximately 2721 kg of nitrogen (Stevenson, 1982). However, the greatest majority of the earth's nitrogen (98%) is in rocks, sediments and soils (Stevenson, 1982). The amount of nitrogen in rocks is about 50 times more than that in the atmosphere, while the amount in the atmosphere is approximately 5,000 times more than that found in soils (Stevenson, 1982). It is an important element in proteins and nucleic acids, which are vital to all living organisms on earth (Kormondy, 1996). Although nitrogen makes up about 78% of the gases in atmosphere, it is often a limiting factor for plants and animals (Kormondy, 1996).

Living organisms cannot use nitrogen in its dinitrogen gaseous form for growth. Plants can only use nitrogen in the form of nitrate or ammonium ions, and animals can only use it in organic forms, which they obtain by consuming plants or other animals (Kormondy, 1996). The weathering of rocks releases these ions so slowly that it has a negligible effect on the availability of fixed nitrogen. So, nitrogen is often the limiting factor for growth and biomass production in all environments where there is suitable climate and availability of water to support life.

Unstable nitrogen moves from the atmosphere to usable forms and back to the atmosphere through a process known as nitrogen cycle. Organisms, both living and dead, and soil act as the reservoir for unstable nitrogen in organic and inorganic forms (Kormondy, 1996)

The deficiency of nitrogen in most tropical soils has contributed to reduced agricultural and forest yields in Africa. The Green Revolution was accompanied by a huge increase in the application of fertilizers, particularly nitrogen (Galloway, 1998). Grain crop yields have constantly increased during the 20th century to remarkable levels in many years. This increase in yield has been primarily facilitated by the introduction of genetically improved cultivars and use of chemical pesticides and fertilizers. Improvement in grain crop production technology from 'slash and burn' practice to the success of the 'Green Revolution' was associated with increasing nitrogen (N) availability to crops. The expanding use of industrially manufactured N fertilizers has been one of the main factors behind the fast growth in agricultural productivity over the last few decades. Between 1950 and 1990, the per capital use of N fertilizer increased about 10 folds from about 1.3 to 15 kg N per person, and leveled off in the last decade of the 20th century (Galloway, 1998)

2.2 The Concept of Nitrogen Fixation

Nitrogen fixation is a process whereby microorganisms help to convert atmospheric nitrogen (N_2) into ammonia and then into protein (Sprent, 1987; Purves *et al.,* 1992). The concept of nitrogen fixation was used by the Haber-Bosch process to industrially produce a relatively small amount of ammonia, using an iron-based catalyst, very high pressures and fairly high temperatures. The major conversion of N_2 into ammonia however is achieved by microorganisms (Sprent, 1987).

Some estimates of the amount of nitrogen fixed on a global scale is represented on a Table 1.

Table 1. Amount of Nitrogen Fixed on a Global Scale

Type of fixation	**N_2 fixed (10^{12}g per year or 10^6 metric tons per year**
Non-Biological	
Industrial	50
Combustion	20
Lightening	10
Total	80
Biological	
Agricultural Land	90
Forest and Non-Agricultural Land	50
Sea	35
Total	175

Source: Bezdicek and Kennedy, (1998).

2.3 Biological Nitrogen Fixation

Approximately 78% of gases in the atmosphere is nitrogen gas (N_2). Unfortunately N_2 is unusable by most living organisms. Plants, animals and micro-organisms can die of nitrogen deficiency, surrounded by the N_2 that they cannot use. All organisms use the ammonia (NH_3) form of nitrogen to manufacture amino acids, proteins, nucleic acids and other nitrogen – containing components necessary for life.

Biological nitrogen fixation is the process by which microorganisms convert inert N_2 into biologically useful NH_3 (Lindermann and Glover, 1998). This process is mediated in nature only by bacteria referred to as nitrogen fixing bacteria. Biological nitrogen fixation is an essential natural process that supports life on this planet. Nitrogen fixation can be both biological and non-biological. Biological nitrogen fixation systems are estimated to fix 100 – 175 million metric tons of nitrogen annually (Burns and Hardy, 1975). Non biological nitrogen fixation occurs through the effects of lightening and in industry primarily by the Haber-Bosch process which requires high levels of fossil fuel. Worldwide, lightening may fix 10 million metric tons of nitrogen per year (Bezdicek and Kennedy, 1998).). Industrial fixation for fertilizer nitrogen has increased from 3.5 million tons in 1950 to 80 million tons in 1989 in response to the needs of high yielding crops. Burns and Hardy (1975) estimated the annual amount of nitrogen fixed biologically in various systems to be between 100 - 175 million metric tons.

Nitrogen fixation in legumes involves a partnership between the plants and bacteria collectively called rhizobia. In legumes and a few other plants, the bacteria live in small pouches on the roots called nodules. Within these nodules, nitrogen fixation takes place by the bacteria, and the NH_3 produced is made available to the plant. Plants may also benefit from nitrogen fixing bacteria when these bacteria die and release nitrogen to the soil environment. In addition to symbiotically fixed nitrogen in plants, biological nitrogen fixation can be accomplished by different free-living organisms including blue-green algae and some soil bacteria. These organisms may contribute significant quantities of NH_3 to natural ecosystems. For example, these algae are particularly important in flooded soils, such as in paddy rice ecosystems although their contributions are less than 2.2 kg of nitrogen per acre per year. This may be contrasted to nitrogen fixation in legumes,

in the range of 11-34 kg N per acre per year in natural ecosystems and several hundred kilograms in some cropping systems (Lindermann and Glover, 1998)

2.4 Mechanisms of Biological Nitrogen Fixation

Biological nitrogen fixation can be represented by the following equation, in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of sixteen moles of ATP and a supply of electrons and protons (Hydrogen ions) (Bezdicek and Kennedy, 1998)

$$N_2 + 8H + 8e^- + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16\ ADP + 16Pi$$

This reaction is performed exclusively by prokaryotes (the bacteria and related organism) using an enzyme complex called nitrogenase. This enzyme consists of two proteins, an iron protein and a molybdenum-iron protein.

The reaction occurs when N_2 is bound to the nitrogenase enzyme complex. The iron protein is first reduced by electrons donated by ferredoxin or flavodoxin released from the plant root hairs. Then the reduced iron protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2 producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin), HN=NH is further reduced to $H_2N\text{-}NH_2$ and in turn reduced to $2NH_3$. Depending on the type of microorganism, the energy required for the reduction of ferredoxin is generated by photosynthesis, respiration or fermentation (Bezdicek and Kennedy, 1998)

2.5 Factors Affecting Biological Nitrogen Fixation

Several factors may affect nitrogen fixation in legumes. Many extensive reviews have already been reported about the factors that affect biological nitrogen fixation in legumes (Vincent, 1962;

Gibson, 1971; Sprent, 1976; Vincent, 1980). The factors can broadly be grouped into four main categories namely: I. Biological II. Environmental III. Nutritional and IV. Management

2.5.1 Biological Factors : Among the biological factors that affect nitrogen fixation include :

2.5.1.1 The Bacteria

An important pre-requisite for effective nodulation and nitrogen fixation is the presence of an effective and competitive *Rhizobium* strain in the soil (Danso and Owiredu, 1988). The survival and persistence of an adequate number of effective rhizobia in soils is essential to ensure nodulation (Dudeja and Khurana, 1989). In spite of *Rhizobium* being a natural inhabitant of the soil, many soils lack effective strains, especially those strains effective for legumes species not previously grown on a particular soil and therefore calls for the need for inoculation with effective strains in most soils (Dudeja and Khurana, 1989).

2.5.1.2 The Host Plant

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

2.5.1.3 Efficiency of Nitrogen Fixation

In a fully functioning nitrogen fixation system, energy utilization in fixing N_2, in developing new nodule and in maintaining existing nodule tissue appears to balance the energy used in taking up nitrates and reducing them to ammonia. Gibson (1971) showed that under certain conditions and with certain legumes this is true. However, considerable variation in the efficiency with which

different host/ bacteria associations utilize carbon to fix N has been shown (Gibson, 1971) and goes a long way to affect nitrogen fixation in legumes (Kang *et al*., 1984).

2.5.1.4 Longevity of Nodules

The amount of N fixed by a leguminous crop depends very much on the longevity of the nodules on its roots (Dudeja and Khurana, 1989). A number of factors affect longevity including the strains of bacteria forming the nodules, environmental effects, the physiological condition of the plant and parasites in the nodules (Dudeja and Khurana, 1989).

2.5.2 Environmental Factors

The main environmental or physical factors that affect nitrogen fixation include:

2.5.2.1 Temperature

Temperature appears to be one of the most important physical factors affecting N_2 fixation. Maximum soil temperatures in the tropics have been reported to exceed 40 ºC at 5 cm and 50 ºC at 1 cm depth (Eaglesham and Ayanaba, 1984; Lal, 1993) and limit nodulation (Day *et al.,* 1978; Graham, 1981; Eaglesham and Ayanaba, 1984; Dudeja and Khurana, 1989).

Although some *Rhizobium* strains have been reported to survive at temperatures around 70 ºC in dry soil (Marshall, 1964), excessive high temperatures in general, can kill the majority of bacteria in the surface layers of soil. Upper temperature limit for nitrogen fixation in tropical legumes vary between 27 ºC and 40 ºC (Gibson, 1971; 1975; Dart, 1974). In relation to rhizobia growth, upper limits range between 32 ºC and 47 ºC, though tolerance varies among species and strains (Gibson, 1971; Dart *et al.,* 1976; Day, *et al.,* 1978; La Favre and Eaglesham, 1986). Day *et al*. (1978) reported that, the population of rhizobia in some Nigerian soils range between 4-40 cells/g in the

surface 5 cm soil, whereas it was up to 10^4 cells/g of a soil at a depth of 20-25 cm below the surface.

High temperatures can prevent nodulation, or if nodulation does occur, can inhibit the activity of nitrogen fixation in legumes (Day *et al.,* 1978) even though root nodules may be insulated from the highest temperatures by the soil. Conversely, cool temperatures lead to delayed development of plants (Day *et al.,* 1978) including delays in the formation of nodules, and so decreased rates of N_2 fixation. Survival of rhizobia in soils at high temperatures appears to be improved by the presence of clay particles and soil organic matter, but many of the soils where temperatures are high are sandy. Eaglesham and Ayanaba (1984) demonstrated the differences in environmental adaptation to high temperatures among rhizobia isolated from different climatic zones. The root infection process is probably the component most affected by high temperatures, with sensitivity located at the nodulation sites (Barrios *et al.,* 1963; Lie, 1974; 1981). High temperatures also inhibit root hair penetration and infection thread formation (Joffe *et al*., 1961; Barrios *et al*., 1963; Dart, 1974.). Nodule initiation, rhizobial release from the infection thread, and bacteriod development are also affected by temperature (Roughley, 1970; Pankhurst and Gibson, 1973; Vincent, 1980).

Indirect effects of high temperature on the metabolism of the host plant and direct effects on nitrogen fixation have been recognized for a long time (Jones and Tisdale, 1921). However the overall balance between photosynthesis and respiration determines levels of N_2 fixation (Hungria and Vargas, 2000).

2.5.2.2 Drought

Water stress generally affects rhizobial survival and growth in soils. It also affects the formation and longevity of nodules, synthesis of leghaemoglobin and nodule function (Hungria and Vargas, 2000). Severe stress may lead to irreversible cessation of nitrogen fixation (Sprent, 1976; Vincent, 1980; Walker and Miller, 1986; Guerin *et al.,* 1991). However, rhizobial strains (Boonkerd and Weaver, 1982) and plant species (Walsh, 1995) vary in relation to drought tolerance. Dixon and Wheeler (1986) reported that, low water supply might affect N_2 fixation by lowering plant carbohydrate supply, carbohydrate transportation to the nodules, or direct impairment of nodule development and activity. Srivastava and Ambasht (1994) found that, Casuarina nodule mass increased by four-folds from the end of the dry season (June) into the wet season (July and August) in Egypt. Binkley and Giardina (1997) reported that, soil moisture variations accounted for about 80% of the variations in nitrogen fixation rates.

In general, it has been noted that, the numbers of rhizobia in soil decline drastically as soil dries. Bushby and Marshall (1977), in their comparative study of the survival of *Rhizobium* and *Bradyrhizobium* in dry soil, indicated that, *Bradyrhizobium* strains are more tolerant of dessication than strains of *Rhizobium* over short periods. Other workers found no simple relationship between the dessication tolerance of fast or slow growing rhizobia but they did find that specific strains of each survived in much greater numbers than others (Chao and Alexander, 1982). Rhizobia generally survive poorly on drying in soils which contain only small amount of clay or organic matter (Chao and Alexander, 1982). Strains which survive under greater water stress are those which retain less water within their cells (Bushby and Marshall, 1977; Al-Rashid *et al.,* 1982)

2.5.2.3 Water logging

Water – logging is also a major environmental stress affecting N_2-fixation. Whilst rhizobia are normally aerobic organisms, some strains of *Bradyrhizobium* and *R. meliloti* possess a dissmilatory nitrate reductase which can function as an electron acceptor, and thus enable rhizobia to survive under anaerobic conditions (Zablotowicz *et al.,* 1978). Because of the aerobic characteristic of rhizobia, the survival of rhizobia during long periods of flooding is of particular importance in cropping systems in which legumes are grown in rotation with rice. Toomson (1990) indicated that, the size of the population of rhizobia sampled from the field was generally larger (10^2 – 10^4 cells/g of soil) when the soil was moist or fully water logged as compared to when the soil was dried (< 10- 10^3 cells/g of soil). In contrast, large reductions in the numbers of rhizobia nodulating chickpea (which are generally fast growing rhizobia and which may not possess a dissmilatory nitrate reductase) have been reported when grown after paddy rice (Toomson *et al*., 1982)

Lack of oxygen is also a major problem for root respiration and can rapidly result in loss of nitrogenase activity (Sprent and Gallacher, 1976; Witty, 1983). Water logging can result in the rapid release into the soil of certain heavy metals, like iron and manganese, which are highly toxic to both rhizobia and plants in high concentration.

2.5.2.4 Light

The effects of light on nitrogen fixation can be ascribed to its effects on photosynthesis and to photoperiodic effects. A certain light intensity is necessary for maximum nodulation of seedlings and further nodule development of established plants. Decreased day length, effects of shading or excessive cloud cover all have adverse effects on nodulation. Gibson (1971) reported that,

transferring legumes from a low to high light intensity results in a rapid increase of both nodule size and number.

2.5.2.5 Soil acidity

Acid soils pose problems for the plant, the bacteria and the symbiosis (Giller and Wilson, 1993). Low pH is often associated with increased Al and Mn toxicity and reduced Ca supply. In addition to Al toxicity, acidity may also be related to phosphorus (P) and molybdenum (Mo) deficiencies (Hungria and Vargas, 2000). These stresses affect the growth of rhizobia (Cooper *et al.,* 1983; Coventry and Evans, 1989; Campo, 1995) of the host legume (Kim *et al.,* 1985) and symbiosis (Murphy *et al.,* 1984; 1990; Campo, 1995)

The optimal pH for rhizobial growth is considered to be between 6.0 and 7.0 (Jordan, 1985) and relatively few rhizobia grow well at pH less than 5 (Graham *et al.,* 1994). However, some rhizobia can tolerate acidity better than others, and tolerance may vary among strains within species (Vargas and Graham, 1988; Brockwell *et al.,* 1995). Acidity affects early steps in the infection process, including the exchange of molecular signals between symbiotic partners and attachment to the roots (Hungria and Vargas, 2000). Other stages of nodule establishment and function are also impacted by acidity, as is the growth of the host plant (Graham, 1981). Liming has been considered the most efficient practice in overcoming soil acidity, with some of the benefits to legume crops not only due to increased soil pH, but also to increased availability of Ca to plant, bacteria and the symbiosis. Calcium may affect rhizobial cell wall integrity (Vincent, 1962) of some enzymes (Norris *et al.,* 1959) or membrane transport systems (O`Hara *et al.,* 1989)

2.5.2.6 Salinity

Some legumes are relatively salt tolerant but high concentrations can damage the nodules indirectly by withdrawing water osmotically from nodules or in situations of high evaporation rates, nodules may be damaged as a result of deposition of salts directly on nodule and roots (Sprent, 1976). Apart from the effects of NaCl salinity, carbonates and bicarbonates of Na have been shown to affect the symbiotic process. In most reforestation programmes of salt affected areas, the approach is to select tree species that are salt tolerant than to inoculate the host plant with a microorganism that would improve the salt tolerance of the plant (Reddell *et al.,* 1986)

2.5.3 Nutritional (chemical) factors

Several of the nutrients essential for growth of plants or bacteria play specific roles in nodulation and or N_2-fixation. Thus the availability or otherwise of these nutrients as well as other nutrients essential for the growth of bacteria or plant can cause reduction in the numbers and sizes of nodules as well as the amount of N_2 fixed (Giller and Wilson, 1991). Among the mineral nutrients that affect nitrogen fixation are:

2.5.3.1 Combined nitrogen

Combined N added to the soil through fertilization can be a limiting factor that affects the symbiotic relationship between rhizobia and their partners (Gibson, 1985). It is widely accepted that, the capacity for nitrogen fixation by a nodulating legume is influenced at least in two ways by mineral nitrogen in the soil in which it is grown. Firstly, the process of nodulation may be promoted by relatively low level of available nitrate or ammonia, higher concentrations of which almost always depress it (Dixon and Wheeler, 1986). Secondly, the rate of N_2 fixation by an active, growing and well nodulated legume is always suppressed by nitrate ions (NO_3^-) (Davidson and Robson, 1986).

It was suggested that nitrogen fertilizers added to soil may delay the symbiotic process through decreased multiplication of free-living rhizobia (Dart, 1974). Recent reports indicate that, N fertilizers inhibit at least four phases of legume nodulation; (i) root hair infection (Abdel-Wahab *et al.,* 1996), (ii) nodule initiation, growth and development (Atkins *et al.,* 1984; Imsandle, 1986) (iii) as well as the level of nitrogenase activity (Sanginga *et al.,* 1989; Arreseigor, 1997) and (iv) promote premature nodule senescence (Gibson and Harper, 1985; Abaidoo *et al.,* 1990).

MacDicken (1994), reported that, nodulation is reduced or eliminated when the soils have high supplies of ammonium and nitrate. Sanginga *et al*. (1989) found N fertilization at a rate of 40-80 kg N/ha on *L. leucocephela* to reduce the nodule mass and N_2 fixation. Goi *et al*. (1993) also found that, both nodulation and growth of *Acacia auriclofomis* were stimulated by ammonium, but impaired by nitrate additions. Peoples *et al.* (1994) reported that, the 15N/ 14N ratio in *L. leucocephela* leaves increase with plantation age and Dommergues (1995) inferred that, this pattern indicated a decline in N_2 fixation as soil N supply increased. Consequently, Dommergues (1995) reported that N_2 fixation in tropical trees decline as soil N supplies increase. Ingestad (1980) on the other hand foumd that frequent and low concentration of nitrate supply increased N_2 fixation in grey alder (*Alnus incana*)

2.5.3.2 Phosphorus

Phosphorus availability is a major limiting factor for plant growth especially in tropical soils where the most common limiting nutrient in many areas is phosphorus (P). Nitrogen fixing plants have a high demand for phosphorus (Israel, 1987) and in general require larger amount of P than any other plant and this is also applicable for tropical N_2 fixing trees. The phosphorus effect is complex, acting on nodulation, nitrogen fixation and plant growth ((Israel, 1987). Phosphorus stimulates root growth and increases the number and density of nodules. It also enhances early

nitrogen fixation and the amount of N accretion per unit of nodule tissue is higher with legumes well supplied with P (Munns, 1977). However, the supply of P to trees may also depend heavily on soil pH and exchangeable aluminum (Binkley and Giardina, 1997).

Deficiencies in nutrients like P essential for the growth of not only plant but bacteria can cause reduction in the numbers and size of nodules as well as the amount of N_2 fixed (Giller and Wilson, 1991). For instance, Beck and Munns (1984) indicated that, there is marked variation in the ability of rhizobia to grow in low concentrations of phosphorus which appears to be due to variation in the efficiency of phosphorus uptake systems (Smart *et al.,* 1984). Strains of *Rhizobium* also differ in their ability to store phosphorus, but even in the most efficient case, the amount of phosphorus stored can only support growth for 3-4 generations after removal of all phosphorus from the medium (Beck and Munns, 1984). Beck and Munns (1984) indicated that, in low concentrations of phosphorus, high concentrations of calcium are required for rhizobial growth. It has been suggested that, as nodulated plants often have less well-developed root systems than unnodulated plants, the ability of nodulated plants to capture nutrients, particularly P is decreased (Cassman *et al.,* 1981). Most legumes therefore depend heavily on mycorrhizae for efficient uptake of phosphorus (Hayman, 1986)

Mycorrhizas assist particularly in the uptake of phosphorus and other nutrients which are not mobile in the soil by increasing the volume of soil effectively explored by the plant. The degree of dependence on mycorrhiza for capture and uptake of phosphorus is partly determined by the root geometry of the legume; legumes with poorly-branched root systems with few root hairs e.g. *Leucaena leucocephela* (Munns and Mosse, 1980) and *Centrosema pubescens* (Crush, 1974) tend to be more dependent on mycorrhizas than those plants with many long root hairs e.g. *Lotus pedunculatus* (Crush, 1974). Many researchers have indicated that nodulation and growth of

legumes growing on phosphorus deficient soil are often stimulated by mycorrhizal inoculation (Arias *et al.,* 1991).

2.5.3.3 Other nutrients

Under adequate conditions of P, potassium stimulates infection and N fixation, but high levels of K are inhibiting if P levels are low. According to Vincent (1962), a higher K requirement is found in older established pastures but again no direct role has been demonstrated for the symbiotic state though it is thought that K may stimulate nodule activity by improving carbohydrate supplies.

Similarly, S deficiency affects nodulation by reducing nodule number, size and N fixation (Munns, 1977). It has been shown that, S deficiency leads to a failure of protein synthesis whether nitrogen is available to the plant symbiotically or in a combined form (Vincent, 1962). A number of disorders due to deficiency of trace elements have been shown to affect the growth of legumes (Cooper *et al*., 1983) including Ca, Mg, Mn, Al, etc. (Brady *et al.,* 1990; Campo, 1995)

2.5.4 Management factors

Management practices are those designed to improve plant growth in general through improved soil structure, irrigation, soil fertility, weed control and plant disease control. These factors increase nitrogen fixation through the improved growth of the host legume.

2.6 Nitrogen fixing trees

Nitrogen fixing trees fall into two main groups, the nodulated tree legumes and nodulated actinorhizal trees. The family leguminosae is divided into three sub-families, the papilonoideae (pea-like flowers), the mimosoideae (compound inflorescence with reduced petals) and the caesalpinoideae (flowers usually with five petals, apparently radially symmetrical) (Polhill *et al.,* 1981).

Within the leguminosae, the majority of the trees used for agroforestry are nodulated members of the papilionoideae and Mimosoideae, but there are few species in the Caesalpinodeae many of which do not form nodules but which have been used experimentally in agroforestry (Giller and Wilson, 1991). Detailed information in many more species have been given by Allen and Allen (1981). Within the leguminoseae, the Mimosoideae and the Papilionoideae are thought to have evolved from Caesalpinodeae, the latter being primitive and less nodulated compared to the other two groups (Polhill *et al.,* 1981)

2.7 Some nodulated tree legumes used in agroforestry

The nodulated tree legumes used in agroforestry are mainly members of the sub-families Mimosoideae and Papilionoideae which are within the family Fabeaceae. They include:

2.7.1 *Acacia spp*. (Mimosoideae)

The genus *Acacia* is one of the largest and most diverse genera in the legume family containing about 1200 species and distributed throughout the tropics and sub-tropics (Brewbaker, 1985; Binkley and Giardina, 1997). Most species are generally resistant to drought, due at least in part, to having very deep root systems. Species of *Acacia* noted for their or actual use in agroforestry include, *A. senegal*, *A. mearnsii*, *A. aneura*, *A. auricloformis*, *A. holoserica*, *A. mangium* and *A. tortillis.*

Several acacia species are important economically. For instance *A. senegal* native to the Sudan region in Africa yields true gum arabic (Brewbaker, 1985), a substance used in adhesives, pharmaceuticals, inks, confections and other products. The bark of most acacias is rich in tannin which is used in tanning and in dyes, ink, pharmaceuticals among others.

In Bangladesh, *A. auricloformis* and *A. mangium* introduced in early 1980`s are getting priority in plantation programmes. *A. auricloformis* grows in a wide range of deep and shallow soils including sand dunes, clay, limestone and lateritic soils. An increment in height of 2- 4 m per year in the first few years is common even on soils of low fertility (Boland, 1989). A mean annual increment of 15-20 m^3/ha is obtainable on relatively fertile soils, but on less fertile soils, the increment is reduced to 8-12 m^3/ha (Wiersum and Ramian, 1982). The recommended rotation for *A. auricloformis* is 4-5 years for fuelwood, 8-10 years for pulp and 12-15 years for timber productions (Domingo, 1983).

Domingo (1983) reported that, *A. auricloformis* produces profuse bundles of nodules and can survive on land very low in nitrogen and organic matter. In Indonesia and Philipines, *A. auricloformis* has been successfully planted on steep and unstable slopes for erosion control (NAS, 1980).

Acacia mangium is extensively used in many Asian countries for the conversion of degraded *Imperata* grasslands to productive forests (NAS, 1979). *A. mangium* annual increments in plantations typically range from 20-40 m^3/ha and up to 50 m^3/ha on the best sites (Binkley and Giardina, 1997).

Acacia trees have been reported to host nitrogen fixing bradyrhizobia. Galiana *et al.* (1990) reported that, *A. mangium* and *A. auricloformis* nodulated effectively with *Bradyrhizobium* strains but formed ineffective nodules with *Rhizobium* strains.

2.7.2 *Albizia spp.* (Mimosoideae)

About 100 species comprise the genus of *Albizia* and *Paraserianthese*, but only a few of them such as: *Albizia procera*, *A. lebbek*, *A. chinensis*, have been extensively used in tropical plantations (MacDicken, 1994). Albizias are important forage, timber and medicinal plants and many are cultivated as ornamentals for their attractive flowers (Lowry, *et al.,* 1994).

Albizias are nodulated effectively by both *Rhizobium* and *Bradyrhizobium strains* (Turk and Keyser, 1992). *Albizia lebbek* is a moderate to large deciduous tree that reaches 30 m in height in rain forest of India, Bangladesh and Myanmar and is naturalized in many other lands under sub-tropical moist and wet forest where rainfall is between 1000-5000 mm/year. The native area of Albizia is South and Southeast Asia (Nielsen, 1979). The species is used for timber, fuel wood, charcoal, folder or shade in plantation and agroforestry programmes. The species is considered a promising source of pulp for high quality paper (Parrotta, 1992), traditional medicines and also vegetables (Hensleigh and Holaway, 1988). *A. procera* is nodulated by *Rhizobium* and thrives on fertile soils. Phosphorus application improves nodulation and nitrogen fixation, particularly on infertile soils.

Albizia saman (*Samanea saman*) is a huge umbrella-shaped canopy of shady leaves. It is sometimes referred to as rain tree and a well known ornamental throughout the tropical and sub-tropical world (F.A.O., 1987). It produces pods with an edible pulp, and its wood is excellent for boats, furniture and crafts.

2.7.3 *Leucaena spp.* (Mimosoideae)

Leucaena leucocephela is the most widely used agroforestry species because of its fast growth and large biomass production, deep rooting and high nitrogen fixing ability (NAS, 1977; Anon,

1984a; F.A.O. 1986). It is one of the most popularnitrogen fixing tree in the tropics, often hailed as a miracle tree (NAS, 1977; Parrota, 1992). The annual stem wood increments range from 10-60 m^3/ha/year (Domingo, 1983).

Growth of *Leucaena* has been improved by rhizobial inoculation into some soils in Australia (Diatloff, 1973), in Colombia (Halliday and Somasegaran, 1983), in Africa (Savory and Thomas, 1977; Sanginga *et al.,* 1986), Malaysia (Chee *et al.,* 1989) and Thailand (Homchan *et al*., 1989a) though in several cases indigenous rhizobia are present which can nodulate leucaena (Bushby, 1982; Homchan *et al.,* 1989a). *Leucaena* is nodulated by fast-growing rhizobia isolated from several tropical hosts but generally not by most temperate *Rhizobium* species or by slow growing rhizobia (Lewin *et al*., 1987). *Leucaena* is reported to grow and establish poorly on acid soils in the tropics (Halliday,1981). Aluminum toxicity and phosphorus deficiency have been identified as the main factors inhibiting *Leucaena* growth in acid soils (Duguma *et al*., 1988; Shelton and Brewbaker, 1994) although improvement of acidity tolerance has been achieved by crossing with acid tolerant accessions of *L. diversifolia* (Hutton, 1990).

2.7.4 *Calliandra spp.* (Mimosoideae)

This genus has over 100 species which are mainly shrubs and trees and occurs in tropical and warm weather temperate regions. *Calliandra* leaf meal is used as supplement for poultry (Domingo, 1983). It is also used as alley-cropping systems as a nurse tree for partially shade tolerant timber trees and as an effective understorey in coconut plantations with about 60% light transmission (F.A.O., 1987). Fast growth, excellent coppicing ability and ability to tolerate infertile soils make it an excellent fuel wood tree (F.A.O., 1987). The high nitrogen fixation ability and heavy litter production by *Calliandra* enables it to improve soil productivity so rapidly that farmers in Indonesia (Java) often rotate or intercrop agricultural crops with *Calliandra*

plantations (Domingo, 1983). *Calliandra* nodulates readily with indigenous rhizobia in soils in Java and is nodulated by both fast and slow growing strains (Giller and Wilson, 1991)

2.7.5 *Prosopsis* (Mimosoideae)

All the 44 species of this genus are found in semi-arid and arid regions throughout the tropics (Anon, 1979). Most of the species are of potential use in agroforestry because of their ability to withstand extreme drought, and the fact that their pods are important animal fodder in many regions (Felker, 1979). *Prosopsis* species is nodulated by fast and slow-growing rhizobia (Jenkinson *et al.,* 1987)

2.7.6 *Gliricidia spp.* (Papilionoideae)

The widely known species of this genus of agricultural importance is *Gliricidia sepium*. The genus *Gliricidia* is indigenous to tropical America (Simons and Stewart, 1994), where the small trees are commonly found at high altitudes of 2,000m. *Gliricidia sepium* has been used as shade tree for coffee and cocoa (Giller and Wilson, 1991). The leaves of *Gliricidia* are toxic to horses but can provide high quality feed for cattle and goats (Giller and Wilson, 1991). The dense wood is an excellent firewood.

Gliricidia sepium can form nodules with both fast and slow growing rhizobia but was found to fix N_2 most effectively with fast growing strains originally isolated from *Gliciridia* nodules (Akkasaeng *et al*., 1986). The species was found successful in an international provenance trial in Bangladesh (Hossain *et al.,* 1996)

2.7.7 *Sesbania* (Papilionoideae)

The over 60 species of this genus are distributed throughout the tropics with representatives in all continents (Evans and Rotar, 1987; Evans, 1990) and vary from annual to short-lived perennials. Several species including *S. bispinosa*, *S. cannabina*, *S. seban*, *S. gradiflora*, *S. speciosa*, and *S. rostrata* have traditionally been investigated for use in agroforestry. *Sesbania gradiflora* is more widely distributed in Southeast Asia, and is used mainly in agroforestry systems (Domingo, 1983). Flowers and fruits of *Sesbania gradiflora* are eaten as a vegetable in Indonesia (F.A.O, 1987; Giller and Wilson, 1991). An important advantage of *Sesbania* is that, many species are tolerant of water logging, saline and alkaline conditions (Giller and Wilson, 1991). The stem nodulating *S. rostrata* has excited intense interest due to its ability to grow and fix N_2 in water – logged conditions and its fast growth rate.

Rhizobia nodulating roots of *Sesbania spp*. are generally fast-growing strains, and often exhibit rapid growth, produce large (74 mm) colonies in less than 24 hours, although a few slow growing isolates have been recorded (Odee, 1990).

2.8 The role of trees in forestry and agroforestry programmes

Alley cropping, also referred to as hedgerow inter-cropping (Nair, 1990) entails managing rows of woody plants (hedgerows) with annual crops planted in the alleys between the hedgerows. The most distinctive component of an agroforestry practice is the multipurpose trees and shrubs (MPTS) (Nair, 1990). Multipurpose trees are all woody perennials that are purposefully grown to provide more than one significant contribution to the production and/or service functions of a land use system. They are also classified according to the attributes of the plant species as well as the plant's functional role in agroforestry practice under consideration (Nair, 1990) Soil improvements under alley cropping may result from the service function as well as the protective

role of MPTS as influenced by management and environmental factors. Nair (1990) has enumerated some points which support the soil improving capabilities of trees and shrubs under natural ecosystems. Even though there are reports of investigations conducted by Kang *et al.* (1981), Sanchez (1987), Juo *et al.* (1994) and Avery *et al.* (1990) on alley cropping's role in soil improvement, the results are quite inconclusive. However, Nair (1990) expresses the hope that, given the substantial volume of scientific information on the soil improving attributes of trees, it is a matter of management to realize the significant contribution that tree based systems like alley cropping can make to soil fertility and overall soil productivity. However some properties are offered by Young (1989) and Kang *et al*. (1981) as guidelines. These properties include high biomass production (below and above ground), nitrogen fixation and mycorrhizal association, a well-developed rooting system and rapid growth and ability to coppice. The other properties are fast or moderate rate of litter decay to suit either nutrient content in the biomass and absence of toxic substances in foliage or root exudates.

Young (1989) has provided a list of 32 genera and 55 species which have been identified to be beneficial for the maintenance or improvement of soil fertility from several sources. Included in the list are species such as *Albizia lebbek*, *Gliricidia sepium*, *Leucaena leucocephela*, *Cajanus cajan*. *Dactyladenia barterii*, *Alchornea cardiflora*, among others, which have been used in hedgerow intercropping trials in the tropics. The possible contributions of the biological nitrogen fixing species among the list should be cherished, especially in most tropical countries where inorganic nitrogen fertilizers are expensive.

Amara (1987) has provided a list of potential trees for use in agroforestry systems. Of importance are: *Albizia lebbek*, *S. siamea)*, *E. cyclocarpum*, *G. sepium* and *L. leucocephela*. There is a wide range of nitrogen fixing plants that have been used in forestry with the objective of raising soil

nitrogen levels and subsequently improving the growth of the non-nitrogen fixing forest species (Turvey and Smethurst, 1983). Still research on nitrogen fixing forest trees in general has lagged behind that on food, feed and forage crops. Cleveland *et al*. (1999) found that, for tropical evergreen forests, estimates of N fixation are extremely rare and highly variable. Some shrub and tree species form a symbiosis with *Frankia*, a nitrogen- fixing actinomycete that only recently has been cultured in vitro. The other nitrogen fixing trees are legumes and form symbiosis with *Rhizobia*. The major research need to quantify nitrogen fixation by trees is still at the methodological stage, though this is very important for developing countries.

A wide variety of nitrogen fixing trees is available for use in plantations, but only few species have received much attention for silviculture and management (Giller and Wilson, 1991; Binkley and Giardina, 1997). Allen and Allen (1981) well covered the many uses of legume trees. Moreover, there are some reports regarding the ecological and silvicultural aspects of biological nitrogen fixation of tropical tree species (MacDicken, 1994) and temperate species of *Alnus rubra* (Hibbs *et al*., 1994)

2.9 Nodulation status of tropical woody legumes

Leguminosae or Rabeaceae consist of about 650 genera and 18,000 species and is considered as the third largest family of flowering plants (Polhill *et al.,* 1981). Although taxonomists are not entirely in agreement, Sprent (1995) followed the classification of Polhill *et al*. (1981), which divides the legumes into three sub-families namely: Caesalpinoideae, Mimosoideae and Papilionoideae. The Caesalpinoideae which consists of about 1900 species are mainly woody and tropical whilst Mimosoideae which also consists of about 2713 mainly woody species which extend into sub-tropical and temperate regions. The Papillionoideae which is considered the largest sub-family consists of about 13,000 species and is largely herbaceous, but about 4000-

5000 species are woody and out of these most are tropical (Sutherland and Sprent, 1993). The nodulation status, nodule structure and nodule nitrogen fixing activity of wild legumes are very important characteristics which should be determined when evaluating the contribution of these legumes to soil fertility. Of the family as a whole, about 57% of the genera and 20% of the species have been examined for nodulation (de Faria *et al*., 1989); most of those which have not been examined are of tropical origin. Within the three sub-families, nodule morphology and anatomy are generally consistent within tribes (Corby, 1981; Sprent *et al.,* 1989; de Faria *et al.,* 1989). Allen and Allen (1981) published a classic book on legumes summarizing not only what was known about nodulation at that time, but also the history and the uses of the various genera. Since then the most systematic search to new nodulating genera and species has taken place in Brazil (de Faria *et al*., 1989; Moreira de Souza *et al.,* 1992). The fundamental position that, nodulation is least common in Caesalpinodeae, but most common in Papillionoideae with Mimosoideae being intermediate remains unchanged. The nodulation status of the sub-families will be considered in detail.

2.9.1 Caesalpinoideae

Within the Caesalpinodeae, nodulation is rare for over 23% of the species examined but consistent within genera groups (Sutherland and Sprent, 1993). Of the eight confirmed nodulating genera within this sub-family, seven are found in the tribe Caesalpinieae. This tribe contains 47 genera, 19 of which are of unknown nodulation status with the other 28 belonging to the type genus Cesalpinia, apparently non-nodulating (de Faria *et al*., 1989). Ecological data on nodulation are very rare. Barrios and Herrera (1993) reported abundant nodules on *Campsiandra laurifolia* in seasonally-flooded areas of the Orinoco basin. Nodules were perennial and had active nitrogenase with the typical structure of other Caesalpinoid nodules (de Faria *et al*., 1989), in which bacteria

are retained in infection threads throughout the nitrogen-fixing period. The other nodulating Caesalpinoid genus is *Chamaecrista*, in the tribe Cassieae. All the nodulated members of the genus *Cassia* listed in Allen and Allen (1981) have been re-assigned to *Chamaecrista*, using the usual taxonomic criteria such as flower characters.

2.9.2 Mimosoideae

Nodulation in this sub-family is by far more common than in the Caesalpinoideae. Over 90% of the species examined can nodulate. Several related groups in the tribe Mimoseae appear not to nodulate (de Faria *et al*., 1989).

By far the largest genus (1200 species) in the Mimisoideae is *Acacia*. At present several sub-genera are recognized (Ross, 1981; Vassal, 1981). While nodulation is very common, there are several well-authenticated non-nodulating species including the American *A. greggii* and African *A. brevispica* (Eskew and Ting, 1978)

Nodules examined in the Mimosoideae are all indeterminate, with distinct apical meristems, their central tissue contains a mixture of infected and uninfected cells. Infection by rhizobia may be via a classical root hair pathway (e.g. Prosopsis) (Barid *et al*., 1985) by direct epidermal entry (eg. *Mimosa scabrella* (de Faria *et al.,* 1988) or via sites of emergence of lateral roots [e.g *Neptunia plena* (James *et al.,* 1992)]. However, in the latter, infection threads are formed shortly after infection, unlike the situation in wound infection of Papillionoid species.

2.9.3 Papilionoideae

In this sub-family, 97% of the species examined can nodulate. In the tribe Swartzieae, only the type genus has been found to nodulate. Of the 11 remaining genera, 6 have been examined and no nodules found (de Faria *et al*., 1989; Moreira de Souza *et al.,* 1992). Barnet (1988) summarized

the reports of nodulation among Australia native legumes. In all 52 genera are listed, 49 from the Papilionoideae, three from Mimosoideae (*Acacia, Albizia, Neptunia*) and none from the Caesalpinodeae. Although Norris (1959) reported nodules on *Intsia bijuga* from Queensland, this and other species of the Caesalpinoid genus *Intsia* have been reported as non-nodulators (Allen and Allen, 1981)

2.10 Some methods for estimating nitrogen fixation in trees

An accurate method of measuring N_2 fixation is essential for any evaluation of the usefulness of different methods for measuring N_2 fixation, yet such a method has remained remarkably elusive (Danso *et al.,* 1992). Despite active research on this subject since the discovery of N_2-fixation, many measurements of the amount of N_2-fixed in the field remain little better than informed guesses. Difficulties encountered in measuring N_2-fixation in grain and pasture legumes are magnified enormously when we consider trees. Of all the N_2-fixing symbiosis, trees are the most problematic for measurement of the amount of N_2-fixed, hence most research has concentrated on annual herbaceous plants than trees. Danso *et al.* (1992) identified two major reasons for this situation;

a. The perennial nature of trees / shrubs,
b. The relatively large size of trees.

Despite the problems associated with nitrogen fixation measurements, several methods for estimating N_2-fixation in plants are available although, all have some degrees of error. Most of these methods have been reviewed by various researchers (e.g. Danso, 1985; Knowles, 1981; Rennie and Rennie, 1983). The methods that have been commonly used for estimating nitrogen fixation in trees are;

1. The total N difference method (TND)
2. Acetylene reduction assay (ARA)
3. ^{15}N isotope dilution method (ID)

2.10.1 Total nitrogen difference method (TND)

This method is the most primitive and simplest of all the methods (Danso *et al*., 1992). It involves quantification of total nitrogen in both the fixing plant and the reference crop plant (Danso *et al.,* 1986). The technique operates upon the assumption that two genotypically similar plants of the same isoline that differ only in the ability to nodulate and fix nitrogen should absorb the same amount of soil N whether or not the nitrogen fixing species is actively fixing under uniform environmental conditions. Thus by analyzing for the total N in the two plants, the amount of fixed N_2 could simply be estimated as the difference between the two (Danso and Herridge, 1987).

i.e.: TN (fi) – TN (nfi) = Ndfa

Where TN (fi) = Total nitrogen in the N_2-fixing isoline

TN (nfi) = Total nitrogen in the non-fixing isoline

Ndfa = Amount of N_2-derived from the atmosphere (fixed N_2)

One major problem with this technique is the difficulty in getting a non-fixing isoline for the reference. Indications are that non-fixing isolines of N_2-fixing trees seem not to exist (Danso and Herridge, 1987). Hence, N_2-fixing trees in non-fixing mode (e.g. uninoculated, inoculated with killed or ineffective rhizobia in soils devoid of homologous *Rhizobium* strains) have frequently been used as nitrogen fixing tree (NFT) references (Gauthier *et al.,* 1985).

In some cases however, unrelated species have been used as NFT controls (Pareek *et al*., 1990) though it has been suggested that this could introduce undesirable error in the N determination and consequently in the accuracy of the BNF estimates (Danso, 1985; Rennie and Rennie, 1983). Furthermore, as a limitation to the assumption upon which the technique operates, it is probable that the fixing and non-fixing reference plants may be taking up different amounts of soil N, particularly in N rich soils, which could be a major source of error (Danso 1985; Rennie and Rennie, 1983). To minimize this error source therefore, it is recommended that soils with marginal N content should be used when applying the TND method (Danso *et al*., 1986). Thus serious problems or errors occur when this method is employed under conditions thought to be free of mineral N but which are in fact contaminated with N. For example, vermiculite was used as an N-free growth medium for the study of associative N_2-fixation (Rennie, 1984), but later research showed that significant quantities of mineral N can be released from vermiculite when it is incubated under warm moist conditions (Giller *et al*., 1986).

Another limitation to this method is the fact that, the method becomes progressively inefficient and impractical as trees mature. Apart from the difficulty in the recovery of all parts of the mature plant for analysis, including roots and litter, it is also difficult to account for the re-cycled N (Danso *et al*., 1992). According to Danso *et al.* (1992), this might be one reason for the observed gross difference between the TND approach and other techniques in BNF estimation.

Despite the above limitations to the TND approach, the method has the advantage of giving a measure of the total amount of N fixed over the length of the experiment and is indispensable for many laboratory based studies (Giller and Wilson, 1991).

2.10.2 Acetylene reduction assay (ARA)

This method has been used widely for estimating BNF in leguminous trees (Danso *et al.,* 1992). In this method, the amount of N_2 fixed is measured indirectly by analysing for the amount of ethylene produced (nitrogenase activity/ acetylene reduction activity) by detached nodules or whole plants in a gas-tight container filled with acetylene gas over a given period of time.

Based on the theoretical electron flow relationship it was established that 3 moles of acetylene reduced are equivalent to 1 mole of N_2 reduced or fixed, and therefore Hardy *et al*. (1973) proposed that, the amount of acetylene reduced to ethylene be multiplied by a conversion factor of 3 to get the amount of reduced N.

There are however, some serious limitations associated with this technique;

a. The conversion factor of 3 does not conveniently apply in all cases (Hansen and Pate, 1987). For example, Turner and Gibson (1980) cautioned that environment and other plant effects acting independently on the nitrogenase reduction of acetylene and nitrogen can sufficiently alter the 3:1 ratio.
b. Also the technique is said to be instantaneous and may not truly reflect BNF over a long duration (Fried *et al.,* 1983).

Application of acetylene reduction assays for measurement of N_2-fixation in soils is complicated by the effect of acetylene on other microbial processes. For example, acetylene has been found to block the last step of denitrification (Balderstone *et al*., 1976) and also affects autotrophic nitrification (Heynes and Knowles, 1978). In addition, acetylene blocks bacterial oxidation of ethylene in soil so that `endogenous' ethylene accumulates (de Bont, 1976; Nohrstedt, 1976). Thus, control treatment used to estimate background concentrations of ethylene in soil in which

ethylene accumulation is measured in the absence of acetylene, greatly underestimate the accumulation of endogenous ethylene which occurs in the absence of acetylene. Witty (1979) demonstrated this clearly by using ^{14}C labeled acetylene for ARA measurements of N_2-fixation in soil cores. Only half of the ethylene which accumulated was from the labeled ^{14}C and the remaining unlabelled ethylene must have come from the soil.

Other problems, in the application of acetylene reduction assays for measurement of low rates of nitrogenase activity in soil result from the differences in solubility and rates of diffusion of acetylene and ethylene in water (van Berkum and Bohlool, 1980).

2.10.3 The N^{15} isotope technique

This method holds promise as an effective and straight forward approach for measuring nitrogen fixation in trees. It is based on the differential dilution of the $^{15}N/\ ^{14}N$ isotope ratios in NFTs with varying N_2-fixing potentials (Fried and Middelboe, 1977). Nitrogen in the atmosphere is virtually all $^{14}N_2$ (99.633%) plus a small amount (0.367%) of the natural $^{15}N_2$. Any substance which has an atom % ^{15}N greater than that of the atmosphere (0.367%) is said to be enriched with ^{15}N and the ^{15}N enrichment is expressed as atom %^{15}N excess. Similarly, a material depleted in ^{15}N has an atom %^{15}N below that of the atmosphere and is said to be a ^{15}N depleted material. If a plant is grown under conditions where its sole source of N is fertilizer, which is entirely composed of ^{15}N (100 atoms % ^{15}N), then all the N in the plant (apart from the amount that is originally present in the bacterial inoculums) will be ^{15}N. If the plant is able to fix dinitrogen ($^{14}N_2$) from the atmosphere then the plant will have an atom %^{15}N which is less than that of the fertilizer (i.e., less than 100 atom %^{15} N). This difference can be used to calculate the proportion of N derived from N_2-fixation and is the underlying principle behind the isotope dilution method for measurement of N_2-fixation (Giller and Wilson, 1991)

Fried and Middelboe (1977) established the following working formula for the calculation of %N dfa in fixing plants.

$\%Ndfa = 1 - \frac{\%Ndff(fixer)}{\%Ndff\ (non\ fixer)} \times 100$Boakye, rewrite the formula. What you have is not correct.

Where;

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

% Ndff (fixer) = percent N derived from the ^{15}N labeled fertilizer by the fixing plant.

%Ndff (Non-fixer) = percent N derived from the ^{15}N labeled fertilizer by the non-fixing plant.

Some basic assumptions using this technique include:

i. That the ^{15}N fertilizer enrichment is even with depth in the soils under text
ii. That the N-fixing and the non-fixing reference plant have similar N-uptake patterns (Witty, 1983).
iii. That the ^{15}N soil enrichments are the same under both the N_2-fixing and non-fixing plant.

2.11 Factors influencing N-15 estimates of BNF in trees

The measurement of biological nitrogen fixation (BNF) in plants is a crucial step toward efforts at increasing the contribution of atmospheric nitrogen to plant nutrition and soil fertility. Although, there are many methods for measuring BNF in plants, none is perfect in terms of measuring N-fixed accurately. The advantages and limitations of each method have been discussed in several reviews (Danso, 1985; Knowles, 1981). However, of the several methods available, the ^{15}N methodologies are the most reliable for measuring N_2 fixed in plants and hence, are often the

methods of choice (Duhoux and Dommerques, 1985; Hardarson *et al.,* 1984b; Legg and Sloger, 1975; Rennie, 1982; 1984; Sanginga *et al*., 1985; West and Wedin, 1985). These ^{15}N methodologies are particularly useful as they can at a single harvest measure the integrated amounts of N_2 assimilated by both greenhouse and field-grown plants in addition to measuring the N contributed from soil or fertilizer sources (Danso, 1985; Fried *et al.,* 1983; Vose and Victoria, 1986) There is therefore an increased use in the ^{15}N methodologies for measuring N_2 fixed in various crops and cropping systems (Ledgard *et al.,* 1985; Weaver, 1987). The increased use of ^{15}N techniques in most N_2-fixation measurements has revealed some practical problems. As suggested by Vose and Victoria (1986), a major problem with the use of ^{15}N techniques has been a general lack of understanding of some of the underlying concepts. It is therefore likely that with better understanding of the concepts, and adequate precautions to reduce some of the avoidable errors, many of the limitations associated with the ^{15}N techniques could be reduced. N_2-fixation measurements in trees are more problematic than for annual legumes, and Danso *et al.* (1992) have suggested some reasons for it. Among the factors likely to affect and limit the use of ^{15}N methodologies in measuring BNF in trees include the following:

2.11.1 Selection of appropriate reference plant

The selection of appropriate reference plant for a particular N_2 fixing tree appears to be the greatest problem encountered using the ^{15}N methodology. Fried *et al.* (1983) suggested the criteria for selecting reference crops for estimating the amounts and proportions of N_2 fixed by the ^{15}N methodology. It is important that, serious attempts are made to ensure that, the reference plant is a non-N_2 fixer, with similar N uptake pattern as the nitrogen fixing tree (NFT), and that, both are obtaining their N from a similar soil horizon, However, the higher the N_2-fixed, the less stringent are the requirements for all the criteria to be met rigidly (Hardarson *et al.,* 1988).

According to Danso *et al.* (1986) and Philips *et al*. (1986), the need for the precise quantification of N_2 fixation may not be compelling in many agronomic trials, e.g., in comparing some treatment effects, or ranking varieties of *Rhizobium* strains for N_2 fixing effectiveness. To achieve such objectives, a reference crop is often not necessary, as the results can be obtained using the relative ^{15}N enrichments of the different trees; the lower the ^{15}N enrichment, the higher the N_2 fixation.

The problem of selecting a suitable reference crop is more serious in trees than in seasonal crops, as the ^{15}N uptake patterns need to be matched over the different seasons and not over one or only a few seasons. Where the ^{15}N is added once, only at the beginning, it is likely that because the $^{15}N/\ ^{14}N$ ratio in soil declines less rapidly with time (Pareek *et al.,* 1990), the errors due to mismatched reference and fixing plants are likely to become smaller with time (Fried *et al.,* 1983; Witty, 1983). Sanginga *et al*. (1990) and Pareek *et al*. (1990) have emphasized on the criteria for the selection of reference plants for NFTs. The results of Sanginga *et al*. (1990) clearly show that, highly erroneous values can be obtained unless efforts are made to select suitable reference crops.

2.11.2 ^{15}N labeling

The problem associated with the ^{15}N labeling include knowledge on what N rate to apply to the fixing and reference plants, the time and frequency of application. Although for most N_2 fixation studies, ^{15}N labeled fertilizers have been used mainly as tracers, the N they contain can affect growth and N_2fixation and hence the N rate to be used in fixation studies must not be overlooked. In many studies, N rates may have been used without prior knowledge of how they would affect N_2 fixation under the local conditions. For the use of the ^{15}N techniques to estimate N_2 fixation, a zero ^{15}N fertilizer rate which would have been ideal is only possible with the ^{15}N natural abundance method. As a rule, the ^{15}N applied must be high enough to ensure satisfactory ^{15}N

detection in plants, without reducing N_2 fixation. Low N rates will therefore necessitate the use of highly enriched ^{15}N fertilizers (West and Wedin, 1985). However, cost consideration may not always make this approach the best (Rennie, 1986; Danso *et al*., 1987). Increasing the quantity of ^{15}N fertilizer (if it does not significantly inhibit N_2 fixation) with a corresponding decrease in ^{15}N enrichment is thus economically more advisable (Danso *et al.,* 1988). It is sometimes advisable to apply high versus low rates of N to the reference and fixing crops respectively, to estimate N_2 fixed. This approach overcomes the limitations of poor growth of the reference crop when soil N is inadequate, while avoiding the suppression of N_2 fixation by high inorganic N (Fried and Broeshart, 1975).

2.11.3 Application rates

A single application of ^{15}N labeled fertilizer satisfactory for N_2 fixation measurements over more than one season, for instance, in perennial crops, may be very expensive and or inhibitory to N_2 fixation. Besides, the greatest problem with this approach, even with annuals is the sharp decline in $^{15}N/\ ^{14}N$ ratio of soil N, often necessitating the rigorous selection of reference crop. To overcome these problems, equal-sized, multiple additions of small amounts of ^{15}N fertilizer during various times of the growth cycle were first used by Vallis *et al*. (1967) to estimate N_2 fixed. Since then, several studies using this approach (Boddey *et al.,* 1983; Danso *et al.,* 1988; Vallis *et al.,* 1967) have been reported. Boddey *et al*. (1983), found that frequent additions of small doses of ^{15}N fertilizer ensured high ^{15}N fertilizer enrichments in soil, and resulted in small declines in $^{15}N/\ ^{14}N$ ratio, compared to the single application. Frequent ^{15}N additions may therefore assist in minimizing errors due to mismatch between reference and fixing crop. Rennie (1985; 1986) has, however, criticized the use of repeated ^{15}N additions on plots. However, the arguments and evidence he adduced in support of his claim appear unconvincing.

2.11.4 Sampling of plant material

The ^{15}N enrichments in various plant parts (e.g. seed, herbage, crowns and roots) frequently differ (Fried *et al.,* 1983; Jensen, 1986; Ladd, 1981; Rennie *et al.,* 1978). Measurements of nitrogen fixed based on only one plant part e.g. seed or leaf (Ruschel *et al*., 1982) may therefore not adequately represent N_2 fixed in the whole plant (Ladd, 1981; Philips *et al*., 1983). While he sampling of whole plant material would not pose much problem in the case of annuals, it may be extremely difficult in trees, because of the usually massive sizes of trees, which makes it difficult to completely sample the whole tree. There is also the difficulty in harvesting roots which in the case of NFTs may contain more than half of the N in the whole plant (Sanginga *et al.,* 1990b). Significant N reserves occur in the roots of trees, and in NFTs, and N reserves may account for a significant proportion of the N used for regrowth. Domenach and Kurdali (1989) reported that, the reserves of N in *Alnus glutinosa* accounted for 10% of the N in leaves at the end of the growing period.

Part of the sampling problem is also due to the heterogeneity in ^{15}N enrichments in different plant parts (Sanginga *et al*., 1990b; Shearer *et al.,* 1983). Leaves generally give estimates of % Ndfa closest to that for the whole tree (Sanginga *et al*., 1990b), and thus where % Ndfa is the most desired estimate, it seems appropriate to sample the leaves if the whole tree cannot be harvested.

Nitrogen cycling from litter decomposition influences the ^{15}N enrichment in the rhizosphere (Jordan, 1985). With trees, litter fall and decomposition can be significant, and can consequently cause large changes in the $^{15}N/\ ^{14}N$ ratios of soil under the fixing and non-fixing crops. With the ^{15}N enrichment in a fixing crop being usually lower than that in a reference crop, the expected net

effect would be a lower $^{15}N/ ^{14}N$ ratio in soil under the fixing crop, and therefore a higher than real estimate of N_2 fixed.

2.12 Rhizobia

Rhizobia are usually defined as nitrogen-fixing soil bacteria capable of inducing the formation of root or stem nodules on leguminous plants in which atmospheric nitrogen is reduced to ammonia for the benefit of the plant. The family leguminosae (fabaceae) comprises about 650 genera and 18,000 species, and includes short-lived annual herbs and woody perennials that are distributed over a wide range of ecological conditions (Doyle, 1994). Many of its members are of considerable agricultural and ecological importance, generally reflecting the beneficial symbiotic association with rhizobia. Currently, there are 44 recognized species of nodule-forming bacteria on legumes, within 12 genera, 10 of which belong to the class Alphaproteobacteria (*Allorhizobium, Azorhizobium, Blastobacter, Bradyrhizobium, Devosia, Ensifer, Mesorhizobium, Methylobacterium, Rhizobium and Sinorhizobium*), and 2 to the *'Betaproteobacteria'* (*Burkholderia and Ralstonia*) (Sawada *et al*., 2003). In the last few years, many studies investigating rhizobia isolated from tree legumes in Kenya and Sudan have revealed considerable phenotypic and genotypic diversity among strains, and several distinct groups have been identified and novel species described (Zhang *et al*., 1991; Odee *et al*., 1997, 2002; Nick *et al.,* 1999; Mclnroy *et al*., 1999). Exploration of new biogeographical regions and the investigation of legumes that have not been checked for nodulation not only helps to uncover unknown rhizobia, but also support research efforts aimed at selecting effective combinations of rhizobium-legume genotype to exploit the enormous potential of increased nitrogen fixation.

2.12.1 Taxonomy and diversity of tree legume *Rhizobium*

In the last few years, many studies have investigated rhizobia associated with legume trees (De Lajudie *et al.,* 1994; Lindstrom *et al.,* 1983; Zhang *et al*., 1991) and various techniques have been used to identify and describe strains. These studies concluded that, there is a large heterogeneity among the strains and accordingly, they were often divided into several groups (Batzli *et al.,* 1992; Crow *et al*., 1981; De Lajudie *et al.,* 1994; Dupuy *et al.,* 1994; Jarvis, 1983; Moreira *et al.,* 1998; Zhang *et al.,* 1991). The low similarity among these groups shows that trees can form nodules and fix nitrogen with several different clusters of rhizobia (Crow *et al.,* 1981; Jarvis, 1983; Lindstrom *et al.,* 1983; Padmonabhan *et al.,* 1990).

Early *Rhizobium* taxonomy used has mainly been based on the nodulation host range (Fred *et al.,* 1932), although overlapping host ranges have already been reported several years ago (Wilson, 1944). Over time, rhizobia have been found to be diverse, both in their symbiotic and physiological properties. Fast-growing and slow growing strains were described and Jordan (1984) proposed a new genus, *Bradyrhizobium*, consisting of slow growing strains.

During the last years, the assessment of diversity within rhizobial natural populations in various regions of the world has received increased attention (Amann *et al*., 1995; Batzli *et al.,* 1992; Brunel *et al*., 1996; Cartwright *et al.,* 1995). Many attempts have been made to determine the actual composition and characteristics of indigenous strains isolated from different cultivated legumes (Chen *et al*., 1991; Crow *et al.,* 1981) and also from less explored plants that have important roles in certain ecosystems (Felsenstein *et al.,* 1985). The development of numerous molecular genetic methods has greatly contributed to these investigations. The availability of several sensitive and accurate PCR- based fingerprinting methods (Galtier *et al*., 1996; Gibson, 1980; Gurtler, 1993) has enabled the differentiation among closely related bacterial strains and

the detection of a higher rhizobial diversity than previously considered (Gurtler *et al.,* 1996; Jarvis, 1983; Jarvis *et al.,* 1982). Consequently, the taxonomy of root and stem nodulating bacteria has been deeply changed in the recent years.

In Ghana, with the exception of Fening (1999) who studied the diversity of cowpea rhizobia in some soils of Ghana there is little or no knowledge on the nature and diversity of rhizobia that nodulate other legumes especially tree legumes.

2.12.2 Some methods for analysing rhizobial diversity

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

2.12.2.1. Cross Inoculation Concept

The cross inoculating group concept is based on the ability of *Rhizobium* strains to specifically nodulate a group of legume host species (Fred *et al.,* 1932). On the basis of this concept, rhizobial strains have long been described as being specific when they are restricted in their host range or promiscuous when they have a very broad host range (Burton, 1972). However, close examination of a wide range of legume species has shown that, many legumes are nodulated by rhizobia outside their own groups (Thies *et al.,* 1991). Promiscuities of some rhizobial strains have also been found to be so broad that it goes beyond closely related legumes, to include legumes that are so distantly related as to be placed in different sub-families within the family leguminosae (Pueppke and Broughton, 1999). A typical example of this being the fast growing *rhizobium* strain NGR 234 which has been shown to nodulate over 110 genera of legumes including members of different sub-families such as *Lablab purpureus* and *Leucaena leucocephela* (Trinick, 1980 and Pueppke and Broughton, 1999). These findings have made many

scientists to question the integrity of the cross inoculation concept as a system for determining relatedness among rhizobia strains (Wilson, 1944; Bromfield and Barran, 1990) and has put the concept in general disrepute. Also the cross inoculation concept like other earlier methods for characterizing *Rhizobium* strains pays no attention to nitrogen fixation abilities. It is thus common to find strains of rhizobia that can form nodules on say 10 different legume host species and yet in association with perhaps five of those host plants fix nitrogen only weakly or not at all (Wilson, *et al.,* 1987). Due to these setbacks associated with the cross inoculation concept, its use in the classification of rhizobia has reduced. According to Graham *et al.* (1991), the continued use of this method by some scientists can only be justified on the basis of convenience and agronomic significance. The concept actually has some practical use for selecting rhizobial strains which have the potential to be used as inoculants for particular legume crops (Mpepereki *et al*., 1990).

2.12.2.2 Cultural and metabolic methods

Cultural and metabolic characteristics have long been described as useful guides for the recognition of rhizobial groups at the species level (Vincent, 1970). Rhizobial strains may be recognized as such by a combination of a large number of traits such as growth characteristics, carbon source utilization, stimulation by sugars or vitamins, limits of pH, temperature tolerance and production of hydrogen sulphide (Vincent, 1970). This variety of tests by which the root nodule bacteria could be characterized was suggested by Lange (1961), as a way to resolving the taxonomic difficulties within the genus *Rhizobium*. Graham and Parker (1964) however, considered the above – mentioned methods as impracticable. Despite this criticism, cultural and metabolic parameters are still used for phenotypic characterization and are frequently carried out in combination with an analysis of the genotype.

A central dogma in rhizobiology is that only two different types of rhizobia exist. The slow – growing strains which are said to largely nodulate tropical legumes and the fast growing strains that nodulate predominantly temperate legume (Jordan, 1984). Evidence has however, now accumulated through a variety of cultural and physiological tests (Broughton and Dilworth, 1971; Lim and Ng, 1977, 1979; Pankhurst, 1977; Keyser *et al*., 1982; Lawrie, 1983; Padmonabhan *et al*., 1990; Mpepereki *et al.,* 1997), which show that some tropical legumes are nodulated by both fast and slow growing rhizobial strains. The results of these reports point to the possible existence of several unique but unidentified *Rhizobium* species that nodulate tropical legumes.

With the exception of work done by Fening (1999) on the cultural and physiological characteristics of the indigenous rhizobial populations of some Ghanaian soils no data exist on the cultural and physiological characteristics of the indigenous rhizobial populations of Ghanaian soils. However, knowledge about the cultural and physiological characteristics of the native rhizobia may serve as guide to species identification (Vincent, 1970)

It also provides information about the performance of rhizobial strains under stress conditions, which gives additional criteria for selection of rhizobial strains for inoculant production.

2.12.2.3 Serological method

The use of serology in studying rhizobial diversity is based on the principle of antigen- antibody reaction. Thus, when an animal is injected with an antigen (composed of basic proteins or polysaccharides) from a particular rhizobial strain, the animal reacts by producing antibodies. Because an antibody is produced to react with a specific antigen, it is used to identify that particular protein-containing strain when linked with enzymes or dyes that make the reaction visible (Beck *et al.,* 1993). The specificity that exists between a strain's antigen and antibody

makes serology a convenient means of investigating rhizobia in ecological studies (Beck *et al.,* 1993).

The most common serological methods currently used are agglutination (Means *et al*., 1964; Wollum, 1987) fluorescent antibody techniques (Bohlool, 1987) and various forms of the enzyme-linked immunosorbent assay (ELISA) (Asanuma *et al.,* 1985; Ayanaba *et al*., 1986; Kishinevsky and Jones, 1987). Serological studies of indigenous rhizobia have revealed considerable diversity within and among geographic locations. In some instances it has been possible to correlate the presence of particular serogroups within a restricted region to soil properties such as pH (Ham, *et al.,* 1971) or total nitrogen content (Bezdicek, 1972).

Apart from using serology to study rhizobial diversity, the practical importance of serology is to identify groups that have practical importance to the management of symbiosis. Although many studies have documented serological diversity within rhizobial populations, relatively few have assessed the value of the resulting groupings in predicting symbiotic performance. One major problem with using serology to characterize rhizobia is the presence of strains that do not react with all antisera tested and the frequency of non-reactive strains is often significant (Mpepereki and Wollum, 1990). Instances of cross-reaction of strains with antiserum derived from reference strains are also common. The best documented example of this is the suite of soybean bradyrhizobia that constitute serocluster 123 (Serogroups 123, 127 and 129) (Schmidt *et al*., 1986). Although related serologically, the serogroups comprising this serocluster are known to exhibit physiological and symbiotic diversity (Hickey *et al.,* 1987; Sadowsky *et al*., 1987)

2.12.2.4 Antibiotic resistance method

Ecological studies of micro-organisms in their natural habitat require the recovery of either the resident population or added cell on a suitable growth medium capable of excluding other contaminating organisms. The lack of a suitable medium that allows the selective, recovery of rhizobia in soil has hindered studies on the behavior of rhizobia in soil (Danso and Alexander, 1974). Rhizobia like other bacteria contain small numbers of naturally occurring mutants that are resistant to high concentrations of certain antibiotics. The growth of antibiotic or toxin resistant mutants in media containing elevated levels of anti-microbial agents has been used for recognizing rhizobial strains in ecological studies (Danso *et al.,* 1973; Alexander, 1977; Brockwell *et al*., 1977; Bushby, 1984) and other bacteria (Nelson and Neal, 1974). Streptomycin (stre), Spectinomycin (spec), and Kanamycin (K) are some of the antibiotics normally used. Repeated culturing on YMA plates containing these antibiotics marks target rhizobial strains. Resistant strains usually retain their N_2 fixing capabilities. Antibiotic-resistant marked strains of rhizobia may be identified by their ability to grow on media containing the antibiotics to which they are resistant, while non-marked ones are unable to grow. The antibiotic marker technique is applied in ecological studies where strain identification is not possible by serology due to cross-reaction of strains or because of unavailability of antisera. Antibiotic markers also provide useful confirmatory data. The popularity of the technique is due partly to ease with which the mutants, in particular those resistant to streptomycin have been obtained (Danso *et al.,* 1973; Alexander, 1977).

2.12.2.5 Symbiotic effectiveness

Population size of indigenous rhizobia and their diversity alone do not provide information on their symbiotic effectiveness. It is therefore important to know to what extent native rhizobial

strains that nodulate a particular legume are effective since low rate of nitrogen fixation can occur even with normal nodulation (Eaglesham, 1985). There exist significant differences in effectiveness of indigenous rhizobial strains within and between soils (Singleton and Tavares, 1986). Differences in effectiveness may be due partly to differences in the sizes and structure of indigenous rhizobial populations (Singleton and Tavares, 1986; Thies *et al*., 1991)

Screening 23 cowpea rhizobia isolates in two field sites in Nigeria, Ahmad *et al.,* (1981) found that only 30% of the isolates were effective. Ferreira and Marques (1992) found great variation in effectiveness among 170 strains isolated from native clover, with a predominance of strains with medium and high effectiveness. Fredericks *et al.* (1990) reported similar findings with clover isolates obtained from Ethiopian soils. Contrary to these results, Gibson *et al*. (1975) and Lie and Goktan (1984) observed only small differences in effectiveness among *R. trifolii* populations from eight regions in Australia and *Rhizobium* population nodulating pea in Europe respectively. Cultivar differences in effectiveness of rhizobia on legume species exist. Robinson (1960) found that rhizobia isolated from *Trifolium pratense* were ineffective on *T. subterraneum*. Ferreira and Marques (1992) reported similar findings.

Many scientists have attributed differences in effectiveness of rhizobia to environmental factors. Holding and King (1963) found that, the mean effectiveness of *R. trifolii* correlated significantly with the base saturation, pH, as well as the exchangeable calcium and magnesium contents of the soil. Hagedom (1978) reported differences in response of rhizobia to phosphorus to be responsible for differences in effectiveness within *R. trifolii* populations. His results however contradicted those of Ferreira and Marques (1992) who showed that soil type and plant origin did not influence general effectiveness of natural populations of *R. leguminosarum* by *trifolii*.

Rhizobia are well adapted to live in the free living state in soil and can survive for over 30 years even in the complete absence of legumes (Martesson and Witter, 1990). However, with time, genomic changes may occur (Weaver and Wright, 1987; Gibson *et al*., 1975). Gibson *et al*. (1990) observed that the symbiotic effectiveness of *R. leguminosarum* bv *trifolii* re-isolated from the field after several years differed from the parent culture. The *Rhizobium* genome carries a high number of repeated sequences (Flores *et al.,* 1988; Martinez-Romero, 1994; Brom *et al.,* 1991), which allow recombination or rearrangements, and deletions (Hahn and Hennecke, 1987), which makes it easier for it to cope with environmental changes.

2.12.2.6 Molecular characterization methods

Recent molecular approaches that analyse the bacterial genome have renewed interest in bacterial systematics and taxonomy, and have broadened the knowledge and understanding that man has on microbes (Martinez-Romero, 1994). Restriction fragment length polymorphism (RFLP) analysis of DNA, DNA-DNA or DNA-RNA hybridization, DNA sequencing as well as other approaches like the electrophoretic analysis of metabolic enzymes and numerical taxonomy have proven valuable in bacterial taxonomy and systematic (Selander *et al*., 1986; Schleifer and Stackebrandt, 1983). Both the conservation of ribosomal RNA due to its constraints in ribosomes, and the existence of variability in some domains render ribosomal RNA gene sequences (5S, 16S and 23S) a very good choice to compare organisms and infer phylogenies (Woese, 1987). In other words, the ribosomal RNA is highly suitable for phylogenetic studies as it is constant in its function, present in all bacteria and contains highly conserved as well as more variable regions (Woese, 1987; Schleifer and Ludwig, 1989). Therefore, the 16S rRNA gene sequences have become an indispensable parameter in *Rhizobium* taxonomy (Young and Haukka, 1996) but also restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA genes

has been proven to be a valuable tool for *Rhizobium* species identification (Laguerre *et al.,* 1994). Nevertheless, the conservative nature of 16S rRNA genes limits its use for discrimination at the strain level. The intergenic spacer (ITS) between the 16S rRNA and the 23S rRNA genes was described to be more variable (Massol-Deya *et al*., 1995) and RFLP of the PCR-amplified ITS was used for the characterization of *Rhizobium* strains. The above characterizations of rhizobia in terms of their phylogeny and diversity have been made possible by the development of the polymerase chain reaction (PCR).

2.12.3. The polymerase chain reaction (PCR)

The polymerase chain reaction was developed over two decades ago (Mullis and Faloona, 1987) PCR is basically a biochemical amplification process where even a single target DNA segment can be amplified a million-fold or more in hours (Mullis and Faloona, 1987). The main feature of PCR is that if the sequences of DNA flanking an unknown region of a DNA can selectively be copied repeatedly to generate large quantities of DNA copies for further analysis (Wilson and Walker, 1995). Analysis of the amplification product is usually done by standard agarose or polyacrylamide gel electrophoresis.

A standard PCR protocol employs DNA primers, usually, 10-20 bases in length, which have been synthesized complimentary to specific known segments of the target DNA. The primers are complimentary to opposite strands of the target DNA. The primers are added to the test sample in a buffered solution containing a balanced mix of the four deoxynucleotides (DATP, DCTP, DGTP and DTTP), magnesium chloride and a heat resistant DNA polymerase (Taq, Amplifaq, Vent etc). A thermocycler is used to provide a strict regime of temperature cycling, and each cycle consists of a denaturation, an annealing and extension steps. For denaturation, the temperature is raised to 94ºC - 95ºC in order to melt or separate the two strands comprising the

target DNA and subsequently lowered to 30ºC - 55ºC to allow the primers to anneal with the corresponding sequence of the template (Innis and Gelfand, 1990). The annealing temperature depends on the melting point of the primer-template complex. Finally, a temperature of usually 72^{o}C is used for the synthesis of the new DNA molecule. A typical single PCR process consists of 30 - 35 cycles. The utilization of PCR in conjunction with either arbitrary or directed primers to differentiate individual strains of *Rhizobium* has proved to be highly useful for ecological studies.

Richardson *et al.* (1995) summarized the potential applications of PCR-based methods in rhizobiology and include: I. The authentication of specific inoculants strains, II.The determination of nodule occupancy in competition trials between native and inoculants strain where mixed inoculants are used, III. The analysis of the recovery and persistence of inoculants strains under field conditions, IV. The identification of predominant nodulating strains from particular sites and V. the assessment of the genetic diversity and relatedness of field populations of rhizobia.

The PCR technique has several distinct advantages over more conventional techniques that have been used for *Rhizobium* strain identification. These advantages comprise a limited requirement to extensively purify and culture large amounts of rhizobial isolates prior to their identification, the precision with which individual strains can be identified, the rapidity of the procedure and the large number of isolates that may be handled at one time (Richardson *et al*., 1995). More importantly, the procedure does not necessarily require a detailed prior knowledge of the individual strains, and there is no need to specifically mark target strains. Therefore, PCR-based fingerprinting techniques allow useful information pertaining to any particular *Rhizobium* strain or isolate, or the rhizobial population itself, to be readily obtained (Richardson *et al*., 1995). However, one main limitation of this methodology is that its huge amplication capacity makes the system very vulnerable to errors from contamination; even a trace of foreign DNA, such as may

be present in dust particles, will be amplified to significant levels and may give misleading results (Wilson and Walker, 1995). Also at low population densities, PCR signals are difficult to interpret, because free DNA can also be amplified. The application is therefore mostly qualitative although attempts are being made to quantify the PCR signals (Simonet *et al.,* 1990; Wimpec *et al.,* 1991)

The development of the PCR has led to a battery of new fingerprinting methods. For instance, it has been shown that DNA primers corresponding to repetitive extragenic palindromic (REP) (Stern *et al.,* 1984) and enterobacterial repetitive intergenic consensus (ERIC) (Hulton *et al*. 1991) sequences, coupled with the polymerase chain reaction technique, can be used to fingerprint the genomes of a variety of gram-negative soil bacteria (Versalovic *et al.,* 1991; Sessitsch *et al*., 1997). The REP and ERIC sequences contain highly conserved central inverted repeats that do not show significant homology to each other, and are normally found in intergenic transcribed but not translated regions (Versalovic *et al.,* 1991; Lupski *et al.,* 1992) REP and ERIC PCR have been demonstrated to be powerful tools for community analysis at the strain level (Judd *et al*., 1993; Laguerre *et al.,* 1996; Sessitsch., 1997).

In addition, PCR based methods using short oligonucleotide primers, that bind to random sequences of the genome, have proven to be a valuable means to generate strain-specific fingerprints of Rhizobium (Dooley *et. al*.,1993; Kay *et al.,* 1994; Selenska-Pobell *et al*., 1995). Based on a reiterated *Rhizobium* nif promoter consensus element, Richardson *et al*. (1995) developed the RPOI-directed primer that also has been demonstrated to be suitable to fingerprint rhizobial genomes (Richardson *et al.,* 1995; Sessitsch *et al.,* 1997)

Various methods employ PCR-amplified DNA as a substrate for a restriction analysis leading to a specific restriction fragment length polymorphism (RFLP). Nitrogen fixation (nif) and nodulation (nod) genes have been analysed for genetic characterization of rhizobial strains (Laguerre *et al.,* 1996). Most of these, however, studied the ribosomal operon most frequently using PCR-RFLP analysis (Nour *et. al*., 1994; Massol-Deya *et al*., 1995; Sessitsch *et al*., 1997). The sequence of the 16S rRNA gene, has been found to be a highly suitable phylogenetic marker (Woese, 1987; Schleifer and Ludwig, 1989) since genetic variation within this molecule gives useful information on microbial taxonomy. RFLP analysis of PCR-amplified 16S rRNA genes has therefore been used frequently for *Rhizobium* species identification (Laguerre *et al.,* 1994; Sessitsch *et al.,* 1997) Greater discrimination can be obtained by analysis of the intergenic spacer (ITS) between the 16S and the 23S rRNA genes and has been applied to examine chromosomal encoded genetic variations at the strain level (Sessitsch *et al*., 1997)

Characterization of rhizobial isolates indigenous to tropical soil using leguminous trees as the host plant has not been exploited especially in Ghana. However, the realization that genetic characterization underlies the diverse expressions of life, led to the use of the PCR-based methods in this study in order to enable a holistic understanding of the relationship among leguminous tree rhizobia.

2.13 Rhizobial population density in soils

Although the usual source of rhizobia inocula is the soil, great variations occur in the population size from soil to soil and within a soil from site to site. The major determinants for the presence of rhizobia are, the presence of appropriate host (Woomer *et al.,* 1988), soil acidity (Damirgri, 1967), seasonal changes (Rupela *et al.,* 1987) and soil texture, (Woomer *et al.,* 1988). Also of

significance is predation by protozoa and antagonism by antibacterial metabolites, (Danso *et al.,* 1974; Roughley, 1985).

Analysis of 305 soil samples from 17 countries by the international centre for Nitrogen Fixation by Tropical Legumes (NIFTAL), indicated that while rhizobia of the cowpea group are common at a large number of sites, their populations are extremely variable in size and ranged from zero to >1000 cells g^{-1} soil (Peoples *et al.,* 1995). More than 50% of the soils according to the authors had fewer than 100 rhizobia g^{-1} soil. The authors concluded that, it would be erroneous to assume that tropical soils have sufficient numbers of resident rhizobia to meet legume demand for N. Populations of cowpea rhizobia in African soils are not better. In Zimbabwe, Mpepereki *et al.,* (1997) counted between zero and 100 cells of cowpea rhizobia per gram soil. Similar population size has been reported for cowpea and groundnut in Egypt (Moawad *et al.,* 1994). Population size of indigenous rhizobia determines success of inoculation. According to Thies *et al*. (1991) the response to inoculation and competitive success of inoculants rhizobia is inversely related to numbers of indigenous rhizobia. The minimum level for response to inoculation however is debatable. While Thies *et al*. (1991) pegged the response level at indigenous soil rhizobial population below 50 cells g^{-1} soil; Singleton and Tavares (1986) pegged the response level below 20 cells g^{-1} soil. It is however, common to observe negative response in the presence of high population densities and high nodulation. Since high specificity exists between *Rhizobium* strains and their host plants, for successful nodulation and fixation, it is essential that the two be compatible. Evaluation of rhizobial inoculants and the development of improved inoculation methods are effective strategies to improve legume productivity and N_2 fixation (Zapata and Baert, 1989; Bromfield and Ayanaba, 1980; Danso, 1988)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Location of the study area

The pot experiments were carried out in small nursery bags (15 x 30 cm) at the screen house of Ecological Laboratory and the laboratory analysis at the Biotechnoly Laboratory, University of Ghana, Legon.

3.2 Soils used and site characteristics

Three soil types taken from the Accra plains were used for the study. The soils belong to the Toje, Hatso and Alajo series (Brammer, 1967). Toje series is classified as Rhodic lixisol and Hatso and Alajo series classified as Haplic lixisol and Calcic vertisol respectively according to F.A.O., (2006). The three soils occur on the same soil catena, Toje series being at the top, Hatso and Alajo series being at the middle and bottom slopes, respectively.

Toje soil consists of more than 75 cm of red sandy loam to light clay, may overlie red ironstone-concretionary clay or iron pan at depth or indirectly overlie ferruginized weathered rock, mainly Togo quartzite schist (Brammer, 1967). The soils are well drained and absorb water readily except when the surface is left bare.

Hatso soil consists of several feet of pale brown sand increasing to sandy clay with depth, humus-stained near the surface and slightly mottled orange in the subsoil, but occasionally found with seepage iron pan at a depth exceeding 60 cm (Brammer, 1967). The soil “packs” on exposure and the ground-surface becomes hard and impermeable. The soil has little retentive capacity and dries out rather deeply during the main dry season.

Alajo series consists of soil developed from alluvium in a marine terrace, is grey-brown, heavy plastic swelling clays containing lime concretions in the subsoil (Brammer, 1967). The soil occurs on valley flats along a tributary of the Odaw stream. The three soil series occur under dry climatic conditions with mean annual rainfall of about 1100 mm which is distributed bi-modally with the long raining season peak in May/ June and short season in September/October. The mean annual temperature is about 31ºC. The vegetation is dominated by grasses.

3.2.1 Soil sampling and preparation

The soil samples were taken as cores from a depth of 0-15 cm after clearing the vegetation cover. The soils were air-dried, pulverized and sieved through a 2 mm diameter sieve.

3.2.2 Soil analysis

The soils were analysed for the following parameters; pH (in water and in $CaCl_2$), total phosphorus, available phosphorus, CEC, organic carbon and total nitrogen.

3.2.2.1 Soil pH

Soil pH was determined in both distilled water and in 0.01M $CaCl_2$. Soil pH was measured in 1:1 soil : water and 1:2 soil : $CaCl_2$ solutions using pH meter. Ten (10) grammes of sieved soil was weighed into a beaker and 10 ml of distilled water or 20 ml of 0.01M $CaCl_2$ solution added. The mixture was vigorously stirred continuously for 30 minutes after which it was left to stand for about 1 hour by which time most of the clay particles had settled and also to allow the mixture to attain room temperature. The glass electrode – pH meter was standardized using two aqueous solutions of pH 4 and 7 and then later used to measure the pH of the prepared soil sample by carefully immersing the glass electrodes in the supernatant. The samples were replicated four times for each soil sample and the average pH recorded.

3.2.2.2 Total phosphorus

Five (5) grammes of sieved soil sample was weighed into 25 ml conical flask. Ten (10) milliliters of concentrated HNO_3 (aq) was added, followed by 15 ml of 60% $HClO_4$. The contents of the flasks were digested until the solution became clear and the dense white fumes of $HClO_4$ had ceased. The digests were then cooled, diluted with distilled water and filtered into 250 ml flasks. The filtrates were then made up to a volume of 250 ml and 5 ml aliquot taken for phosphorus content determination using the molybdate ascorbic acid method of Watanabe and Olsen (1965) involving calorimetry.

The formula below was used to calculate the phosphorus content.

$$P\ (ppm) = \frac{(R - B)}{W \times Va} \times Ve$$

Where: R= spectrometer reading of sample

B= spectrometer reading of standard

Ve = Volume of extractant

W = weight of soil

Va = volume of aliquot

3.2.2.3. Available phosphorus

Five (5) grammes of each sieved soil sample was weighed into an extraction bottle followed by addition of 30 ml of the extractant (0.3 HN_4F + 0.1 HCl). The bottles were then shaken thoroughly for 2 minutes on a mechanical shaker. The soil suspensions were then filtered and 5 ml aliquots of each filtrate taken for available phosphorus analysis. The phosphorus determination was done calorimetrically using the Watanabe and Olsen (1965) modified technique.

3.2.2.4 Total nitrogen

Ten (10) grammes of each sieved soil samples was weighed into a 300 ml Kjedahl flask and moistened with distilled water. Tablets of selenium were added into the content of each flask, followed by 20 mls of concentrated H_2SO_4 (aq).

The mixture was then cooled and transferred with distilled water into 250 ml volumetric flask and made up to volume. Five (5) milliliters aliquot was taken into a Markham distillation apparatus followed by 5 ml of 40% NaOH (aq). The mixture was distilled and the distillate collected into a 50 ml flask containing 5 ml of 2% H_3BO_4(aq) to which three drops of methyl red-methyl blue indicator was added. The green solution formed was titrated against 0.01N HCl to a purplish-red end point. The percent total nitrogen was calculated using the following formula:

$$\% \text{ N } = \frac{0.01 \times 50 \times 0.014 \text{ (Titre value)}}{\text{Weight of soil} \times \text{aliquot of digest use}}$$

3.2.2.5 Cation exchange capacity (CEC)

3.2.2.6 Extraction of exchangeable bases.

Ten (10) grammes of soil was weighed into a 200 ml extraction bottle and 100 ml of 1M neutral ammonium acetate (NH_4OAc, pH 7.0) solution was added. The bottle and its content was placed on a mechanical shaker and shaken for 1 hour and centrifuged at 3000 rpm for 20 min. The supernatant solution was then filtered and the filtrate (aliquot) was used for the determination of Ca, Mg, K and Na.

3.2.2.6.1 Calcium.

To a 10 ml aliquot of the sample solution, 10 ml of 10% KOH and 1 ml 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.

$$Ca\ meq/100\ g\ soil\ = \frac{Titre\ value \times N \times Vol.of\ extract \times 100)}{\text{Aliquot} \times \text{Weight of soil}}$$

Where N = Normality of EDTA

3.2.2.6.2 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 Eriochrome 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.

$$Mg\ meq/100\ g\ soil\ = \frac{Titre\ value \times N \times Vol.of\ extract \times 100)}{\text{Aliquot} \times \text{Weight of soil}}$$

Where N = Normality of EDTA

3.2.2.6.3 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.

$$K\ meq/100\ g\ soil\ = \frac{R \times Vol.of\ extract \times 100)}{\text{Weight of soil} \times 39.1}$$

Where,

R is the flame photometer reading (ppm)

39.1 = Atomic weight of K

3.2.2.6.4 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.

$$K\ meq/100\ g\ soil\ = \frac{R \times Vol.of\ extract \times 100)}{\text{Weight of soil} \times 23}$$

Where,

R (ppm) = flame photometer reading

23 = Atomic weight of Na

3.2.2.7. Organic Carbon

The soil organic carbon content was determined using the wet combustion method of Walkley and Black (1934). Half a gram (0.05 g) of soil was weighed into a 250 ml flask and ten millilitres of 1N potassium dichromate ($K_2Cr_2O_7$) solution and 20 ml of concentrated sulphuric acid (H_2SO_4)

were added. The flask was swirled to ensure full contact of the soil with the solution after which it was allowed to stand for 30 min. Two hundred (200) millilitres of distilled water and 10 ml of orthophosphoric acid were also added. The unreduced $K_2Cr_2O_7$ 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 percent organic carbon was calculated as:

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

Where % C = Percent organic carbon

X = Titre value (mL)

N = Normality of Fe $(NH_4)_2(SO_4)_2$

W = Weight of soil sample

3.3 Plant growth media

Nursery bags were used for growing the test trees. Each nursery bag had a length of 30 cm with an interior opening of diameter 15 cm and posterior sealed at ends but with three perforated holes to allow free drainage of excess water. Each nursery bag was filled with 2 kg of soil type and placed in a plastic saucer after which the soil was kept moist at field capacity with distilled water prior to planting.

3.4 Planting materials

Seeds of 18 leguminous trees and shrubs were collected from the Botanical Gardens, University of Ghana, Legon and the Forest Research Institute of Ghana (FORIG) in Kumasi. The tree /shrub species used for the initial screening exercise included: *Bohemia spp, Sammania sammia*

(Pithecelobium spp.), Acacia albida, Senna nodusa, Leucaena leucocephela, Erythreopleum spp, Acacia auricloformis, Tetrapleura tetraptera, Senna occidentalis, Albizia zygia, Crotalaria ochloleuca, Melletia thonningi, Acacia mangium, Acacia farresiana, Albizia adianthifolia, Tamarindus indica, Albizia lebbek and Cajanus cajan.

3.5 Seed pretreatment

The seeds were initially acid-scarified using concentrated H_2SO_4 (aq). The length of time allowed for the scarification of the seeds varied from 5 minutes to 45 minutes depending on the degree of hardness of the seed coats. The seeds were then thoroughly rinsed in distilled water and later soaked overnight in distilled water. The seeds were then pre-germinated on moist cotton wool in petri dishes and seedlings with equal vigour and size were then used for the experiments.

3.6 Initial nodulation screening

The 18 leguminous trees and shrubs were initially grown with or without P (60 mg P/kg soil) in perforated black polythene bags filled with 1kg of soil type for 10 weeks after which the plants were assessed for the presence or absence of nodules. Cowpea was also grown in the three soils as a standard for 10 weeks after which *Rhizobium* isolates were taken from active nodules of the tree legumes that nodulated and that of cowpea.

3.7 Watering

Watering was done twice daily using distilled water to keep the soil always moistened. A stock solution of the under-listed nutrient elements was prepared and applied at weekly intervals at a rate of 5 ml aliquot/ pot.

H_3BO_4 – 0.5g
$MnSO_4.4H_2O$ – 0.31g
$CaCl_2.6H_2O$ – 8×10^{-4} g

3.8 Isolation of *Rhizobium*

Representative nodule samples were taken from the initial screening study. The nodules were surface sterilized with 70% alcohol for 3 minutes and then with 0.1 % mercuric chloride for another 3 minutes and rinsed with several washes in sterile distilled water (Somasegaran and Hoben, 1994). The nodules were each crushed in a drop of sterile distilled water in a petri dish with a sterile rod. A loopful of the suspension was then streaked on yeast extract mannitol (YEM) agar plates and incubated at 28 ºC. A total of 440 *Rhizobium* isolates made up of 40 *Rhizobium* isolates per plant were obtained from the 10 tree legumes that nodulated as well as 40 *Rhizobium* isolates from cowpea.

3.8.1 Authentication of *Rhizobium* isolates

All the *Rhizobium* isolates were evaluated as pure culture since they could form nodules on their respective host plants. Seeds of the cowpea and the leguminous plants were pre-germinated in petri-dish after scarification with conc. H_2SO_4 in the case of the tree legumes. The pre-germinated seeds were planted in growth pouches containing N-free nutrient solution. Seven days after planting, the growth pouches were inoculated with 1ml YEM broth culture of each isolate. Uninoculated pouches served as control. The pouches were placed on racks and kept in the greenhouse. The plants were harvested 10 weeks after planting and their roots assessed for the presence of nodules. Two hundred out of a total of 400 initial isolates were randomly selected for characterization and further studies.

3.8.2 Culture maintenance

All the isolates (440) were maintained on YEM agar slopes and stored in the refrigerator at 4ºC. All the isolates were re-streaked on YEM agar and checked for purity at least every 3 months.

3.9 Cross inoculation studies

The ability of each isolate to nodulate different leguminous plant species was assessed using the cross inoculation concept (Wilson, 1944). The following plants were used for the cross inoculation studies; *Pithecelobium spp, Leucaena leucocephela, A. auricloformis, Crotalaria ochloleuca, Melletia thonningi, Acacia mangium, Albizia lebbek, Cajanus cajan* and cowpea. Seeds of the plants were surface sterilized in 70% alcohol for 3 minutes and rinsed thoroughly in several changes of distilled water (Somasegaran and Hoben, 1994). The seeds were scarified as discussed earlier under seed pre-treatment and pre-germinated on 1% water agar until the radicles were about 2 cm long. The seedlings were planted two per petri dish.

A total of 24 isolates from each plant was randomly selected and bulked as single inoculants for the cross inoculation studies.

A suspension of each of the isolates was prepared to contain about 10^{8} cells/ ml. One milliliter of each inoculant (suspension) was then used to inoculate a growth pouch containing N-free nutrient solution (Somasegaran and Hoben, 1994). Inoculation was done close to the base of the germinating plant using a calibrated pipette. As a standard or control, each leguminous plant was also inoculated with NGR 234 isolate obtained from IITA Vienna. The pouches were randomly arranged in wooden racks and kept at the screen house behind Ecological Laboratory. The plants were whenever necessary supplied with N-free solution (Somasegaran and Hoben, 1994). The plants were assessed for nodules 10 weeks after inoculation.

3.10 Enumeration of Rhizobia

The population of rhizobia in the three soils capable of nodulating the following tree legumes; *Pithecelobium spp, Leucaena leucocephela, Acacia auricloformis, Crotalaria ochloleuca, Melletia thonningi, Acacia mangium, Albizia lebbek and Cajanus cajan* were enumerated by the

most probable number (MPN) method (Vincent, 1970) using plastic growth pouches (Weaver and Frederick, 1974). Clean seeds of the tree legumes were surface sterilized in 70% alcohol for 3 minutes and rinsed thoroughly in several changes of distilled water (Somasegaran and Hoben, 1994). The seeds were scarified and pre-germinated on 1% water agar until the radicles were about 2 cm long. Seedlings were planted two per pouch. Ten-fold dilutions of each soil sample with four replicates per dilution were used to inoculate the pouches containing N-free nutrient solution (Somasegaran and Hoben, 1994). One milliliter of soil suspension was used to inoculate each pouch. The pouches were randomly arranged in wooden racks and kept at the green house. The plants were supplied with enough N-free nutrient solution when necessary. The plants were assessed for nodules 10 weeks after planting and the most probable number of rhizobia per soil calculated (Vincent, 1970).

3.11 Phosphorus-response studies

The response of six of the tree legumes, namely, *Leucaena leucocephela, Acacia auricloformis, Melletia thonningi, Acacia mangium, Albizia lebbek and Albizia zygia* to phosphorus fertilizer was evaluated in Toje and Hatso soil series.

The experiment was conducted in nursery bags (30 cm high, 15 cm at top and bottom). Each nursery bag was filled with 2 kg of soil. The treatments were:

i. No P fertilizer

ii. P fertilizer at 30, 60, 90 and 120 kg P/ha in the form of TSP. The fertilizers were thoroughly mixed with each soil before filling into the nursery bags.

Two pre-germinated seeds were planted in each nursery bag and later thinned to one after germination.

The experiment was arranged in a randomized complete block design with four replicates. The plants were watered daily until harvest. The plants were harvested 12 weeks after planting by cutting the stem at the soil level. The plant roots were carefully washed from the soil in excess water. Nodules were then separated from the roots and counted and the shoot and root dry weights determined after drying in an oven at a temperature of 70 ºC until constant weights were achieved. Total P in the shoot was determined and the phosphorus use efficiency (PUE) calculated using the formular below:

$$\text{PUE} = \frac{\text{Shoot dry weight (g)}}{\text{Shoot P content (mg)}} \text{x} \ \ 100$$

3.12 Nitrogen response studies

The response of six of the tree legumes to nitrogen fertilizer was evaluated in two different experiments. The first experiment involved using phosphorus as a basal treatment for all the pots at a rate of 90 kg P/ha, whilst the second experiment did not include P treatment.

In the first experiment, six of the tree legumes; *Leucaena leucocephela, Acacia auricloformis, Crotalaria ochloleuca, Melletia thonningi, Acacia mangium and Cajanus cajan* were used. Each nursery bag was filled with 2 kg of soil and mixed thoroughly with 90 kg P/ha as basal treatment. The treatments were:

i. No P fertilizer and no nitrogen fertilizer control
ii. 90 kg P/ha fertilizer and no nitrogen fertilizer
iii. 90 kg P/ha fertilizer and five different levels of nitrogen fertilizers at 40, 80, 120, 180 and 240 kg N/ha.

Pre-germinated seeds of each legume tree were planted in the nursery bags. Nitrogen fertilizer in the form of urea (21%N) was applied to the nursery bags at a depth of 1cm deep. The nitrogen

fertilizer was applied one week after emergence of the tree legumes. The tree legumes were allowed to grow for 8 weeks and watered daily after which they were cut at the soil surface and the shoot dry weights determined after drying in an oven at a temperature of 70 ºC until constant weights were obtained.

The roots were carefully washed of sand and the nodule numbers determined after excising from the roots. The root dry weights were also determined after drying in an oven at 70 ºC ºC until constant weights were achieved.

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The second nitrogen response studied was carried out just like the earlier work (section 3.12) but in this case, phosphorus was not applied as basal treatment.

The treatments were:

i. No N fertilizer control

ii. Five levels of N fertilizer at 40, 80,120,180 and 240 kg N/ha.

3.13 Effectiveness studies

A total of 200 rhizobia isolates from five leguminous trees (*Leucaena leucocephela*, *Acacia mangium*, *Acacia auricloformis*, *Albizia lebbek* and *Melletia thonningi*) were assessed for their effectiveness on their respective host plants. In all, 40 isolates from each tree legume were assessed for their effectiveness.

The experiment was carried out in Leonard jars filled with acid washed and dried river sand and free of any nitrogen. The bases of the Leonard jars were filled with stock solutions devoid of nitrogen. Two pre-germinated seeds were sown into each jar and thinned to one after emergence. The plants were inoculated with their respective isolates after they had been cultured and grown in a broth medium for one week. Inoculation was done at the base of the plant using 1 ml of the

inoculum. As a control, the same experiment was set up as above but in one case inoculation was not applied but was instead supplied with nitrogen fertilizer in the form of KNO_3 at a rate of 200 µg N/ml nutrient solution. In another case, the plants were not inoculated and did not receive nitrogen fertilizer.

The experiment was allowed to run for a period of 10 weeks after which the harvested plants were cut and shoot dry weights and nitrogen contents determined using Kjedhal method. Nodule numbers and root dry weights were also determined after which the effectiveness of each rhizobia isolate determined using shoot dry matter yield. Effectiveness was calculated for each rhizobia isolate using the formular below:

$$E = \frac{Xi - Xu}{Xfert - Xu} \times 100$$

Where E = Effectiveness

Xi = Inoculated dry matter yield

Xu = Uninoculated control dry matter yield

Xfert = Dry matter yield of fertilized plant

3.14 Inoculation and N-15 studies

Three of the tree species namely, *Acacia mangium, Albizia lebbek and Acacia auricloformis* which were found to nodulate and showed good growth in the three soils and have high potential for use in forestry and agroforestry systems were used in this study. *Senna occidentalis* was used as a reference plant.

The seeds of the legume species were scarified using the procedure described in section 3.5. Four seeds were sown in each nursery bag and thinned to two after emergence. Three days after emergence, pots requiring inoculation treatment were inoculated with mixture (24 isolates) of

inoculants prepared from isolates obtained from the initial screening study. The set up was replicated four times. A week after planting, ^{15}N labeled fertilizer was applied in solution at a rate equivalent to 20 kg N/ha. The ^{15}N fertilizer was applied in four equal split doses at weekly intervals starting from a week after emergence of the plants.

Watering was done on daily basis to keep the soil moistened. A stock solution of the under-listed nutrient elements was prepared and applied at weekly interval at a rate of 5 ml aliquot per litre of distilled water.

H_3BO_4 – 0.5g

$MnSO_4.4H_2O$ – 0.31g

$CaCl_2.6H_2O$ – 8×10^{-4}g

Three sequential harvesting of the plants were made at 2, 4 and 6 months after planting.

3.15 Physiological and metabolic characterization of rhizobia isolates

Two hundred isolates from an initial 400 indigenous tree/shrub rhizobia isolates made up of twenty (20) rhizobia isolates each from the ten (10) tree legumes that nodulated in the initial nodulation screening studies from the three soils were randomly selected and used for this studies. The studies include the following:

3.15.1 Growth rate, reaction to Bromothymol blue (BTB) and colony morphology

The growth of the rhizobium was observed for 7 days in a broth culture and viable counts estimated each day by the Miles and Mistra drop count method (Collins and Lyne, 1985). A growth curve was obtained by plotting the viable count verse time.

The abilities of the isolates to change the pH of their growth medium was scored on YEM agar plates amended with 0.25 mg/l bromothymol blue. The agar plates were incubated for 7 days at 30 ºC and scored daily for change in colour. Freshly prepared YEM agar plates containing BTB

had a pH of 6.8 and were green in colour. The isolates that changed the colour of the medium to yellow were scored as acid producers and classified as fast growers. Isolates that changed the medium to blue were considered alkaline producers and classified as slow growers. Colony appearance was scored as dry where the surface was smooth and firm, and wet for those which were watery or slimy.

3.15.2 Growth of tree legume rhizobia in different pH media

The pHs of tubes containing 20 ml of YEM broth with KH_2PO_4 omitted (Zablotowicz and Focht, 1981), were adjusted before sterilization by the addition of HCl or NaOH. The tubes were inoculated with 100 µL aliquots of each isolate and incubated at 28 ºC for 7 days before scoring for growth. Growth was scored as positive if the broth changes to milky and negative if the broth remains clear. Growth was determined at pH 3.5, 4.5, 5.5 in the acid range and pH 7.5, 8.5 and 9.5 in the alkaline range.

3.15.3 Ability of tree legume rhizobia to utilize different carbon sources

The rhizobia isolates were tested for their ability to grow when provided with different carbohydrates as the sole carbon source. The test was carried out in a standard basal medium without mannitol (Zablotowicz and Focht, 1981). Each carbohydrate was added to give 10% (W/V) solution. The carbohydrates were sterilized by Millipore membrane filtration and then added to the sterilized liquefied medium just before plates were poured. Each isolate was scored in duplicate plates and growth scored after incubating for 7 days at a temperature of 28ºC. The following carbohydrate sources were tested: L-arabinose, D-glucose, D-galactose, Fructose, Lactose, Maltose, Mannitol and Sucrose.

3.16 Molecular characterization of some tree legume rhizobia isolates

A total of 60 tree legume rhizobia isolates of the 200 isolates were selected for further molecular studies. The isolates were initially characterized based on the physiological and metabolic traits as well as their effectiveness in nodulation.

3.16.1 DNA extraction

The rhizobia isolates were cultivated on YEM agar plates at 28 ^{o}C for 5-7 days. Single colonies of the isolates were picked and washed in 100 ul TE at pH 7.5 to obtain pelleted cells (Sally *et al.,* 2010). 250 ul of CTAB buffer was added to the washed pelleted cells; vortex for 30 seconds, and incubated at 65^{o}C for 15 minutes and then cooled to room temperature (Sally *et al.,* 2010). 250 ul of 24:1 chloroform: isoamyl alcohol was added to the samples and vortex until the solution was homogenous with the suspension appearing white in colour. The suspension was then centrifuge for 10 minutes at 12000 rpm using a fixed angle rotor. The aqueous phase was transferred to a new sterile 1.5 ml microcentrifuge tube and equal amounts of cold isopropanol added and mixed gently (Sally *et al.,* 2010).

The DNA was precipitated at -20^{o}C for 30 minutes and centrifuged for 10 minutes at 12,000 rpm. The DNA was re-suspended in 30 ul TE buffer at pH 7.4 and the concentration of the extracted DNA assessed at 260 nm using the Nanodrop Spectrophotometer (Sally *et al.,* 2010).

3.16.2 PCR amplication of 16S rRNA gene

The universal primers fD1 (5^{1}- AGAGTTTGATCCTGGCTCAG-3^{1}) and rD1 (5^{1}- AAGGAGGTGATCCAGCC-3^{1}) was used for PCR amplification of the 16S rRNA Weisburg *et al.,* 1991). These primers were derived from conserved regions of the 16S rRNA genes and amplified nearly full length of 16S rRNA genes (Weisburg *et al.,* 1991). Amplification reactions were performed in a total volume of 25 ul and contained the following: 1x reaction buffer (10 Mm

Tris-Hcl, 50 Mm Kcl) with 1.5 mM Magnesium chloride, 2.5 units Taq, 200 uM of each dNTPs (dATP, dCTP, dGTP, and dTTP), 5 pmol of each forward and reverse primers and 100 ng of genomic DNA. The temperature profile was as follows: Initial denaturation at 95 ^{0}C for 3 minutes; 35 cycles of denaturation at 94 ^{0}C for 1 minute, annealing at 55 ^{0}C for 1 minute, extension at 72 ^{0}C for 2 minutes and final extension at 72 ^{0}C for 3 minutes. The amplified products were kept at a temperature of 4^{0}C. All amplifications were carried out in Thermocycler (Bio-rad).

3.16.3 PCR amplification of 16S-23S intergenic spacer (ITS)

The intergenic region between the 16S and the 23S rRNAs was amplified by PCR with primers derived from the 3^1 end of the 16S rRNA (FGPS1490-72; 5^1-TGCGGCTGGATCCCCTCCTT-3^1) (Navarro *et al.,* 1992) and from the 5^1 end of the 23S rRNA (FGPL132-38; 5^1-CCGGGTTTCCCCATTCGG-3^1) (Ponsonnet *et al.,* 1994). The amplification reactions and conditions were the same as those used for 16S rRNA amplification.

3.16.4 Gel electrophoresis and imaging

The resulting amplicons (5 ul) of the 16S, and ITS genes were mixed with loading buffer (2 ul) and analysed electrophoretically through 1% agarose gel stained with ethidium bromide (10 mg/ml) at 100 volts for 1 hour. The bands were observed and photographed under UV light using BioDoc-IT imaging system.

3.16.5 Restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA and ITS genes.

Aliquots (10 ul) of the 16S and ITS PCR products were digested with restriction endonucleases as specified by the manufacturer (Bioron GmbH) with an excess of enzyme (5 U per reaction) in a total volume of 25 ul. The following enzymes were used for the 16S gene digestion: Hae III, Alu

I, HPaII, HPa I and Rsa I. The ITS genes were digested with three enzymes namely Hae III, Alu I and HPa II.

The restriction fragments were separated by horizontal electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8.0) on a 2% (wt/vol) agarose gel containing 10 mg of ethidium bromide per ml. The gels were run at 80V for 2 hrs and immediately photographed under UV light using BioDoc-IT imaging system.

CHAPTER FOUR

4.0 RESULTS

4.1 Soil properties

The characteristics of the soils used in this study are presented in Table 2. Alajo series with a clay loam texture had the highest values for all parameters measured, while Hatso series, a sandy soil was lowest in % N, organic matter and available P values with Toje series a sandy clay loam being lowest in pH and CEC values.

Table 2 Some chemical properties of the soils used

PARAMETER	SOIL TYPES		
	TOJE	**HATSO**	**ALAJO**
pHwater (1:1)	5.3	6.0	6.8
$pHCaCl_2$ (2:1)	4.9	5.2	6.4
Total N (%)	0.059	0.034	0.134
Organic Carbon (% C)	0.65	0.37	1.39
CEC (Cmol/kg)	5.84	7.40	25.20
Available P (ppm)	7.52	3.76	12.4
Texture	Sandy clay loam	Sand	Clay loam

4.2 Ability of 18 tree and shrub species to nodulate in three soil types

The result of the initial screening study to assess the capability of 18 selected leguminous multipurpose trees and shrubs to nodulate with indigenous soil rhizobia in Toje, Hatso and Alajo soils is presented in Table 3. About 55% of the tree legumes nodulated with indigenous rhizobia in all the three soils with about 45% of the legume species not nodulating in any of the three soils.

Nodulation was generally best in Hatso soil followed by Toje soil and lowest in Alajo soil. Three species, namely *Pithecelobium spp*, *Melletia thonningi* and *Albizia lebbek* formed on average the highest number of nodules per plant (>20) in each of the three soils, in contrast to *Acacia mangium, Albizia zygia and Erythreopleum spp* which nodulated poorly in the three soils (< 5 nodules/ plant) with *Acacia auricloformis, Cajanus cajan, Crotalaria ochloleuca and Leucaena leucocephela* being intermediate (10-20 nodules/ plant).

Eight species, namely *Bohemia spp, Tetrapleura tetraptera, Senna occidentalis, Senna nodusa, Acacia albida, Acacia farresiana, Albizia adianthifolia* and *Tamarindus indica* did not nodulate in any of the three soils.

Table 3 Ability of eighteen tree legumes to nodulate with indigenous rhizobia in three soil types

	SOIL TYPES		
Legume species	**TOJE**	**HATSO**	**ALAJO**
Bohemia spp	-	-	-
Pithecelobium spp	+++	++++	+++
Leucaena leucocephela	++	+++	+
Erythreopleum spp	+	++	+
Tetrapleura tetraptera	-	-	-
Crotalaria ochloleuca	++	+++	+

Table 3 continued

SOIL TYPES			
Legume species	**TOJE**	**HATSO**	**ALAJO**
Melletia thonningi	+++	++++	++
Cajanus cajan	+++	+++	++
Senna occidentalis	-	-	-
Senna nodusa	-	-	-
Acacia albida	-	-	-
Acacia auricloformis	++	+++	+
Acacia mangium	+	++	+
Acacia farresiana	-	-	-
Albizia lebbek	+++	++++	++
Albizia zygia	+	++	+
Albizia adianthifolia	-	-	-
Tamarindus indica	-	-	-

KEY:

-	no nodulation
+	poor nodulation, i.e., nodules < 5/plt
++	good nodulation, i.e., nodules > 10/plt
+++	very good nodulation i.e., nodules > 10/plt and big in size
++++	Excellent nodulation, i.e., nodules > 20/ plt and big in size

4.3 Enumeration of native rhizobia capable of nodulating eight tree legumes

Table 4 provides information on the population densities of native rhizobia capable of nodulating eight of the tree legumes in the three soil types. In support of the data reported in Table 3, native rhizobia capable of nodulating each of the selected eight legumes was found in all the three soils used for the study, with the native rhizobia population ranging from 22 cells /g soil for *Crotalaria ochloleuca* and *Acacia mangium* in Alajo soil to 5200 cells /g soil for *Pithecelobium spp* in all the three soils and for *Melletia thonningi* in Hatso and Toje soils and for *Albizia lebbek* in Hatso soil. Each soil series on the average contained more than 1000 cells /g soil for each of the selected eight tree legumes indicating that in general, the three soils harbour satisfactory levels of rhizobia capable of nodulating all the eight tree legumes. In general, the three soils could be ranked as Hatso > Toje > Alajo in terms of population of rhizobia capable of nodulating the eight selected tree legumes.

Table 4 Indigenous rhizobia population /g soil in the Toje, Hatso and Alajo soils

TREE SPECIES	RHIZOBIA POPULATION /g OF SOIL		
	TOJE	HATSO	ALAJO
Pithecelobium spp	5200	5200	5200
Leucaena leucocephela	780	1800	650
A. auricloformis	520	1800	180
Crotalaria ochloleuca	210	210	22
Melletia thonningi	5200	5200	780
Acacia mangium	180	210	22
Albizia lebbek	780	5200	1800
Cajanus cajan	520	780	180
Soil Average	1700	2600	1100

4.4 Cross inoculation studies.

Table 5 provides information on the ability of the rhizobia isolates from each of the selected tree legume to nodulate one another and cowpea as well as the ability of these legumes to be nodulated by NGR 234. The results indicate great variability among the isolates in terms of their ability to induce nodulation on a host of legume plants. Isolates obtained from *Pithecelobium spp* and *Melletia thonningi* appeared to be highly specific as each nodulated only the host from which it was isolated. Isolates from *Acacia mangium, Albizia lebbek* and *Acacia auricloformis* were slightly promiscuous as they nodulated 12% of the other legumes besides their respective host plants. In addition to their respective host plants, rhizobia isolates from *Acacia mangium* and *Albizia lebbek* isolates nodulated *Acacia auricloformis* and *Cajanus cajan* plants, respectively, whilst those from *Acacia auricloformis* nodulated *Albizia lebbek*. *Leucaena leucocephela* and *Crotalaria ochloleuca* isolates appeared to be moderately promiscuous as in addition to their respective host plants, nodulated 25% of the other legumes. In addition to their respective host legumes, isolates from *Leucaena leucocephela* nodulated *Albizia lebbek* and *Cajanus cajan*, whilst those of *Crotalaria ochloleuca* nodulated *Cowpea* and *Cajanus cajan* plants. *Cowpea* and *Cajanus cajan* isolates were also considered to be moderately promiscuous as they nodulated almost 32% of the host legumes in addition to their respective host. *Cowpea* isolates nodulated *Albizia lebbek, Cajanus cajan* and *Crotalaria ochloleuca* whilst *Cajanus cajan* isolates nodulated *Leucaena leucocephela*, *Cowpea* and *Crotalaria ochloleuca* host legumes. Isolates from *Leucaena leucocephela* and *Acacia mangium* nodulated *Albizia lebbek* and *Acacia auricloformis* respectively but isolates from *Albizia lebbek* and *Acacia auricloformis* could not nodulate *Leucaena leucocephela* and *Acacia mangium* respectively. Also isolates from cowpea nodulated *Albizia lebbek* but not the reverse. NGR which is considered to be highly promiscuous nodulated

almost 67% of the host legumes under study. It nodulated all the host legumes except *Acacia auricloformis*, *Melletia thonningi and Acacia mangium.*

Table 5 The ability of *Rhizobium* isolates from nine tree/shrub legumes to nodulate each other.

Source of *Rhizobium*	Tree / shrub legume species								
	Pithecelobium spp	***L. leucocephela***	***A. auricloforms***	***C. ochloleuca***	***M. thonningi***	***A. mangim***	***A. lebbek***	***C. cajan***	***Vigna unguiculata***
Pithecelobium spp.	+++	---	---	---		---	---	---	---
L. leucocephela	---	+++	---	---		---	+++	+++	---
A.auricloforms	---	---	+++	---		---	+++	---	---
C. ochloleuca	---	---	---	+++		---	---	+++	+++
Melletia thonningi	---	---	---	---	+++	---	---	---	---
A. mangium	---	---	+++	---	---	+++	---	---	---
A.lebbek	---	---	---	---	---	---	+++	+++	---
C. cajan	---	+++	---	+++	---	---	---	+++	+++
Vigna unguiculata	---	---	---	+++	---	---	+++	+++	+++
NGR	+++	+++	---	+++	---	---	+++	+++	+++

--- Absence of nodules

+++ presence of nodules

4.5 Classification of 200 rhizobia isolates from five multipurpose tree legumes base on their symbiotic effectiveness.

Tables 6.1, 6.2, 6.3, 6.4 and 6.5 provide detailed information on the symbiotic effectiveness of 200 rhizobia isolates on five leguminous trees in a sterile N free sand medium, while Figs. 1, 2, 3, 4, 5 and 6 provide respective summaries of data derived from the above Tables.

Most of the 40 isolates from each of the five legumes were found to be ineffective on their respective host plants with ineffectiveness ranging from almost 43% in the case of *Acacia auricloformis* (Fig. 5.3) to 88% in the case of *Acacia mangium* isolates (Fig. 5.2).

Also, on their respective host legumes, the proportions of highly effective isolates were relatively low, ranging from 5% in the case of *Acacia mangium* (Fig. 5.2) to 28% in the case of *Albizia lebbek* (Fig. 5.1)

Over 63% of the 200 isolates were found to be ineffective on the five legume trees (*Albizia lebbek, Acacia mangium, Acacia auricloformis, Melletia thonning*i and *Leucaena leucocephela*) tested, whilst almost 18% were either found to be moderately effective or effective (Fig. 5.6)

Table 6. 1 Relative comparison of 40 rhizobia isolates of *Albizia lebbek* for their influence on plant growth, number of nodules formed and dry weight of nodules.

ISOLATE NUMBER	EFFFECTIVENESS INDEX (%) BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT(mg)
1	4	0.18	2	1.6
2	22	0.38	1	1.2
3	11	0.25	2	1.1
4	4	0.18	3	1.5
5	11	0.26	3	2.1
6	30	0.37	1	1.1
7	6	0.20	2	112.4
8	1	0.14	4	2.8
9	11	0.26	6	3.1
10	101	1.28	43	103.9
11	105	1.33	32	147.8
12	1	0.14	1	2.2
13	11	0.26	1	1.6
14	107	1.35	5	153.0
15	177	2.18	39	175.0
16	14	0.29	3	1.7
17	8	0.22	1	1.0
18	147	1.84	52	257.8
19	203	2.44	96	224.8
20	8	0.22	2	2.1
21	88	1.13	3	2.4
22	21	0.37	25	2.8
23	18	0.33	18	84.7
24	23	0.39	1	1.2
25	113	1.42	29	64.6
26	18	0.33	2	4.9
27	16	0.31	5	6.2
28	27	0.44	1	1.1
29	20	0.36	7	8.5

Table 6. 1 continued

ISOLATE NUMBER	EFFFECTIVENESS INDEX (%) BY DM	DRY MATTER (g) / plant	NODULE	
			NUMBER	DRY WEIGHT(mg)
30	11	0.26	1	1.2
31	189	2.28	34	85.7
32	148	1.82	71	152.7
33	9	0.23	3	2.3
34	121	1.51	84	104.6
35	13	0.28	6	64.6
36	9	0.23	1	1.0
37	9	0.23	3	4.1
38	27	0.44	2	1.3
39	38	0.56	8	62.9
40	220	2.64	38	250.9
NO–N FERT	0	0.13	0	0
+N (200kgN/ha)	100	1.27	0	0
LSD (5%)	34.5	0.27	13.2	59.5

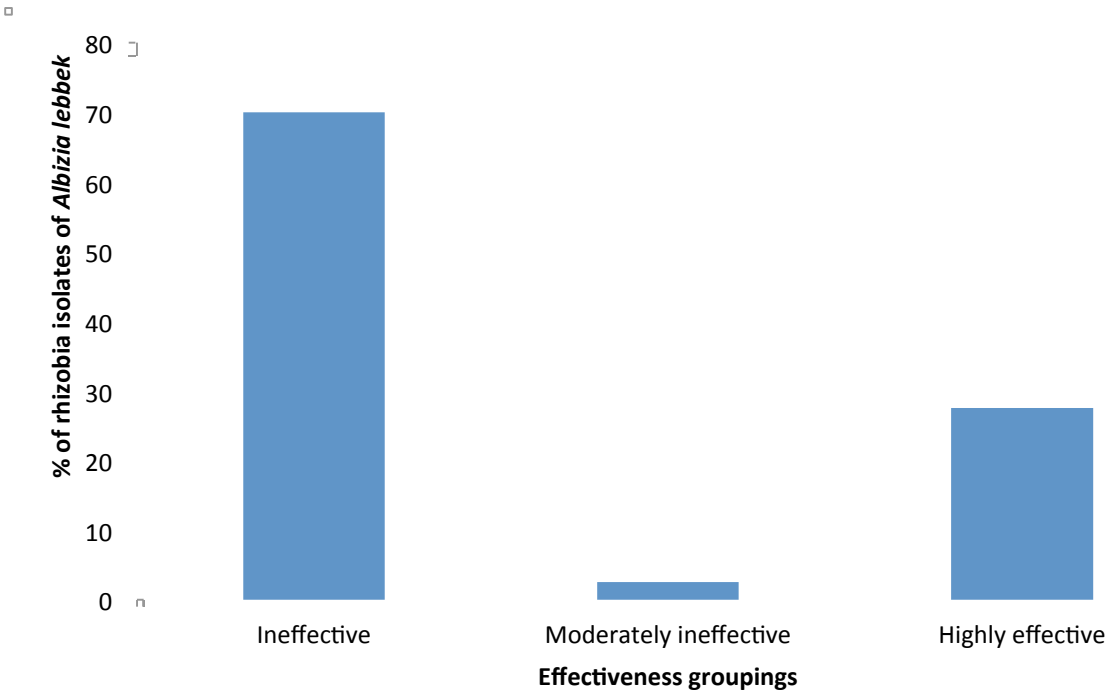

Fig. 5. 1 Classification of 40 isolates of rhizobia obtained from *Albizia lebbek* on the basis of their effectiveness in promoting growth on N-free sand medium relative to plants relying on fertilizer N.

Key

GROUP		CRITERION
Ineffective	»	Isolates with effectiveness index ≤ 50%
Moderately effective	»	Isolates with effectiveness index between 51 – 99%
Highly effective	»	Isolates with effectiveness index ≥ 100%

Table 6. 2 Relative comparison of 40 *Rhizobium* isolates of *Acacia mangium* for their influence on plant growth, number of nodules formed and dry weight of nodules.

ISOLATE NUMBER	FFFECTIVENESS INDEX(%) BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT(mg)
41	5	0.10	3	7.1
42	5	0.10	1	1.3
43	0	0.08	1	1.1
44	3	0.09	2	3.1
45	3	0.09	3	6.8
46	38	0.22	5	8.2
47	5	0.10	10	8.8
48	5	0.10	2	6.1
49	14	0.13	4	8.7
50	0	0.08	2	1.5
51	38	0.22	6	6.9
52	5	0.10	2	6.6
53	8	0.11	13	47.6
54	0	0.08	4	8.1
55	62	0.31	6	18.2
56	19	0.15	8	2.5
57	11	0.12	4	12.3
58	27	0.18	11	16.1
59	0	0.08	4	14.2
60	22	0.16	2	2.6
61	8	0.11	21	16.4
62	11	0.12	17	47.8
63	5	0.10	3	7.8
64	0	0.08	2	1.2
65	92	0.42	9	36.4

Table 6. 2 continued

ISOLATE NUMBER	EFFFECTIVENESS INDEX(%) BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT(mg)
66	135	0.58	17	44.5
67	8	0.11	3	9.8
68	0	0.08	3	2.1
69	5	0.10	1	6.5
70	19	0.15	4	3.6
71	68	0.33	6	29.1
72	38	0.22	12	15.3
73	0	0.08	2	3.7
74	0	0.08	1	1.1
75	200	0.82	19	105.6
76	35	0.21	9	5.7
77	0	0.08	1	1.3
78	27	0.18	4	3.4
79	3	0.09	1	1.0
80	0	0.08	7	1.7
NO – N	0	0.08	0	0
+ N	100	0.45	0	0
LSD (5%)	34.5	0.27	13.2	59.5

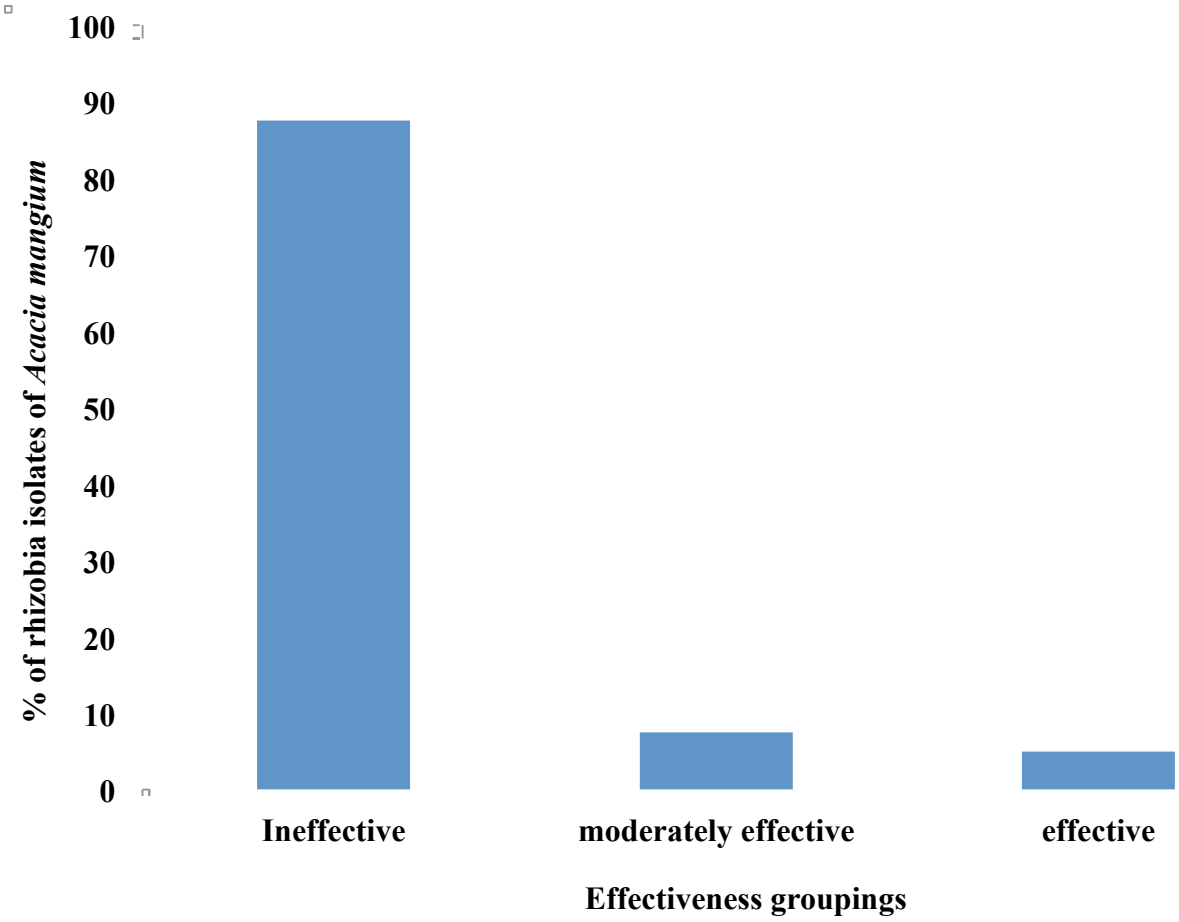

Fig. 5. 2 Classification of 40 isolates of rhizobia obtained from *Acacia mangium* on the basis of their effectiveness in promoting growth on N-free sand medium relative to plants relying on fertilizer N.

GROUP		CRITERION
Ineffective	»	Isolates with effectiveness index ≤ 50%
Moderately effective	»	Isolates with effectiveness index between 51 – 99%
Highly effective	»	Isolates with effectiveness index ≥ 100%

Table 6. 3 Relative comparison of 40 rhizobia isolates of *Acacia auricloformis* for their influence on plant growth, number of nodules formed and dry weight of nodules.

ISOLATE NUMBER	EFFECTIVENESS INDEX (%)BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT (mg)
81	80	0.30	9	7.1
82	97	0.35	12	21.8
83	70	0.27	6	9.7
84	143	0.49	17	52.3
85	160	0.60	21	92.4
86	137	0.47	13	19.6
87	6	0.08	1	1.2
88	70	0.27	11	31.4
89	273	0.88	14	56.5
90	200	0.66	18	89.4
91	37	0.17	1	2.1
92	83	0.31	4	7.9
93	260	0.84	24	101.7
94	150	0.51	19	102.3
95	17	0.11	6	11.9
96	23	0.13	3	5.8
97	57	0.23	5	9.1
98	50	0.21	2	3.7
99	110	0.39	10	16.3
100	140	0.48	16	42.3
101	80	0.30	5	8.1
102	40	0.18	3	3.6
103	117	0.41	11	25.8
104	40	0.18	2	4.2
105	27	0.14	2	6.1

Table 6.3 continued

ISOLATE NUMBER	EFFECTIVENESS INDEX (%)BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT (mg)
106	53	0.22	6	10.1
107	83	0.31	13	52.4
108	100	0.36	6	9.7
109	50	0.21	2	3.2
110	37	0.17	1	1.3
111	17	0.11	1	1.1
112	57	0.23	2	1.9
113	100	0.36	9	7.8
114	43	0.19	5	6.4
115	63	0.25	6	9.1
116	20	0.12	3	4.1
117	27	0.14	5	5.2
118	17	0.11	4	3.9
119	37	0.17	3	4.7
120	23	0.13	3	5.1
NO- N	0	0.06	0	0
200 kgN/ha	100	0.36	0	0
LSD (5%)	34.5	0.27	13.2	59.5

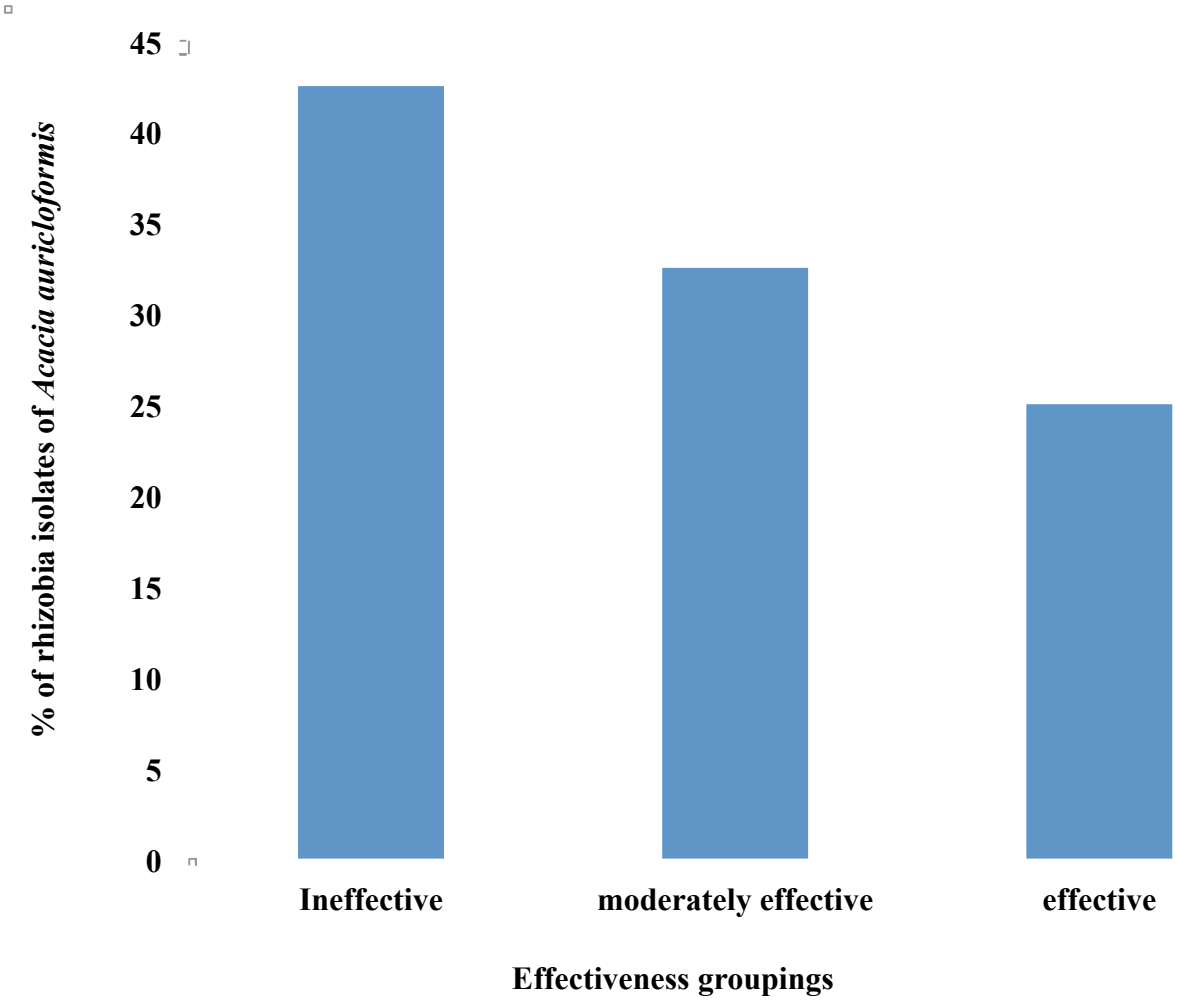

Fig. 5. 3 Classification of 40 isolates of rhizobia obtained from *Acacia auricloformis* on the basis of their effectiveness in promoting growth on N-free sand medium relative to plants relying on fertilizer N.

GROUP	CRITERION
Ineffective »	Isolates with effectiveness index ≤ 50%
Moderately effective »	Isolates with effectiveness index between 51 – 99%
Highly effective »	Isolates with effectiveness index ≥ 100%

Table 6.4 Relative comparison of 40 rhizobia isolates of *Melletia thonningi* for their influence on plant growth, number of nodules formed and dry weight of nodules.

ISOLATE NUMBER	FFFECTIVENESS INDEX (%)BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT(mg)
121	24	0.23	1	4.1
122	0	0.15	1	3.7
123	18	0.21	3	10.1
124	27	0.24	1	1.8
125	42	0.29	2	4.9
126	9	0.18	2	3.9
127	289	1.10	32	221.7
128	116	0.65	18	101.3
129	124	0.56	6	12.4
130	48	0.31	5	10.8
131	3	0.16	2	4.6
132	0	0.15	1	2.1
133	97	0.47	4	9.3
134	21	0.22	1	3.7
135	58	0.34	5	16.9
136	279	1.07	29	97.3
137	6	0.17	2	6.2
138	258	1.00	17	156.1
139	12	0.19	2	2.8
140	0	0.15	1	1.7
141	52	0.32	6	4.1

Table 6.4 continued

ISOLATE NUMBER	FFFECTIVENESS INDEX (%)BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT(mg)
142	18	0.21	2	2.9
143	70	0.38	9	14.2
144	21	0.22	2	5.2
145	258	1.00	33	197.4
146	33	0.26	4	7.1
147	48	0.31	5	11.2
148	3	0.16	2	1.4
149	9	0.18	2	2.8
150	42	0.29	4	8.7
151	61	0.35	6	11.9
152	239	0.94	25	156.8
153	18	0.21	1	4.1
154	21	0.22	1	3.7
155	58	0.34	4	9.1
156	9	0.18	2	3.1
157	12	0.19	1	1.6
158	142	0.62	7	16.4
159	79	0.41	8	10.1
160	36	0.27	9	7.5
NO-N	0	0.15	0	0
200Kg N/ ha	100	0.48	0	0
LSD (5%)	34.5	0.27	13.2	59.5

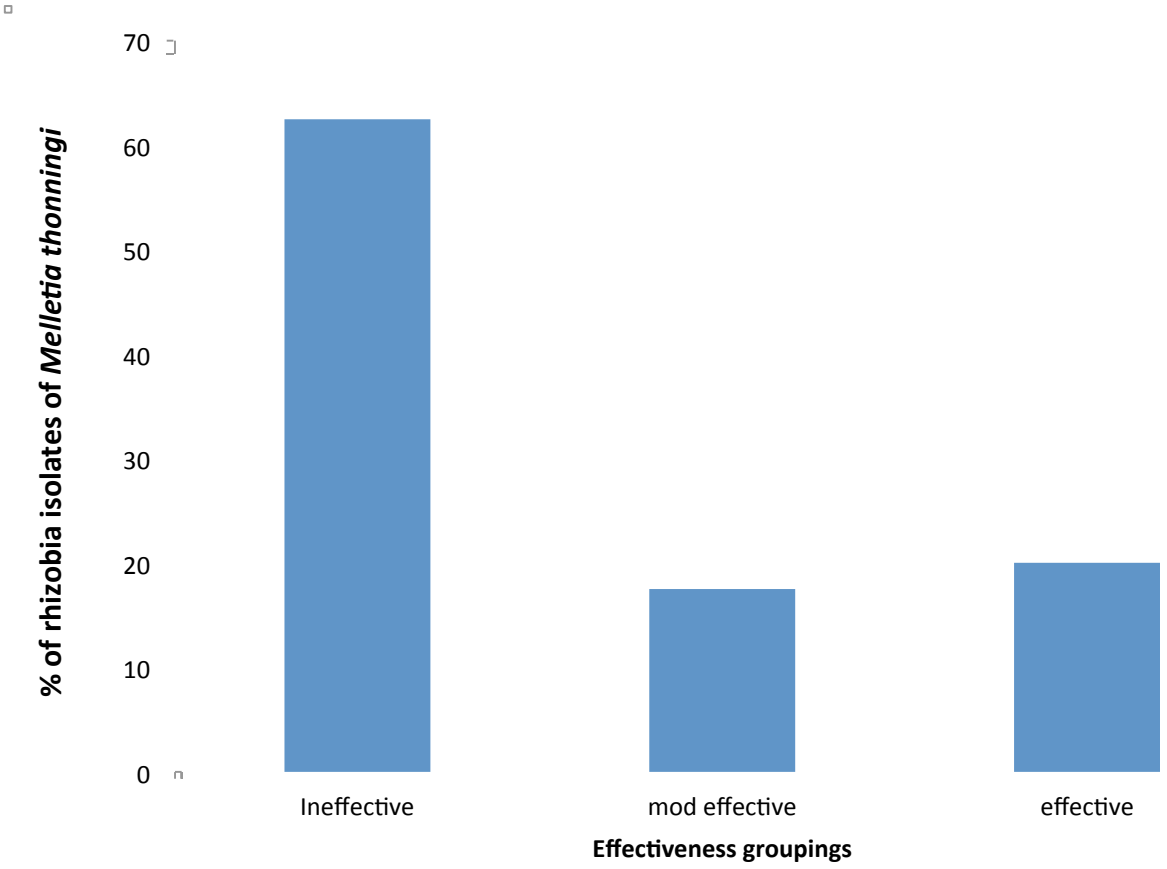

Fig. 5. 4 Classification of 40 isolates of rhizobia obtained from *Melletia thonningi* on the basis of their effectiveness in promoting growth on N-free sand medium relative to plants relying on fertilizer N.

GROUP		CRITERION
Ineffective	»	Isolates with effectiveness index ≤ 50%
Moderately effective	»	Isolates with effectiveness index between 51 – 99%
Highly effective	»	Isolates with effectiveness index ≥ 100%

Table 6.5 Relative comparison of 40 rhizobia isolates of *Leucaena leucocephela* for their influence on plant growth, number of nodules formed and dry weight of nodules.

ISOLATE NUMBER	FFFECTIVENESS INDEX (%)BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT(mg)
161	10	0.44	3	6.8
162	2	0.39	1	3.1
163	0	0.38	1	2.8
164	52	0.71	4	16.4
165	25	0.54	3	9.1
166	89	0.94	9	62.4
167	5	0.41	4	19.7
168	13	0.46	3	11.5
169	32	0.58	4	16.8
170	0	0.38	1	2.1
171	5	0.41	2	5.4
172	133	1.22	17	13.7
173	78	0.87	11	81.5
174	98	1.00	12	74.3
175	273	2.10	23	112.5
176	37	0.61	2	7.3
177	27	0.55	2	6.9
178	19	0.48	3	10.1
179	52	0.71	6	33.9
180	76	0.86	9	77.4
181	37	0.61	3	8.4
182	33	0.59	3	11.3
183	2	0.39	1	2.2
184	6	0.42	2	5.1
185	79	0.88	11	78.4

Table 6.5 continued

ISOLATE NUMBER	EFFECTIVENESS INDEX (%)BY DM	DRY BIOMASS (g) / plant	NODULE	
			NUMBER	DRY WEIGHT(mg)
186	0	0.38	1	3.7
187	87	0.93	6	42.3
188	21	0.51	3	10.6
189	81	0.89	10	77.1
190	56	0.73	4	17.3
191	63	0.78	4	16.8
192	157	1.37	16	120.4
193	113	1.09	12	93.4
194	5	0.41	2	6.3
195	24	0.53	3	7.1
196	10	0.44	3	6.6
197	84	0.91	13	101.3
198	100	1.01	15	131.1
199	157	1.37	21	119.8
200	2	0.39	1	2.8
CONTROL NO-N	0.38	0.38	0	0
+N	1.01	1.01	0	0
LSD (5%)	34.5	0.27	13.2	59.5

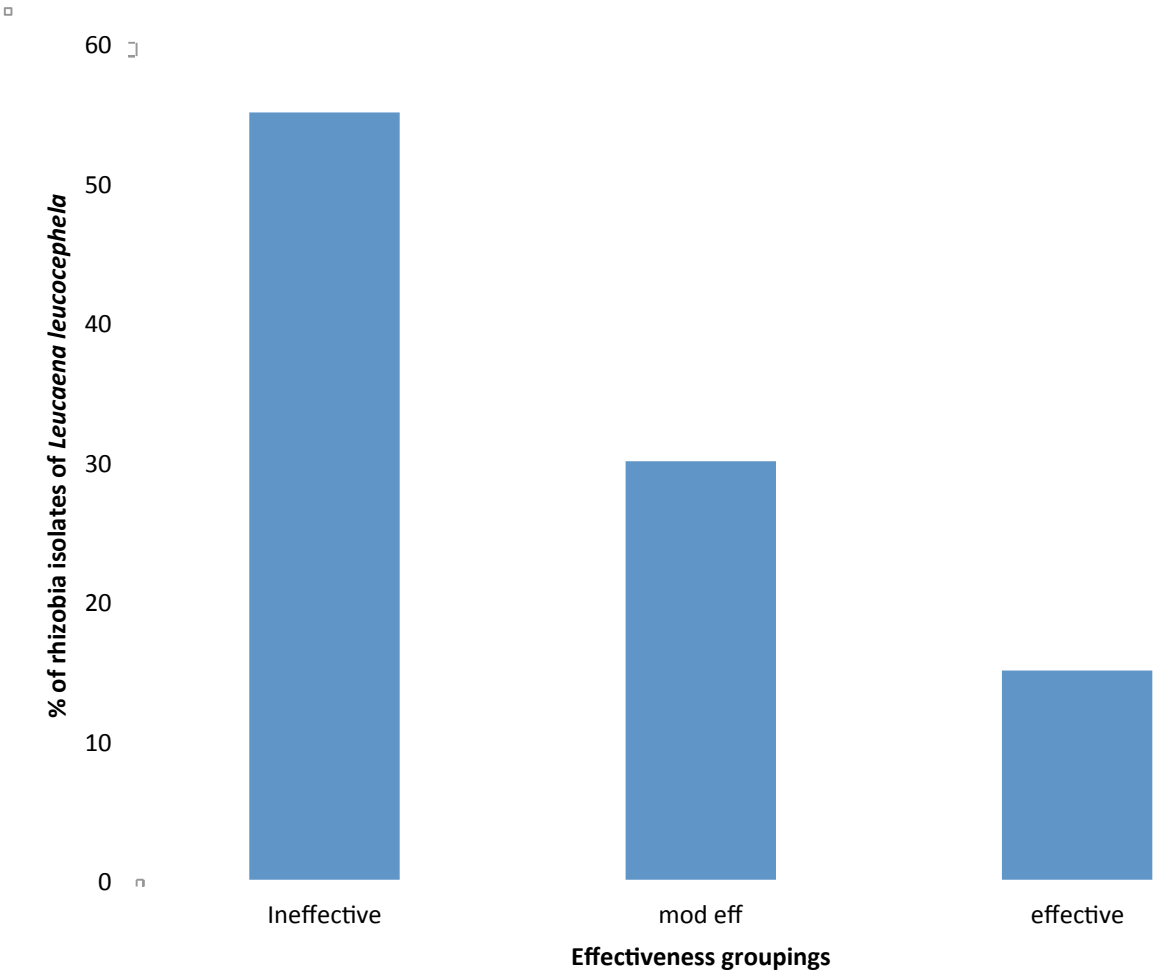

Fig. 5.5 Classification of 40 isolates of rhizobia obtained from *Leucaena leucocephela* on the basis of their effectiveness in promoting growth on N-free sand medium relative to plants relying on fertilizer N.

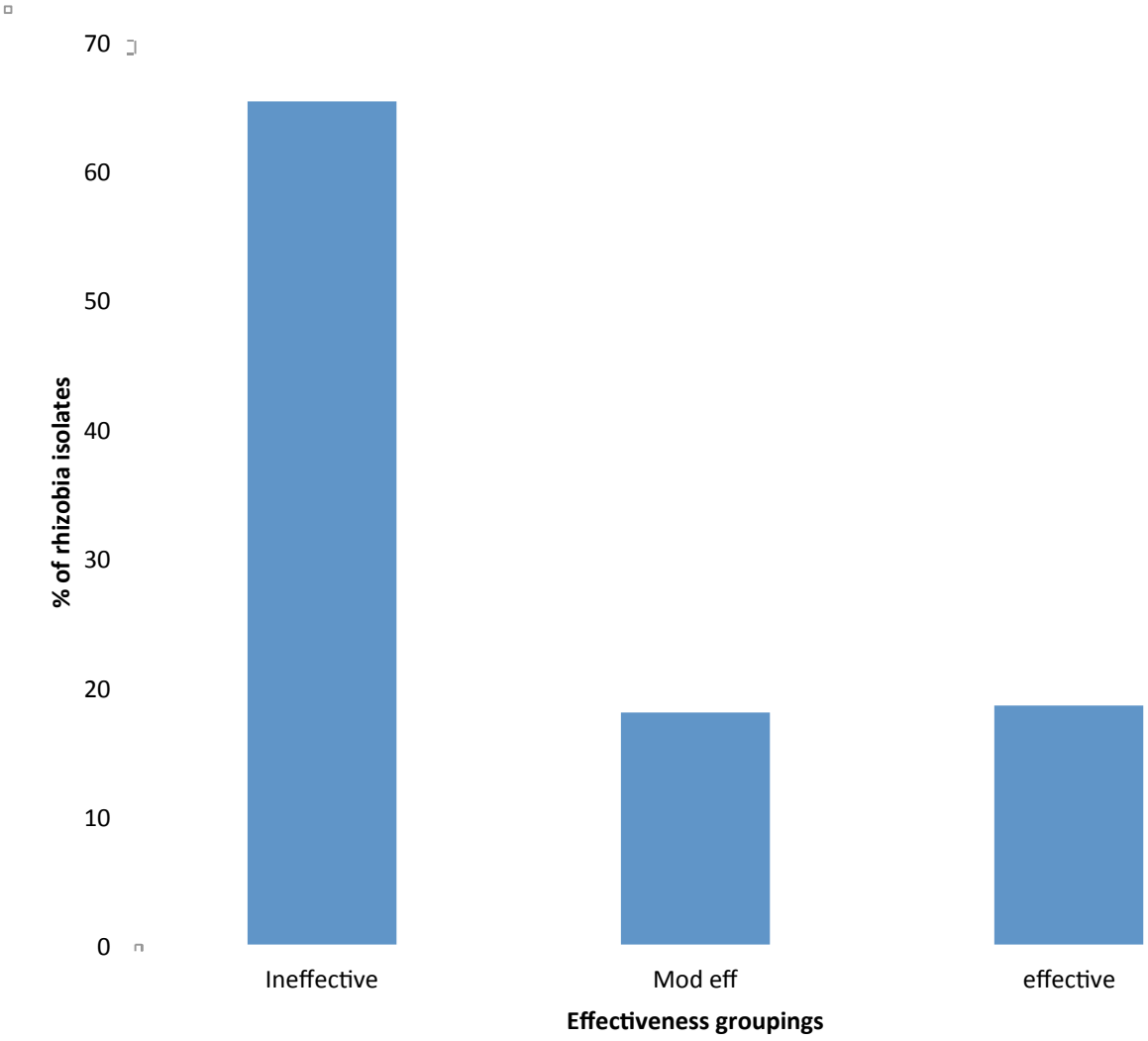

Fig. 5.6 Summary of classification made for all the 200 rhizobia isolates obtained from five multi-purpose tree legumes on the basis of their ability to stimulate plant growth on N-free sand medium relative to dry matter yield of plants that relied on fertilizer

GROUP		CRITERION
Ineffective	»	Isolates with effectiveness index ≤ 50%
Moderately effective	»	Isolates with effectiveness index between 51 – 99%
Highly effective	»	Isolates with effectiveness index ≥ 100%

4.6 Nodulation response of six tree legumes to phosphorus fertilizer application

Figures 6.1 and 6.2 provide information on the effect of phosphorus on the number of nodules formed on six tree legumes in two soil types.

Phosphorus application generally improved upon the formation of nodules on the various tree legumes in the two soil types. Increasing the rates of phosphorus application, especially up to 90 kg P/ha resulted in significant increases in number of nodules compared to their respective controls. However, increasing the rate of phosphorus application beyond 90 kg P/ha resulted in significant decreases in the number of nodules formed in all six tree legume species.Thus in the soils, highest nodulation for any of the tree legumes was reached at the 90 kg P/ha rate. *Albizia lebbek* on average gave the highest nodulation in both soils, while *Acacia mangium* was the poorest in nodulation. The tree legumes varied in terms of their nodulation response to phosphorus . For example, while the application of 90 kg P/ha to *Leucaena leucocephela* resulted in over 850% and 113% increases in nodules formed in Hatso and Toje soils respectively, *Melletia thonningi* at that same rate experienced only 48% and 29% increases in the Hatso and Toje soils, respectively.

The effect of phosphorus in stimulating formation of nodules on the tree legumes in the two soils was more pronounced in Hatso soil than Toje soil.

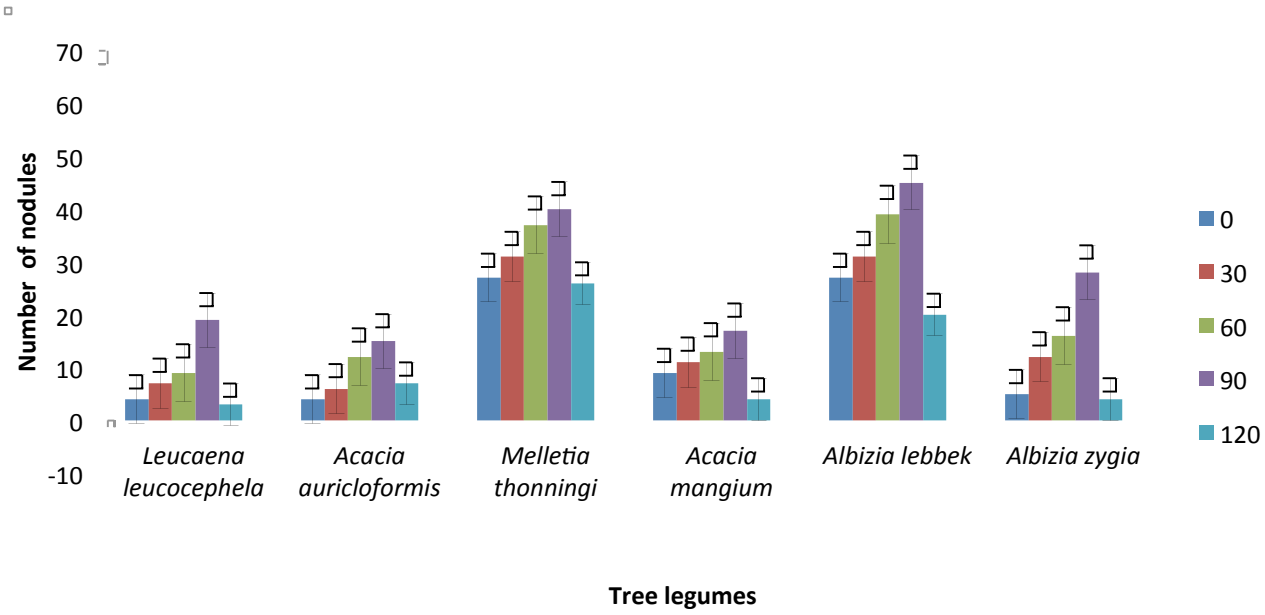

Fig. 6. 1 Effect of five application levels of phosphorus on number of nodules formed on six tree legumes in Hatso soils

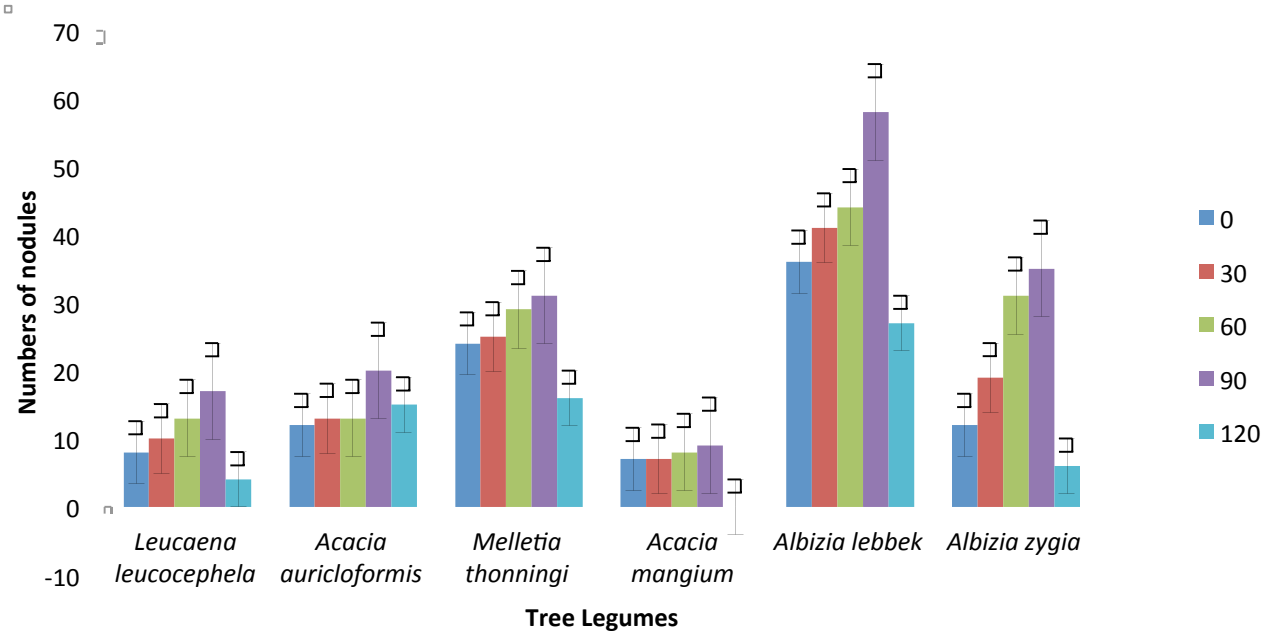

Fig. 6. 2 Effect of five application levels of phosphorus on number of nodules formed on six tree legumes in Toje soil

4.7 Shoot and root dry matter yield (g) response of six selected tree legumes to phosphorus fertilizer in two soil types.

Phosphorus application to the six tree legumes at all rates resulted in significant (p=0.05) increases in shoot dry matter yield of the tree legumes except in the case of *Albizia zygia* in both soils (Table 7 and 8). Just like the number of nodules, increasing the level of phosphorus application resulted in increases in shoot dry matter yield up to 90 kg P/ha, beyond which shoot dry matter yield decreased significantly (p=0.05). Of all the five rates tested, the application of 30kg P/ha in the two soils resulted in the highest percent shoot dry matter yield response which ranged from 6% in the case of *Albizia zygia* in Toje soil to 112% in the case of *Leucaena leucocephela* in Hatso soil. Application of 120 kg P/ha to *Acacia mangium* and *Albizia lebbek* in the two soils resulted in decrease in dry matter yields to levels that were even lower than their respective controls (Table 7 and 8). A similar result was observed for *Albizia zygia* in Toje soil (Table 8). In both soils, the dry matter of *Acacia mangium* and *Albizia lebbek* appeared to be most affected compared to the other tree legumes with the application of 120 kg P/ha. Compared to 90 kg P/ha in Hatso soil, the dry matter of *Acacia mangium* and *Albizia lebbek* decreased by 75% and 62%, respectively, and by 63% and 72%, respectively in Toje soil. In all cases, shoot dry matter yield was higher in Toje soil than in Hatso soil.

Just like shoot dry matter, phosphorus application generally resulted in significant (p=0.05) increase in root dry matter of the tree legumes. In all cases, increasing P rates resulted in increased root biomass from 0 kg P/ha to 90 kg P/ha beyond which further increase in P to 120 kg P/ha resulted in significant (p=0.05) reduction in root biomass for all the tree legumes. In Hatso soil, application of 120 kg P/ha to the tree legumes resulted in 50% of the tree legumes root biomasses being lower than their respective controls (Table 9). In Toje soil however, application of 120 kg P/ha resulted in all the root biomasses of the tree legumes being less than

their respective controls except in the case of *Leucaena leucocephela* and *Melletia thonningi* (Table 10). *Albizia lebbek* on average gave the highest root biomass (Table 10) with the least being *Acacia mangium* (Table 8). On average, growth on Toje soil gave higher average root biomass for the tree legumes than on Hatso soil.

Table 7 Effect of phosphorus application on shoot dry matter yield (g) of six tree legumes in Hatso soil

P-rates Kg/ha	*Leucaena leucocephela*	*Acacia auricloformis*	*Melletia thonningi*	*Acacia mangium*	*Albizia lebbek*	*Albizia* zygia	*Treatment* average
0	0.34	0.24	0.78	0.12	0.57	0.11	0.36
30	0.72	0.50	0.89	0.24	1.02	0.17	0.59
60	0.89	0.54	0.94	0.27	1.11	0.26	0.67
90	1.08	0.62	1.05	0.32	1.21	0.31	0.77
120	0.41	0.25	1.05	0.08	0.46	0.13	0.40
Plant average	0.69	0.43	0.94	0.21	0.87	0.20	

Lsd (5%): Phosphorus = 0.1, Tree = 0.6, Soil = 0.6, Pho x Tree = 0.12

Table 8. Effect of phosphorus application on shoot dry matter (g) yield of six tree legumes in Toje soil.

P-rates Kg/ha	*Leucaena leucocephela*	*Acacia auricloformis*	*Melletia thonningi*	*Acacia mangium*	*Albizia lebbek*	*Albizia* zygia	*Treatment* average
0	0.52	0.37	0.81	0.21	0.66	0.30	0.48
30	0.95	0.55	0.98	0.37	1.21	0.32	0.73
60	0.99	0.59	1.05	0.43	1.34	0.33	0.79
90	1.41	0.64	1.23	0.51	1.44	0.44	0.95
120	0.76	0.48	1.05	0.19	0.41	0.24	0.52
Plant average	0.93	0.53	1.02	0.34	1.01	0.33	

Lsd (5%) : Phosphorus = 0.1, Tree = 0.6, Soil = 0.6, Pho x Tree = 0.12

Table 9. Effect of phosphorus application on root dry matter (g) of six tree legumes in Hatso soil.

P-rates Kg/ha	*Leucaena leucocephela*	*Acacia auricloformis*	*Melletia thonningi*	*Acacia mangium*	*Albizia lebbek*	*Albizia* zygia	*Treatment* average
0	0.23	0.07	0.36	0.02	0.40	0.12	0.20
30	0.28	0.10	0.37	0.03	0.44	0.17	0.23
60	0.35	0.12	0.43	0.07	0.47	0.26	0.28
90	0.36	0.18	0.52	0.09	0.64	0.37	0.36
120	0.21	0.05	0.40	0.01	0.41	0.18	0.21
Root average	0.29	0.10	0.42	0.04	0.47	0.22	

Lsd(5%) = Phosphorus = 0.02, Trees = 0.01, Soil = 0.1, Phosp x Tree = 0.02

Table 10. Effect of phosphorus application on root dry matter yield (g) of six tree legumes in Toje soil.

P-rates Kg/ha	*Leucaena leucocephela*	*Acacia auricloformis*	*Melletia thonningi*	*Acacia mangium*	*Albizia lebbek*	*Albizia* zygia	*Treatment* average
0	0.30	0.08	0.41	0.03	0.44	0.14	0.23
30	0.46	0.11	0.57	0.05	0.72	0.18	0.35
60	0.62	0.14	0.59	0.08	0.81	0.29	0.42
90	0.66	0.19	0.69	0.11	0.83	0.44	0.49
120	0.38	0.06	0.53	0.02	0.42	0.09	0.25
Root average	0.40	0.10	0.50	0.04	0.64	0.23	

Lsd (5%) = Phosphorus = 0.02, Trees = 0.01, Soil = 0.1, Phosp x Tree = 0.02

4.8 Effect of phosphorus application rates on P uptake and phosphorus utilization efficiency (PUE) by six tree legumes

Tables 11, 12, 13 and 14 contain data on how various rates of phosphorus application influenced P uptake and PUE by six tree legumes in two soil types.

Higher P rates in general resulted in significant (p=0.05) increases in P uptake by all the six tree legumes in the two soil types with the highest P uptake occurring at an application rate of 120 kg P/ha for all the soils (Table 11 and 13). The tree legumes also varied in terms of their P uptake capabilities with increasing P rates. For example, while application of 30 kg P/ha to *Leucaena leucocephela* resulted in over 142% and 244% improvement in P uptake in Toje and Hatso soils respectively, the corresponding improvements in P uptake with *Melletia thonningi* were, 60% and 74%, respectively. In general, P uptake was significantly (p=0.05) higher in Toje soil than Hatso soil (Table 11 and 13).

Phosphorus utilization efficiency (PUE) for all the tree legumes in the soils generally decreased significantly (p=0.05) with increasing P rates (Table 12 and 14) except for *Leucaena leucocephela* and *Albizia zygia* at rates of 60 kg P/ha and 90 kg P/ha in both soils and *Acacia mangium* at rates 30, 60 and 90 kg P/ha in Toje soil. The decrease in PUE was also not significant (p=0.05) in *Acacia mangium* at rate 60 kg P/ha and 90 kg P/ha and also *Albizia zygia* at rate 30 kg P/ha and 60 kg P/ha in Hatso soil (Table 12 and 14).

Melletia thonningi on average gave the highest PUE in both soils with the least being *Acacia mangium* in both soils.

Table 11. Influence of five levels of applied P fertilizer on shoot P uptake (mg P/plant) in six tree legumes in Hatso soil

P-rates	*Leucaena*	*Acacia*	*Melletia*	*Acacia*	*Albizia*	*Albizia*	
Kg P/ha	*leucocephela*	*auricloformis*	*thonningi*	*mangium*	*lebbek*	*zygia*	Average
0	5.05	4.51	9.20	4.21	8.75	4.28	6.00
30	17.40	9.73	16.02	10.11	15.91	9.77	13.16
60	24.20	13.14	21.71	14.24	26.24	15.04	19.10
90	31.14	16.21	28.22	18.13	33.81	19.11	24.44
120	34.23	19.98	30.36	23.09	33.97	24.27	27.65
Plt. Ave	17.56	12.71	21.10	13.96	23.74	14.49	

Lsd (5%) = Phosphorus = 2.2, Trees = 3.1, Soil = 3.1, Phos x Trees = 4.1.

Table 12. Influence of five levels of P applied on the efficiency with which available P was utilized for growth (PUE) in Hatso soil

P-rates	*Leucaena*	*Acacia*	*Melletia*	*Acacia*	*Albizia*	*Albizia*	
Kg P/ha	*leucocephela*	*auricloformis*	*thonningi*	*mangium*	*lebbek*	*zygia*	Average
0	67	53	85	29	65	26	54.17
30	41	51	56	23	64	17	42.00
60	37	41	43	19	42	17	33.17
90	35	38	37	18	35	16	29.83
120	12	13	24	3	14	5	11.83
Plt. Ave	38.40	39.20	49.00	18.40	44 .00	16.20	

Lsd(5%) = Phosphorus =2.0, Trees = 2.0, Soil =1.0, Phos x Trees = 3.0.

Table 13. Influence of five levels of P applied on shoot P uptake (mg P/plant) in six tree legumes in Toje soil

P-Rates	*Leucaena*	*Acacia*	*Melletia*	*Acacia*	*Albizia*	*Albizia*	
Kg P/ha	*leucocephela*	*auricloformis*	*thonningi*	*mangium*	*lebbek*	*zygia*	Average
0	8.35	7.01	11.33	5.01	10.22	6.11	7.51
30	20.13	12.24	18.11	13.31	19.71	12.13	15.94
60	29.14	15.28	23.37	16.44	28.11	17.41	21.63
90	40.86	20.39	34.11	21.02	37.22	22.98	29.43
120	48.41	24.94	38.24	28.47	41.38	27.81	34.88
Plt. Ave	29.38	15.97	25.03	16.85	27.32	17.29	

Lsd (5%) = Phosphorus = 2.2, Trees = 3.1, Soil = 3.1, Phos x Trees = 4.1

Table 14. Influence of five levels of P applied on how efficiently six trees utilized available P (PUE) in Toje soil

P-Rates	*Leucaena*	*Acacia*	*Melletia*	*Acacia*	*Albizia*	*Albizia*	
Kg P/ha	*leucocephela*	*auricloformis*	*thonningi*	*mangium*	*lebbek*	*zygia*	Average
0	62	53	72	42	65	29	53.83
30	47	50	54	28	61	26	44.33
60	34	39	45	26	48	19	35.17
90	34	31	36	24	39	19	30.50
120	16	19	28	7	11	7	14.67
Plt. Ave	38.60	38.40	47.00	25.40	44 .80	20.00	

Lsd (5%) = Phosphorus = 2.0, Trees = 2.0, Soi l= 1.0, Phosphorus x Trees = 3.0.

4.9. Influence of six levels of nitrogen application on nodulation and dry matter yield of six tree legumes in two soil types.

Figures 8 and 9 provide information on the nodulation response of six tree legumes to six levels of nitrogen (0, 40, 80, 120, 160 and 240 kg N/ha) in two soil types. Nitrogen application generally resulted in significant (p=0.05) decrease in number of nodules formed on the tree legumes in the two soil types. Nodulation of the tree legumes responded variably to the N levels applied. For instance, the application of 40 kg N/ha completely inhibited nodulation in *Leucaena leucocephela, Acacia auricloformis* and *Acacia mangium* in both Toje and Hatso soils. Similar finding was observed for *Leucaena leucocephela* and *Acacia auricloformis* in Hatso soil. This is in contrast to the 50% and 20% reductions in number of nodules of *Cajanus cajan* and *Crotalaria ochloleuca*, respectively, when grown in Hatso soil with 40 kg N/ha applied or the 20% and 60% reductions in the nodulation of *Cajanus cajan* and *Crotalaria ochloleuca*, respectively, grown in Toje soil with 40 kg N/ha.

The effect of six levels of nitrogen fertilizer on dry matter yield of six tree legumes is presented in Tables 14 and 15. Nitrogen fertilizer application generally resulted in significant (p=0.05) increases in shoot dry matter yield of all the tree legumes at all treatment levels except *Crotalaria ochloleuca* and *Cajanus cajan* which recorded significant (p=0.05) decreases in dry matter beyond an application of 80 kg N/ha.

The effect of nitrogen fertilizer on dry matter though significant at all treatment levels, it decreases with increasing N application in all the soils. On average, application of 40 kg N/ha to the tree legumes resulted in the highest increase in dry matter yield ranging from 11% in *Acacia auricloformis* to 32% in *Leucaena leucocephela* in Hatso soil and 5% to 35% in *Leucaena leucocephela* and *Acacia mangium* respectively, in Toje soil. Application of 240 kg N/ha to the tree legumes in the two soils resulted in the dry matters of *Crotalaria ochloleuca* and *Cajanus*

cajan being lower than their respective controls (Tables 15 and 16). Nitrogen application at all treatment levels for the tree legumes gave significant ($p=0.05$) higher dry matter yield increases in Toje soil than Hatso soil. There was also significant ($p=0.05$) difference among the tree legumes and the interactions among tree, soil and nitrogen treatments.

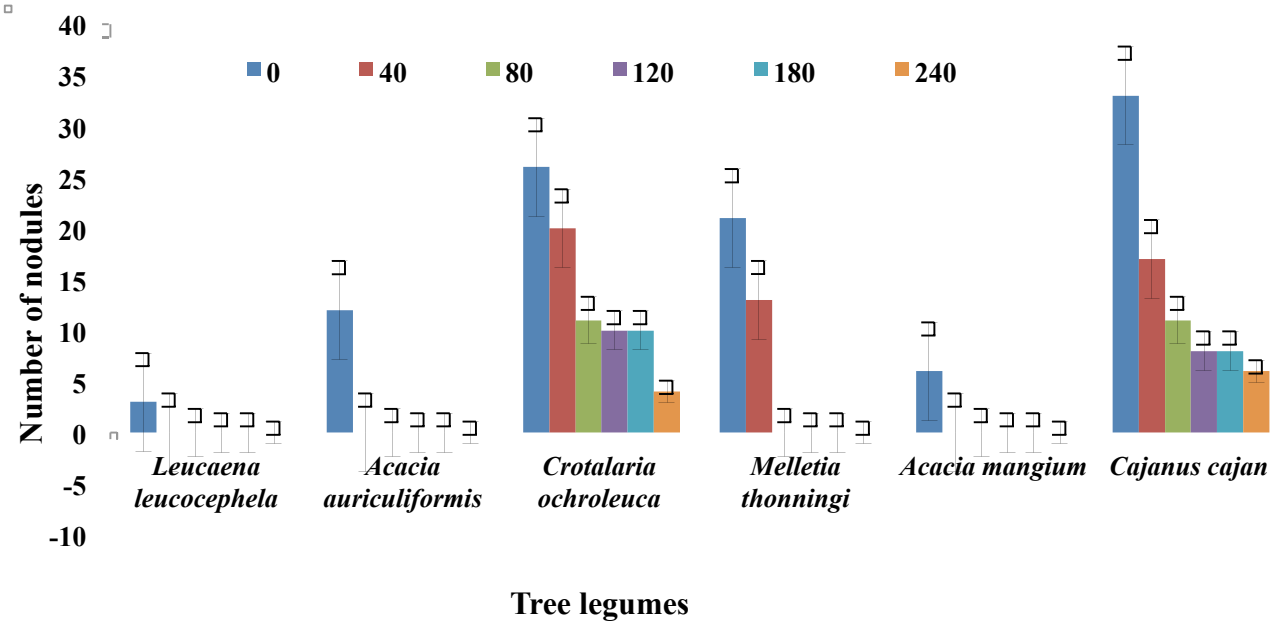

Fig. 8 Effect of six levels of nitrogen fertilizer on nodulation of six tree legumes in Hatso soil

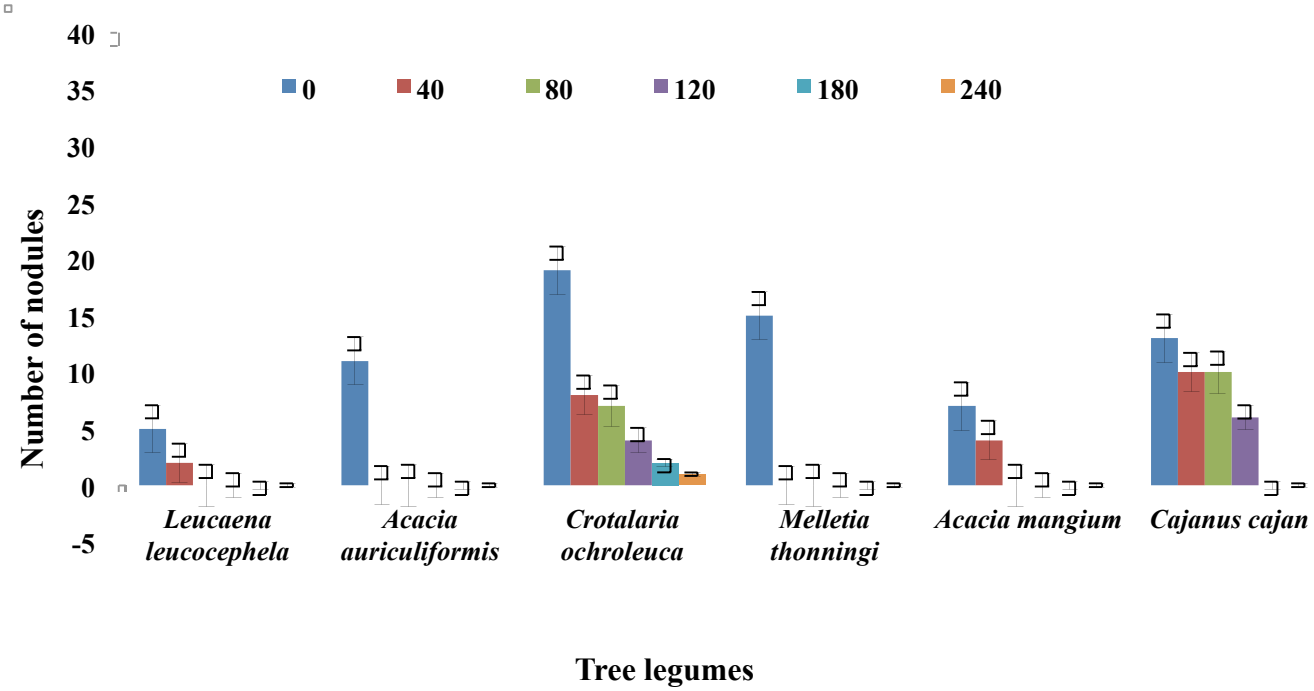

Fig. 9 Effect of six levels of nitrogen fertilizer on nodulation of six tree legumes in Toje soil

Table 15. Response of shoot dry matter yield (g/plant) of six tree legumes to six levels of nitrogen fertilizer in Hatso soil.

N-rates	*Leucaena*	*Acacia*	*Crotalaria*	*Melletia*	*Acacia*	*Cajanus*	*Treat.*
Kg N/ha	*leucocephela*	*auricloformis*	*ochloleuca*	*thonningi*	*mangium*	*cajan*	average
0	0.19	0.09	0.13	0.38	0.11	0.21	0.19
40	0.25	0.10	0.17	0.44	0.14	0.25	0.23
80	0.26	0.11	0.26	0.51	0.14	0.35	0.27
120	0.27	0.15	0.24	0.64	0.16	0.34	0.30
180	0.27	0.18	0.14	0.69	0.17	0.23	0.28
240	0.30	0.23	0.10	0.78	0.20	0.16	0.30
Plt. Ave	0.26	0.14	0.17	0.57	0.15	0.26	

LSD (5%) : Treatment = 0.02, Tree = 0.02, Soil = 0.01,

Soil x treatment = 0.04, Soil x tree = 0.04, Treatment x tree = 0.06,

Soil x treatment x soil = 0.1

Table 16. Response of shoot dry matter yield (g/plant) of six tree legumes to six levels of nitrogen fertilizer in Toje soil.

N- rates.	*Leucaena*	*Acacia*	*Crotalaria*	*Melletia*	*Acacia*	*Cajanus*	*Treat.*
Kg N/ha	*leucocephela*	*auricloformis*	*ochloleuca*	*thonningi*	*mangium*	*cajan*	average
0	0.38	0.32	0.20	0.50	0.23	0.26	0.32
40	0.40	0.35	0.22	0.62	0.31	0.29	0.37
80	0.42	0.37	0.23	0.70	0.48	0.30	0.42
120	0.43	0.42	0.24	1.72	0.55	0.39	0.63
180	0.52	0.43	0.19	1.75	0.62	0.29	0.63
240	0.81	0.45	0.13	1.78	0.69	0.16	0.67
Plt. Ave	0.43	0.39	0.20	1.18	0.48	0.28	

LSD (5%) : Treatment = 0.02, Tree = 0.02, Soil = 0.01,

Soil x treatment = 0.04, Soil x tree = 0.04, Treatment x tree = 0.06,

Soil x treatment x soil = 0.1

4.10 Nodulation and Shoot dry matter yield response of six tree legumes to six levels of nitrogen fertilizer in two soil types amended with 90 kg P/ha.

Figures 10 and 11 show nodulation responses of six tree legumes to six levels of nitrogen fertilizer in the two soils types amended with 90 kg P/ha. In general, nitrogen application resulted in decreases in number of nodules formed on all the tree legumes at all succeeding N levels in the two soils. The results indicate significant (p=0.05) differences in sensitivity of the trees legumes

number of nodules formed to nitrogen fertilizer when P was present. Number of nodules formed in *Melletia thonningi* was the most sensitive to N levels in the two soils with the lowest being *Acacia auricloformis* and *Cajanus cajan* in Hatso and Toje soils, respectively (Fig. 10 and 11). Application of 40 kg N/ha to *Melletia thonningi* resulted in more than 70% and 62% reduction in number of nodules in Hatso and Toje soils, respectively. There was significant (p=0.05) difference between the two soils in terms of their abilities to support nodulation of the tree legumes with increasing N application (Table 17 and 18).

Tables 17 and 18 provide information on the influence of six levels of nitrogen fertilizer on the shoot dry matter yield of six tree legumes in two soil types amended with 90 kg P/ha. Nitrogen application resulted in significant (p=0.05) increases in shoot dry matter yield of all the tree legumes at all treatment levels except *Crotalaria ochloleuca* and *Cajanus cajan* for which a further nitrogen application beyond 120 kg N/ha resulted in significant (p=0.05) decrease in shoot dry matter in Hatso soil (Table 17) but in Toje soil the decrease occurred beyond 120 kg N/ha in the case of *Crotalaria ochloleuca* and beyond 180 kg N/ha in the case of *Cajanus cajan* (Table 18).

The tree legumes responded variably to nitrogen application in the two soils. For instance, an application of 40 kg N/ha to *Cajanus cajan* resulted in more than 125% and 142% increases in dry matter in Hatso and Toje soils, respectively and also less than 2% and more than 72% in the case of *Melletia thonningi* in Hatso and Toje, respectively.

There were also significant (p=0.05) difference among tree legumes and between soils as well as all the interactions between soil, tree legumes and nitrogen treatments.

Comparing the results of the two nitrogen studies on nodulation and dry matter yield response of the tree legumes to nitrogen fertilizer on the two soils with or without phosphorus, nitrogen effect

on dry matter yield was more pronounced when P was added to the soils than not; but nodulation effect was also more pronounced when P was not added to the soils. The results further show that, P application allowed for nodulation at levels of N not otherwise possible. The responses were however variable, for example being high for *A. auricloformis* in both soils, and greater in Toje soil than Hatso soil for *Melletia thonningi*, with *A. mangium* showing less differences to the effect of N in the presence of P, but this effect being still better in Hatso than Toje.

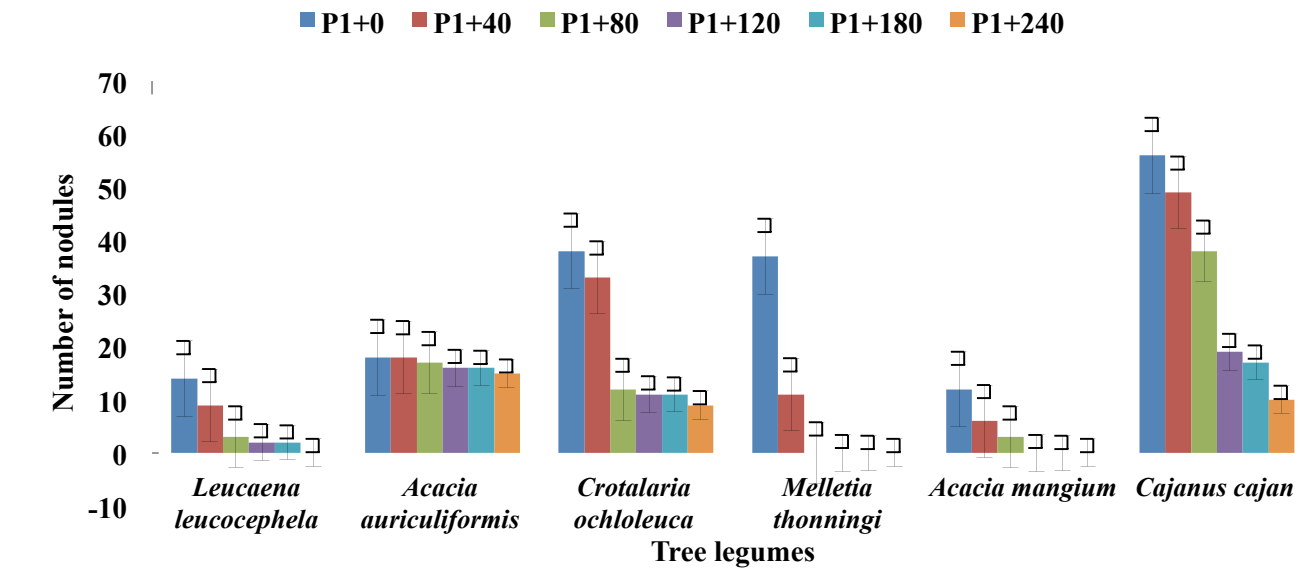

Fig. 10.Effect of six levels of nitrogen fertilizer on nodulation of six tree legumes in Hatso soil amended with 90 kg P/ha.

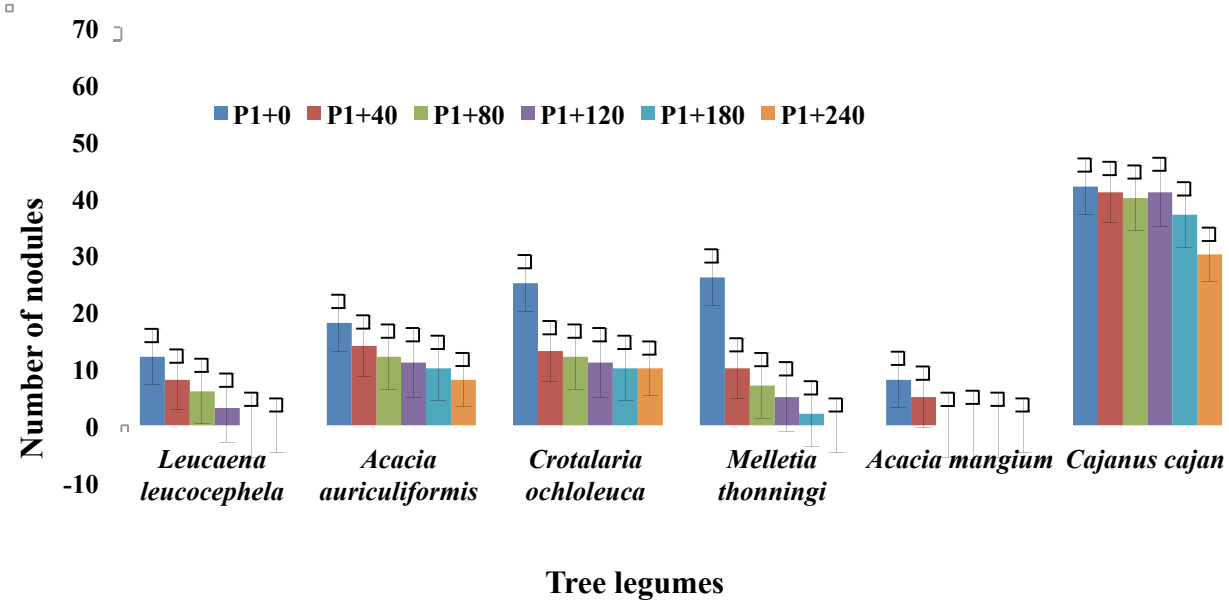

Fig. 11. Effect of six levels of nitrogen fertilizer on nodulation of six tree legumes in Toje soil amended with 90 kg P/ha.

Table 17. Effect of six levels of nitrogen fertilizer in Hatso soil amended with 90 kg P/ha on shoot dry matter yield (g/plant) of six tree legumes.

N-rates Kg N/ha	*Leucaena leucocephela*	*Acacia auricloformis*	*Crotalaria ochloleuca*	*Melletia thonningi*	*Acacia mangium*	*Cajanus cajan*	*Treat.* average
P1	0.48	0.23	0.68	0.57	0.25	1.30	0.59
P1+40	0.63	0.31	1.30	0.58	0.30	2.93	1.01
P1+80	0.75	0.37	1.49	0.61	0.49	2.94	1.11
P1+120	0.79	0.43	1.57	0.69	0.93	2.37	1.13
P1+180	0.80	0.46	0.86	0.76	1.08	1.97	0.99
P1+240	0.84	0.48	0.78	0.81	1.19	1.48	0.88
Plt.average	0.72	0.38	1.06	0.67	0.71	2.17	

P1 = 90 kg P/ha,

LSD (5%) : Treatment = 0.04, Tree = 0.04, Soil = 0.03, Soil x treatment = 0.06,

Soil x tree = 0.06, Treatment x tree = 0.1, Soil x treatment x soil = 0.14

Table 18. Effect of six levels of nitrogen fertilizer in Toje soil amended with 90 kg P/ha on shoot dry matter yield (g/plant) of six tree legumes.

N-rates Kg N/ha	*Leucaena leucocephela*	*Acacia auricloformis*	*Crotalaria ochloleuca*	*Melletia thonningi*	*Acacia mangium*	*Cajanus cajan*	*Treat.* average
P1	0.55	0.43	0.67	0.57	0.46	0.64	0.55
P1+40	0.79	0.53	1.50	0.98	0.54	1.55	0.98
P1+80	0.88	0.71	1.68	1.46	0.57	1.67	1.16
P1+120	1.19	0.82	2.75	2.00	1.01	3.02	1.80
P1+180	1.32	0.95	2.54	2.03	1.46	3.28	1.93
P1 +240	1.76	1.25	2.17	2.05	1.48	3.20	1.99
Plt.average	1.08	0.78	1.89	1.52	0.92	2.23	

P1 = 90 kg P/ha

LSD (5%) : Treatment = 0.04 Tree = 0.04 Soil = 0.03

Soil x treatment = 0.06 Soil x tree = 0.06 Treatment x tree = 0.1

Soil x treatment x tree = 0.14

4.11 Influence of phosphorus, rhizobial inoculation and harvesting times on nodulation, shoot and root dry matter yields of *Acacia mangium*, *Albizia lebbek* and *Acacia auricloformis* in two soil types.

Tables 19, 20 and 21 provide information on the effect of phosphorus, rhizobial inoculation and times of harvest on nodulation, shoot and root dry matter yields of three tree legumes in two soil types.

Phosphorus application at 90 kg P/ha as in the previous phosphorus studies (Page 94), generally, resulted in significant (p=0.05) increases in number of nodules of all the tree legumes at all the three harvest times in the two soil types (Table 19 and 20). Application of P alone resulted in over 117%, 350% and 750% increases in number of nodules formed on *Acacia mangium*, *Albizia lebbek* and *Acacia auricloformis,* respectively in Hatso soil. In Toje soil, application of P to the tree legumes resulted in over 200%, 14% and 211% increases in number of nodules of *Acacia mangium*, *Albizia lebbek* and *Acacia auricloformis,* respectively. The percentage increases in number of nodules due to phosphorus application however decreased with increasing harvest times from 0-2 months to 4-6 months. For instance, in Hatso soil, the percentage increase in the number of nodules on *Acacia auricloformis* from 0-2 months to 4-6 months harvests ranged from 750% between planting and 2 months harvest to 45% between 4 months and 6 months harvest. Also in the case of *Albizia lebbek*, the percentage increase in number of nodules formed due to phosphorus application ranged from 350% between planting and 2 months harvest to 21% between 4 months and 6 months harvest.

Inoculation application generally resulted in significant (p=0.05) improvement in number of nodules of all the tree legumes in Hatso soil, ranging from 100% in *Acacia mangium* to 300% in both *Albizia lebbek* and *Acacia auricloformis* (Table 19). However, inoculation did not result in significant (p=0.05) improvement in number of nodules in Toje soil (Table 20) for all the tree

legumes. Increasing harvesting times from 0-2 months to 4-6 months resulted in a decrease in percentage increase in number of nodules due to *Rhizobium* inoculation in Hatso soil, but in Toje soil, the percentage increase in number of nodules for all the tree legumes increased from 0-2 months harvest to 2-4 months harvest and decreased at 4-6 month harvest. For example, in Hatso soil the percentage increase in number of nodules of *Acacia mangium* due to *Rhizobium* inoculation ranged from 100% at 0-2 months harvest to 3% at 4-6 months harvest. In *Albizia lebbek* and *Acacia auricloformis*, the percentage increase in number of nodules ranged from 300% each to 29% and 8%, respectively.

Harvesting times significantly improved number of nodules of the tree legumes at all treatment levels in both soils. The percentage increase in number of nodules on the tree legumes for all treatments was highest at the 2-4 month harvest in both soils.

In all the soils and for all treatments, number of nodules of the tree legumes was in the order $P_+I_+ > P_+I_0 > P_0I_+ > P_0I_0$. The data indicates a greater influence of P on nodulation than inoculation.

Table 19. Number of nodules formed on three tree legumes in Hatso soil at three harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Albizia lebbek*			*Acacia auricloformis*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	6	27	67	2	16	38	2	21	38
P_+I_0	13	33	85	9	42	46	17	39	55
P_0I_+	12	59	69	8	44	49	8	34	41
P_+I_+	31	91	94	10	57	58	18	63	72
Treat. Av.	15	53	78	7	40	48	10	39	52

Lsd (5 %) Soils = 5.2 Trees = 6.3 Phosphorus = 5.2 Inoculation = 5.2 Harvesting time = 6.3 Phosphorus x Tree = 8.5

Phosph x Harv =10.7 Inoc x Harv =10.7 Phosphorus x Inoc. x Harv = 12.5

Table 20. Number of nodules formed on three tree legumes in Toje soil at three harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Albizia lebbek*			*Acacia auricloformis*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	5	34	55	7	33	36	9	51	71
P_+I_0	6	107	144	16	39	53	28	101	164
P_0I_+	5	83	88	9	42	43	11	92	101
P_+I_+	8	101	173	17	54	110	32	113	193
Treat. Av.	6	82	115	12	42	61	20	89	132

Lsd (5 %) Soils = 5.2 Trees = 6.3 Phosphorus = 5.2 Inoculation = 5.2 Harvesting time = 6.3 Phosph x Tree = 8.5

Phosph x Harv =10.7 Inoc x Harv =10.7 Phosph x Ino X Harv =12.5

The influence of phosphorus, inoculation and time of harvest on shoot dry matter yield of three tree legumes in two soil types is presented in Tables 21 and 22.

Phosphorus application resulted in significant (p=0.05) improvement in shoot dry matter yield of the tree legumes. Application of phosphorus to the tree legumes in Hatso soil resulted in 175%, 61% and 160% increases in shoot dry matter of *Acacia mangium*, *Albizia lebbek* and *Acacia auricloformis* respectively, over 2 months growing period. In Toje soil however, P application resulted in 83%, 142% and 402% increases in shoot dry matter yields of the tree legumes over the same period.

The percentage increases in shoot dry matter yield of the tree legumes due to phosphorus application however varied with increasing harvesting periods in both soils. For instance, in Hatso soil, the percentage increase in dry matter yields from planting to 2 months harvest to 4 month planting to 6 months harvest for *Acacia mangium* and *Acacia auricloformis* decreased from 175% to 19% and from 160% to 41% respectively, whiles in the case of *Albizia lebbek* in Hatso soil, the percentage increase in dry matter yields increased from 61% from planting to 2 month harvest to 144% from 4 month planting to 6 month harvest.

Rhizobium inoculation of the tree legumes resulted in significant (p=0.05) improvements in shoot dry matter yield of all the tree legumes except *Albizia lebbek* in both soils. Inoculation of *Acacia mangium* and *Acacia auricloformis* in Hatso soil resulted in increases of more than 33% and 100% in dry matter yield, respectively, and in Toje soil, inoculation application resulted in increases of 45% and 11%, respectively, in shoot dry matter yield. Increasing harvest times resulted in a decrease in percentage increase in dry matter yields of *Acacia mangium* in both soils but in Toje soil, percentage increase in shoot dry matter yield of *Acacia auricloformis* increased with increased harvest times.

Shoot dry matter yields of all the tree legumes increased significantly ($p=0.05$) with increasing time of harvest for all the treatments. The highest dry matter yield for all the tree legumes occurred at the 6^{th} month harvest, however on the average and for all treatments, the percentage increase in shoot dry matter yield was highest at the 2 months after planting and 4 months after planting.

In both soils and for all treatments, shoot dry matter yield was in the order $P_+I_+ > P_+I_0 > P_0I_+ > P_0I_0$.

The data indicates a greater influence of P on shoot dry matter yields than inoculation.

Table 21. Shoot dry matter yields (g/plant) of three tree legumes in Hatso soil at three harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Albizia lebbek*			*Acacia auricloformis*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	0.12	3.50	11.65	1.60	5.93	5.98	0.30	2.63	8.12
P_+I_0	0.33	8.37	13.85	2.58	13.77	14.60	0.78	6.73	11.44
P_0I_+	0.16	4.74	14.16	1.57	8.01	9.67	0.60	4.18	11.21
P_+I_+	0.51	8.55	17.77	3.59	16.20	18.59	0.89	7.73	17.18
Treat. Av.	0.28	6.29	14.36	2.34	10.98	12.21	0.64	5.32	11.99

Lsd (5 %) Soils = 0.1Trees = 0.2Phosphorus = 0.1Inoculation = 0.1Harvesting time = 0.2

Tree xPhosph =0.4 Tree x Inoc =0.3 Inoc x Harv Time = 0.2 Tree x Inoc x Harv Time = 0.4

Tree x Phosph x Harv Time = 0.4

Table 22. Shoot dry matter yields (g/plant) of three tree legumes in Toje soil at three Harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Albizia lebbek*			*Acacia auricloformis*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	0.29	2.95	12.02	1.63	2.83	3.52	0.38	3.12	9.13
P_+I_0	0.53	9.19	17.16	3.94	8.63	10.21	1.91	7.47	13.41
P_0I_+	0.42	5.85	12.77	1.71	3.70	5.33	0.42	3.95	11.77
P_+I_+	1.24	10.81	19.16	4.06	11.64	14.50	2.53	8.11	18.47
Treat. Av.	0.62	7.2	15.28	2.84	6.70	8.39	1.31	5.66	13.20

Lsd (5 %) Soils = 0.1 Trees = 0.2 Phosphorus = 0.1 Inoculation = 0.1 Harvesting time = 0.2

Tree xPhosph = 0.4 Tree x Inoc = 0.3 Inoc x Harv Time = 0.2 Tree x Inoc x Harv Time = 0.4

Tree x Phosph x Harv Time = 0.4

Table 23 and 24 provide information on the influence of phosphorus, inoculation and times of harvest on root dry matter yield of some tree legumes.

Root dry matter yields of the tree legumes increased significantly ($p=0.05$) with application of phosphorus, inoculation and increasing time of harvest. On average, the highest root biomass for all the tree legumes occurred in Hatso soil. In all the soils and for all treatments, root dry matter yield was in the order $P_+I_+ > P_+I_0 > P_0I_+ > P_0I_0$. The data indicates a greater influence of P on root dry matter yields than inoculation.

Table 23. Root dry matter yields (g/plant) of three tree legumes in Hatso soil at three Harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Albizia lebbek*			*Acacia auricloformis*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	0.08	3.45	6.43	0.72	5.65	5.98	0.08	2.15	3.01
P_+I_0	0.31	4.44	7.44	0.89	9.53	12.03	0.36	2.74	4.71
P_0I_+	0.12	3.49	7.32	1.57	8.01	9.67	1.93	5.73	9.38
P_+I_+	0.45	5.31	8.58	2.63	9.70	13.84	0.45	5.12	8.11
Treat. Av.	0.24	4.17	7.44	1.45	8.32	10.38	0.71	3.94	6.30

Lsd (5 %) Soils = 0.12 Trees = 0.14 Phosphorus = 0.12 Inoculation = 0.12 Harvesting time = 0.14

Tree x Phosph = 0.18 Tree x Inoc =0.16 Tree x Harv Time = 0.16 Tree x Inoc x Harv Time=0.2

Tree x Inoc x Phosph = 0.22

Table 24. Root dry matter yields (g/plant) of three tree legumes in Toje soil at three Harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Albizia lebbek*			*Acacia auricloformis*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	0.11	2.87	3.46	0.81	4.18	5.74	0.17	3.16	4.13
P_+I_0	0.22	3.91	8.80	3.04	7.61	10.86	0.59	3.88	5.36
P_0I_+	0.17	4.20	5.93	1.68	6.13	6.48	0.18	4.01	6.31
P_+I_+	0.43	8.33	8.91	3.29	7.95	12.01	0.61	5.89	7.93
Treat. Av.	0.23	4.83	6.78	2.21	6.47	8.77	0.39	4.24	5.93

Lsd (5 %) Soils = 0.12 Trees = 0.14 Phosphorus = 0.12 Inoculation = 0.12 Harvesting time = 0.14

Tree x Phosph = 0.18 Tree x Inoc = 0.16 Tree x Harv Time = 0.16 Tree x Inoc x Harv Time = 0.2

Tree x Inoc x Phosph = 0.22

4.12 Influence of phosphorus, inoculation and harvesting periods on % and total N fixed in three tree legumes in two soil types.

Tables 25 and 26 provide information on the proportion of N fixed by three tree legumes in two soil types.

The proportion of N fixed by all the tree legumes at all treatment levels increased significantly (p=0.05) from the 1st harvest (2 months) to the 2nd harvest (4 months) but decreased at the 3rd harvest (6 months) in Hatso soil, especially in the case of *Albizia lebbek* across all treatments (Table 25). However, in Toje soil, increasing harvesting time from 4 months to 6 months resulted in further increase in % N fixed especially in *Albizia lebbek* and *Acacia auricloformis* across treatments. For the 6 months growing period, *Acacia mangium* derived 90% (where no P and Inoculation were applied) to 100% (where both P and inoculation were applied) of its N requirements from fixation (Table 25 and 26) as against 50% to 90% in the case of *Albizia lebbek* and 75 to100% in the case of *Acacia auricloformis* in both soils.

In general, phosphorus fertilizer application resulted in significant (p=0.05) increases in the proportion of N fixed by all the tree legumes at all harvesting times in both soils except for *Albizia lebbek* at 2 months harvest in Hatso soil (Table 25). The effect of P on the proportion of N fixed by tree legumes decreased with increasing harvesting periods in both soils.

Inoculation of the tree legumes resulted in significant increases in % N fixed in *Acacia mangium* and *Acacia auricloformis* in the first 2 months of growth and also at the 6 months harvest in Hatso soil, whilst % N fixed in *Albizia lebbek* in Hatso soil was significantly (p=0.05) different at both 4 months and 6 months harvesting periods. In Toje soil however, % N fixed increased

significantly (p=0.05) at all harvesting times except for *Acacia mangium* at the 6 months after planting and *Albizia lebbek* at the 2 months after planting (Table 26).

Application of phosphorus and inoculation together further enhanced the proportion of N fixed significantly in both soils. For instance, combined application of phosphorus and inoculation almost quadrupled the % N fixed by *Acacia mangium* in Hatso soil just after 2 months (Table 25.). In general, for all the tree legumes and for all treatments, *Acacia mangium* fixed the highest proportion of its N requirement followed by *Acacia auricloformis* and least by *Albizia lebbek*. Also for all the soils and the tree legumes, the treatments were of the trend $P_+I_+ > P_+I_0 > P_0I_+ > P_0I_0$. These findings indicate the greater influence of P on % and total N fixed compared to inoculation.

Table 25. Percent N fixed in three tree legumes in Hatso soil at three Harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Acacia auricloformis*			*Albizia lebbek*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	20.95	90.55	89.25	19.57	69.80	74.29	14.24	82.14	50.29
P_+I_0	68.81	95.94	94.71	32.82	92.92	97.18	13.68	92.34	78.38
P_0I_+	67.02	93.15	93.72	20.31	83.16	87.89	10.78	85.03	79.84
P_+I_+	76.54	95.94	98.83	66.98	95.20	98.55	20.47	92.84	90.34
Treat. Av.	58.33	93.90	94.13	34.92	85.27	89.48	14.79	88.09	74.71

Lsd (5 %) Soils = 4.6 Trees = 5.6 Phosphorus = 4.6 Inoculation = 4.6 Harvesting times = 5.6 Phosph x Inoc. =6.4 Phosph x Harv =7.9 Inoc x Harv =10.7 Phosph x Inoc x Harv =11.1

Table 26. Percent N fixed in three tree legumes in Toje soil at three Harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Acacia auricloformis*			*Albizia lebbek*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	39.51	79.10	88.17	45.27	75.87	83.40	34.24	47.77	47.17
P_+I_0	55.81	95.51	98.26	63.31	89.82	90.22	60.32	65.34	75.47
P_0I_+	68.91	97.78	89.58	53.19	90.21	92.54	32.93	57.42	60.47
P_+I_+	60.02	97.61	94.92	75.53	97.78	98.50	46.37	69.50	86.85
Treat. Av.	56.06	92.50	92.73	59.33	88.42	91.17	46.85	56.59	67.49

Lsd (5 %) Soils = 4.6 Trees = 5.6 Phosphorus = 4.6 Inoculation = 4.6 Harvesting time = 5.6 Phosh x Inoc. =6.4 Phosph x Harv =7.9 Inoc x Harv =10.7 Phosph x Inoc x Harv =11.1

The total amounts of N fixed by the tree legumes in response to phosphorus application and inoculation in the two soil types are provided in Tables 27 and 28.

The amount of N fixed by the tree legumes increased significantly(p=0.05) with increasing harvesting periods for all treatments in both soils with the highest amount of N fixed occurring at the 6th month for all the tree legumes in both soils. The harvesting period from 2 months to 4 months resulted in the highest proportional increase in the amount of N fixed in *Acacia mangium* for all treatments, in both soils, whilst increasing the harvesting period from 4 months to 6 months resulted in the highest increase in the amount of N fixed in *Albizia lebbek* and *Acacia auricloformis* in both soils.

Phosphorus fertilizer application, generally, resulted in significant increase in amount of N fixed in the tree legumes in both soils except *Acacia auricloformis* at the 2 months harvest in Hatso soil (Table 27). Phosphorus response to amount N fixed was more pronounced in *Acacia mangium* and *Acacia auricloformis* at the 4th month harvest but at 6th month harvest in the case of *Albizia lebbek* in Hatso soil, whiles in Toje soil, phosphorus response to amount N fixed by the tree legumes was more pronounced at 2 months harvest for *Acacia auricloformis* and *Albizia lebbek* but at 4 months harvest for *Acacia mangium* (Table 28).

Rhizobium inoculation did not result in significant (p=0.05) increase in amount of N fixed for all the tree legumes at 2 months harvest in both soils, except for *Albizia lebbek* in Toje soil (Table 28). However, increasing harvesting time to 4 month and 6 month resulted in significant (p=0.05) increases in amount of N fixed in all the tree legumes in both soils.

In general, the total N fixed for the tree legumes for both soils was of the trend $P_+I_+ > P_+I_0 > P_0I_+ > P_0I_0$

By examining the nodulation, growth and nitrogen fixing potential of the tree legumes, *Acacia mangium* was found to have better nitrogen fixing potential followed by *Acacia auricloformis* and *Albizia lebbek* being the least. This is because of the ability of *Acacia mangium* to combine high biomass production with high proportion of nitrogen fixed.

Table 27. Total N fixed (mg N/plant) in three tree legumes in Hatso soil at three Harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Acacia auricloformis*			*Albizia lebbek*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	44.53	1696.43	4776.73	119.22	191.53	4105.08	427.10	890.30	2570.48
P_+I_0	185.77	3497.28	5760.56	255.36	357.09	4467.48	675.13	1201.62	7449.41
P_0I_+	84.57	2104.75	5765.25	157.33	334.88	4892.64	338.35	849.03	4810.60
P_+I_+	269.37	3570.32	6679.02	475.57	368.80	6551.90	962.55	1640.07	8716.58
Av.	146.06	2717.20	5745.39	251.87	313.08	5004.28	600.78	1145.26	5886.77

Lsd (5 %) Soils = 147.5 Trees = 180.6 Phosphorus = 147.5 Inoculation =147.5 Harvesting time = 180.59 Phosh x Inoc. =208.5 Phosph x Harv =255.4 Inoc x Harv =255.4 Phosph x Inoc X Harv =361.2

Table 28. Total N fixed (mg N/plant) in three tree legumes in Toje soil at three Harvest times with or without the application of phosphorus and *Rhizobium* inoculation.

	Acacia mangium			*Acacia auricloformis*			*Albizia lebbek*		
	Harvesting Times (Months)								
Treatments	2	4	6	2	4	6	2	4	6
P_0I_0	163.73	1709.77	5049.23	211.50	1839.99	4160.59	7851.16	1367.65	1551.48
P_+I_0	311.78	3895.99	6480.50	863.35	3638.71	5608.78	2523.02	3380.12	4738.99
P_0I_+	246.10	2319.60	6311.88	211.22	1913.82	4733.44	962.44	1102.05	2582.78
P_+I_+	711.18	4316.30	7524.01	1259.66	3219.14	6965.69	2200.00	2491.58	6413.06
Av.	477.5	4080.5	8455.2	8485.5	3537.2	7156.1	4512.2	8338.4	5095.4

Lsd (5 %) Soils = 147.5 Trees = 180.6 Phosphorus = 147.5 Inoculation =147.5 Harvesting time = 180.59 Phosh x Inoc. =208.5

Phosph x Harv =255.4 Inoc x Harv =255.4 Phosph x Inoc X Harv =361

4.13 Physiologic and metabolic properties of 200 indigenous rhizobia isolated from five tree legumes (*Albizia lebbek, Acacia mangium, Acacia auricloformis, Leucaena leucocephela* and *Melletia thonningi*).

Table 29 summarizes the physiological and metabolic properties of 200 indigenous tree legume rhizobia in three soil types.

The tree legume rhizobia were diverse in terms of their colony morphology, reaction with bromothymol blue (BTB), growth at different pH ranges as well as in their ability to utilize different carbon sources.

Fifty four percent (54%) of the total isolates acidified the YEM medium between 3 to 5 days of growth and were considered as fast growers and thus classified as *Rhizobium* while 46% of the total isolates alkalined the medium between 5-7 days and were considered as slow growers and classified as *Bradyrhizobium* (Table 29). None of the tree legumes was nodulated by only a single type of *Rhizobium* in terms of either fast or slow growing rhizobia. Each tree legume was however predominantly nodulated by either *Rhizobium* or *Bradyrhizobium.* Apart from *Acacia mangium* and *Acacia auricloformis* which were predominantly nodulated by 57.5% and 51.5% *Bradyrhizobium* strains respectively, the rest of the tree legumes were predominantly nodulated by *Rhizobium* (Fig. 12). For example, 70% of rhizobia that nodulated *Albizia lebbek* belonged to the genus *Rhizobium* (Fig. 13).

Morphologically, colonies of the isolates were classified as dry (D), wet gummy firm (WGF), or wet gummy soft (WGS) based on their texture. All the tree legumes were nodulated by all three colony types of rhizobia, thus dry, wet gummy firm and wet gummy soft rhizobia isolates except *Acacia mangium* that was nodulated by only wet gummy firm and wet gummy soft rhizobia

isolates (Fig.14). Apart from *Acacia auricloformis* and *Melletia thonningi* which were predominantly nodulated by wet gummy firm rhizobia isolates, all the other tree legumes were predominantly nodulated by wet gummy soft rhizobia isolates (Fig. 14). For instance, *Albizia lebbek*, *Leucaena leucocephela* and *Acacia mangium* were predominantly nodulated by 50%, 62.5% and 80% respectively of wet gummy soft rhizobia isolates. In all, only 15% of the total isolates were considered dry while 32.5% and 52.5% were considered wet gummy firm and wet gummy soft, respectively (Fig.15). All the isolates from Alajo soil series that nodulated the tree legumes were considered wet gummy soft but Toje and Hatso soil series harboured all the three types of isolates (i.e., dry, wet gummy firm and wet gummy soft).

Each of the 40 isolates from each tree legume varied in terms of its ability to grow in media at different pH levels. In general, the inability of the tree legume rhizobia isolates to grow in media at different pH levels increased with decreasing pH levels. For instance, at pH of 3.5, 40% and 35%, respectively, of *Albizia lebbek* and *Acacia mangium* rhizobia isolates could not grow and also 30% each of *Acacia auricloformis* and *Leucaena leucocephela* rhizobia isolates could not grow (Fig. 16). In contrast, almost all the tree legume isolates could grow in the alkaline pH range from 7.5 to 9.5 (Fig.17).

Seventy percent (70%) of the total isolate of 200 could grow at all the pH ranges (3.5 - 9.5) tested. However, thirty percent of the total of 200 isolates could not grow at a pH of 3.5 while 13.5% and 7% could not grow at pH 4.5 and 5.5, respectively. Majority of the isolates that could not grow at acidic pH ranges (pH 3.5, 4.5 and 5.5) were considered to be fast growers or *Rhizobium*. More than 66% of the isolates that could not grow at pH 3.5 were *Rhizobium* while more than 77% and 71% of the isolates that could not grow at pH 4.5 and 5.5, respectively were *Bradyrhizobium*.

Table 29. Physiological and metabolic characterization of some indigenous tree legume rhizobia in three soil types.

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
1	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
3	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
4	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
5	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
6	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
7	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
8	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
9	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
10	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	WGF
11	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D

Key

(+) Means positive response (-) Means negative response

ARA = Arabinose FRU = Fructose GAL = Galactose GLU = Glucose MAN = Mannitol

SUC = Sucrose

WGS = Wet gummy soft; WGF = Wet gummy firm; and D = Dry

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
12	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
13	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
14	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
15	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
16	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
17	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
18	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGS
19	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
20	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
21	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGF
22	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
23	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	D
24	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
25	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
26	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
27	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
28	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
29	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
30	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGF
31	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGF
32	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
33	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
34	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
35	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
36	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
37	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
38	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
39	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
40	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
41	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
42	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
43	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
44	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
45	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
46	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
47	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
48	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
49	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
50	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
51	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
52	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
53	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
54	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
55	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
56	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
57	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
58	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
59	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
60	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
61	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS

Table 29 continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
62	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
63	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
64	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
65	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
66	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
67	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
68	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
69	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
70	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
71	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
72	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
73	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
74	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
75	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
76	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
77	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
78	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
79	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
80	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
81	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
82	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
83	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
84	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGS
85	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
86	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
87	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
88	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
89	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
90	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
91	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
92	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
93	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
94	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
95	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
96	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
97	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
98	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
99	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
100	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
101	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
102	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	WGF
103	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
104	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
105	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
106	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
107	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
108	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
109	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	D

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
110	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
111	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
112	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
113	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
114	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
115	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
116	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
117	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
118	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
119	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
120	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
121	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
122	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
123	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGF
124	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGF
125	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
126	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
127	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
128	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
129	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
130	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
131	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
132	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
133	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
134	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
135	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
136	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	WGF
137	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
138	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
139	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGS
140	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	WGS
141	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
142	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
143	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
144	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
145	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
146	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
147	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
148	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
149	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
150	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
151	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
152	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
153	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
154	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
155	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
156	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
157	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
158	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
159	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
160	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
161	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
162	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
163	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
164	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGS
165	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGF
166	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGF
167	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	WGS
168	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	WGS
169	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
170	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
171	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
172	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
173	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
174	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
175	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
176	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
177	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
178	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
179	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
180	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGS
181	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
182	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	WGF
183	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
184	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
185	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
186	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
187	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
188	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
189	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
190	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF

Table 29 Continued

Isolates	Growth		pH						Carbon Sources							Colony Morph.
	Fast	Slow	3.5	4.5	5.5	7.5	8.5	9.5	ARA	FRU	GAL	GLU	MAL	MAN	SUC	
191	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
192	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	D
193	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	D
194	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	D
195	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
196	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	D
197	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
198	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
199	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF
200	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	WGF

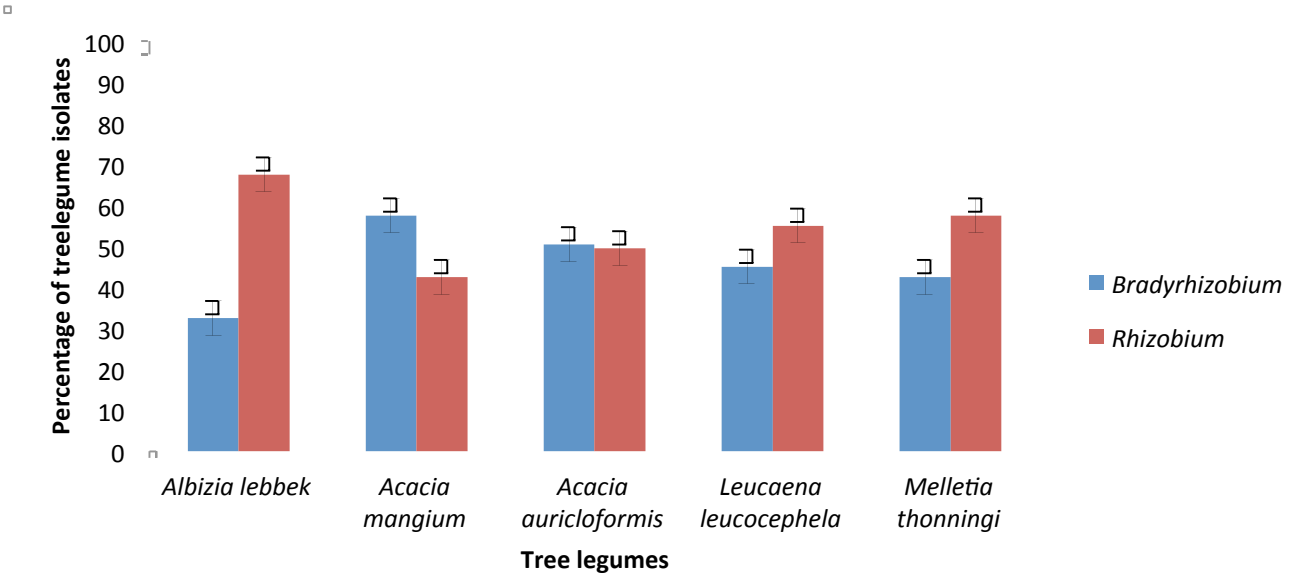

Fig. 12. Classification of 40 rhizobia isolates obtained from each of the five tree legumes, based on their reactions with Bromothymol blue.

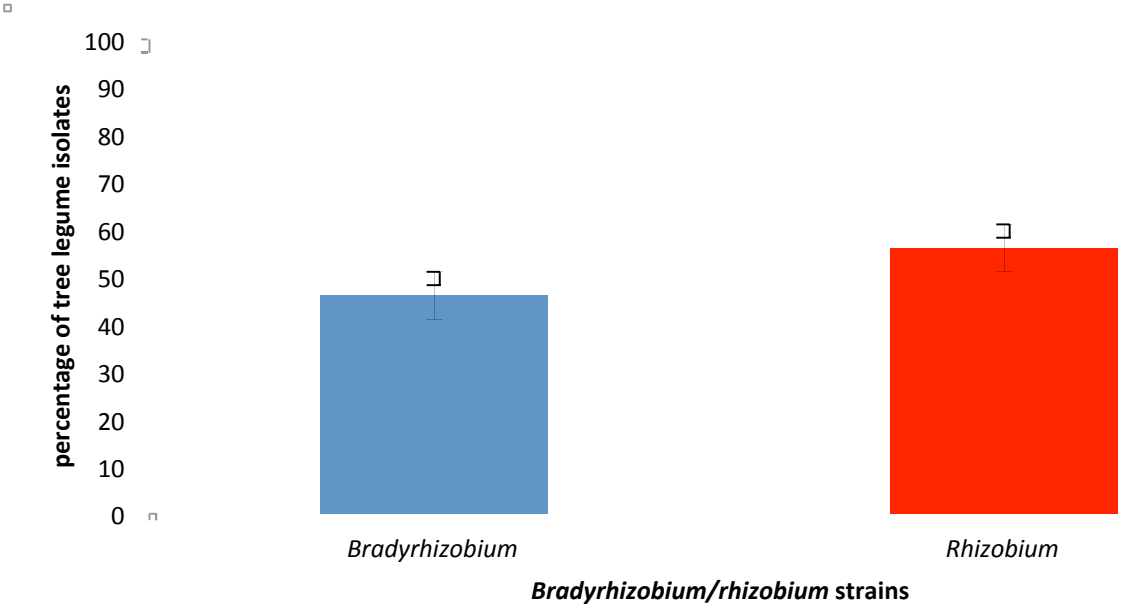

Fig. 13. Classification of 200 rhizobia isolates obtained from five tree legumes, based on their reactions with Bromothymol blue.

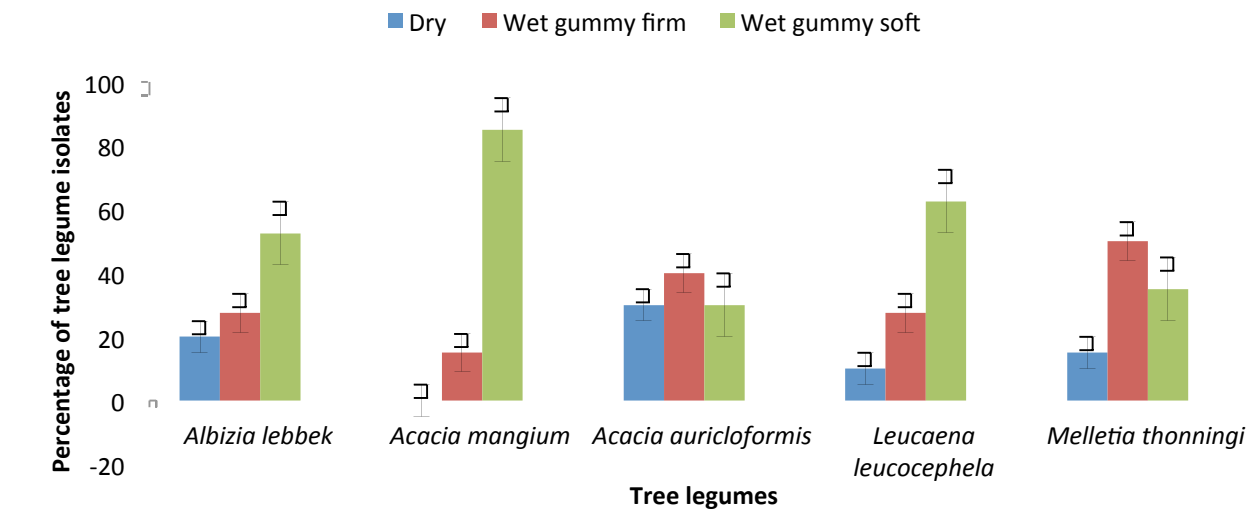

Fig. 14. Classification based on colony morphology of 40 rhizobia isolates that nodulated each of the five tree legumes.

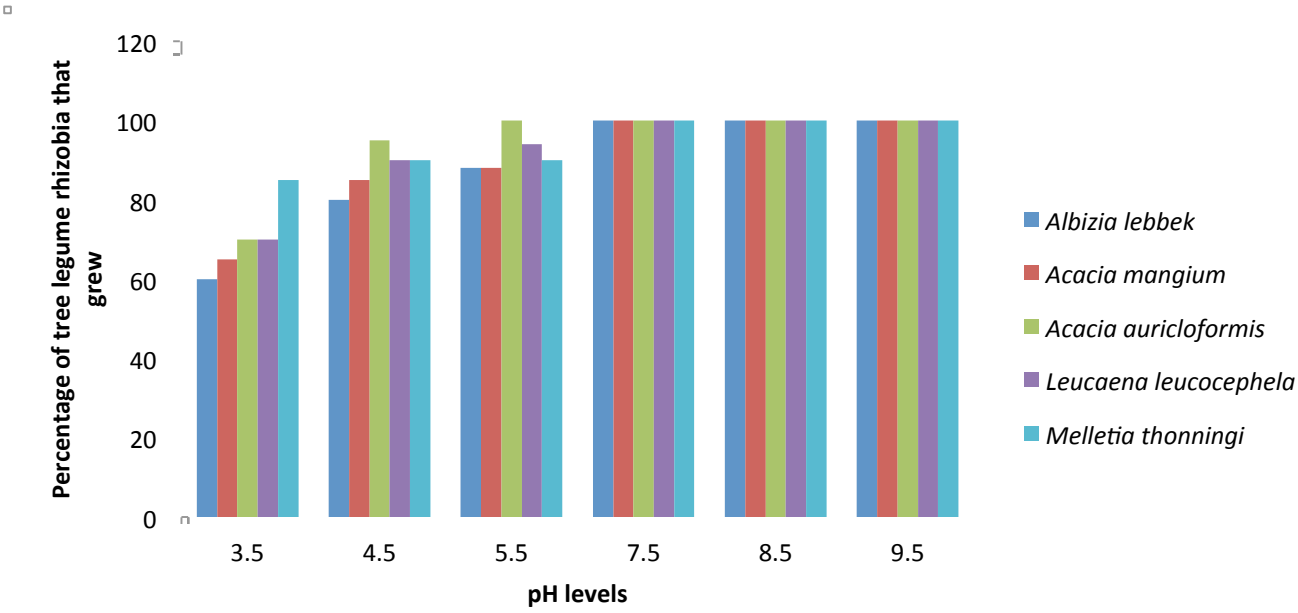

Fig. 15. Effect of six pH levels on 40 rhizobia isolated from each of the five tree legumes

4.14 Molecular Characterization of 60 rhizobia isolated from five tree legumes.

Sixty of the rhizobia isolates were randomly selected as representative strains of the tree legumes rhizobia after phenotypic and metabolic characterization. The relationships among the isolates were then assessed using molecular tools. Table 30 provides a list of strains used for this study and their host legumes.

Table 30. List of *Rhizobium* isolates used in the study and their respective host legumes

Host Tree Legumes	Isolate numbers
Albizia lebbek	2, 3, 4, 6, 7, 8, 9, 10, 15, 30, 42, 45, 48, 51.
Acacia mangium	5, 11, 12, 13, 14, 17, 20, 53, 58, 61, 62.
Acacia auricloformis	1, 16, 21, 23, 24, 25, 26, 28, 52, 54, 55, 56, 57.
Leucaena leucocephela	19, 22, 27, 31, 33, 35, 37, 38, 39, 40, 50
Melletia thonningi	18, 29, 32, 34, 36, 43, 44, 47, 49, 59, 60

4.14.1 PCR amplification and RFLP analysis of 16S rRNA genes of five tree legumes rhizobia.

PCR amplification of 16S rRNA genes of almost all the 60 rhizobia isolates of the tree legumes produced a single band of size 1.5 kb.

Restriction of the 16S rRNA genes of the tree legumes rhizobia with each of the following restriction enzymes, Hae111, Alu 1, Hpa I, Hpa II and Rsa 1 produced multiple bands (fig.20). Although each restriction enzyme produced polymorphic banding patterns, the most discriminative ones were those obtained with Rsa 1 and Hpa II. The RFLP patterns of the 16S rRNA obtained with Hpa II is shown

in Fig.18. The results delineated 60 different 16S rRNA genotypic patterns with all the five endonucleases (Table 31)

Combined cluster analysis of all the 16S rRNA restriction patterns showed that, the isolates can be grouped into two distinct clusters A and B at about 45% similarity level and five major sub-clusters at 70% similarity level (Fig. 18). Almost 62% of the isolates in cluster A were those predominantly obtained from *Albizia lebbek* with cluster B containing a mixture of isolates obtained from all the tree legumes. At about 45% level of similarity, isolates from *Leucaena leucocephela* appeared distinct from those of *Albizia lebbek*.

Cluster B was further sub-grouped into sub-clusters at higher similarity levels and the data indicated that all the sub-groups consisted of a mixture of isolates from all the tree legumes. Majority of the isolates from *Acacia mangium* appeared to be closely related to isolates from *Acacia auricloformis* at 68% similarity level as most of their respective isolates clustered in one sub-group. Majority of the isolates were separated at a similarity level between 80% and 90%.

Table 31.The 16S rRNA restriction patterns of 60 *(Brady)rhizobium* isolates obtained from five tree legumes in three soil types.

Isolate Number	Host Legume	soil type (series)	16S rRNA patterns (genotypes)1
1	*Acacia auricloformis*	Toje	Aaaaa
2	*Albizia lebbek*	Toje	Abbab
3	*Albizia lebbek*	Hatso	Bccbc
4	*Albizia lebbek*	Hatso	Bcccc
5	*Acacia mangium*	Toje	Cdccc
6	*Albizia lebbek*	Toje	Cedcd
7	*Albizia lebbek*	Alajo	Ccede
8	*Albizia lebbek*	Toje	Cdfee
9	*Albizia lebbek*	Hatso	Bfcff
10	*Albizia lebbek*	Toje	Aecgf
11	*Acacia mangium*	Toje	Bdghd
12	*Acacia mangium*	Hatso	Bdhic
13	*Acacia mangium*	Hatso	Cdfic
14	*Acacia mangium*	Hatso	Cehfc
15	*Albizia lebbek*	Alajo	Cdgec
16	*Acacia auricloformis*	Toje	Bdikf
17	*Acacia mangium*	Hatso	Bgggg
18	*Melletia thonningi*	Hatso	Dccgc
19	*Leucaena leucocephela*	Hatso	Bcfkc
20	*Acacia mangium*	Toje	Degkc
21	*Acacia auricloformis*	Toje	Bhgkh
22	*Leucaena leucocephela*	Alajo	Bhjgd
23	*Acacia auricloformis*	Toje	Dedle
24	*Acacia auricloformis*	Toje	Cagkf

Table 31 continued

Isolate Number	Host Legume	soil type (series)	16S rRNA patterns (genotypes)1
25	*Acacia auricloformis*	Toje	Ciggd
26	*Acacia auricloformis*	Hatso	Begci
27	*Leucaena leucocephela*	Hatso	Cjged
28	*Acacia auricloformis*	Toje	Ceded
29	*Melletia thonningi*	Hatso	Eegbf
30	*Albizia lebbek*	Hatso	Begcj
31	*Leucaena leucocephela*	Hatso	Eifdj
32	*Melletia thonningi*	Toje	Feded
33	*Leucaena leucocephela*	Hatso	Ajgef
34	*Melletia thonningi*	Alajo	Gkbdf
35	*Leucaena leucocephela*	Toje	Hegdj
36	*Melletia thonningi*	Toje	Bafhj
37	*Leucaena leucocephela*	Hatso	Aacij
38	*Leucaena leucocephela*	Alajo	Fechj
39	*Leucaena leucocephela*	Toje	Befik
40	*Leucaena leucocephela*	Alajo	Bgdbd
42	*Albizia lebbek*	Hatso	Aekbd
43	*Melletia thonningi*	Hatso	Fefcc
44	*Melletia thonningi*	Toje	Bgccc
45	*Albizia lebbek*	Toje	Iefdc
47	*Melletia thonningi*	Toje	Bicef
48	*Albizia lebbek*	Hatso	Cefkc
49	*Melletia thonningi*	Hatso	Blgeb
50	*Leucaena leucocephela*	Hatso	Cedef

Table 31 continued

Isolate Number	Host Legume	soil type (series)	16S rRNA patterns (genotypes)1
51	*Albizia lebbek*	Toje	Blgkf
52	*Acacia auricloformis*	Toje	Cmblf
53	*Acacia mangium*	Toje	Jdglc
54	*Acacia auricloformis*	Toje	Cedgd
55	*Acacia auricloformis*	Toje	Bdggb
56	*Acacia auricloformis*	Hatso	Bdgbb
57	*Acacia auricloformis*	Toje	Cagcc
58	*Acacia mangium*	Hatso	Bagbg
59	*Melletia thonningi*	Toje	Cegbh
60	*Melletia thonningi*	Toje	Bagce
61	*Acacia mangium*	Toje	Bdgae
62	*Acacia mangium*	Hatso	Cagbe

[1] Letters refer to the type of restriction pattern of 16S rRNA digested with Hae III, Rsa I, Hpa I, Hpa II and Alu I

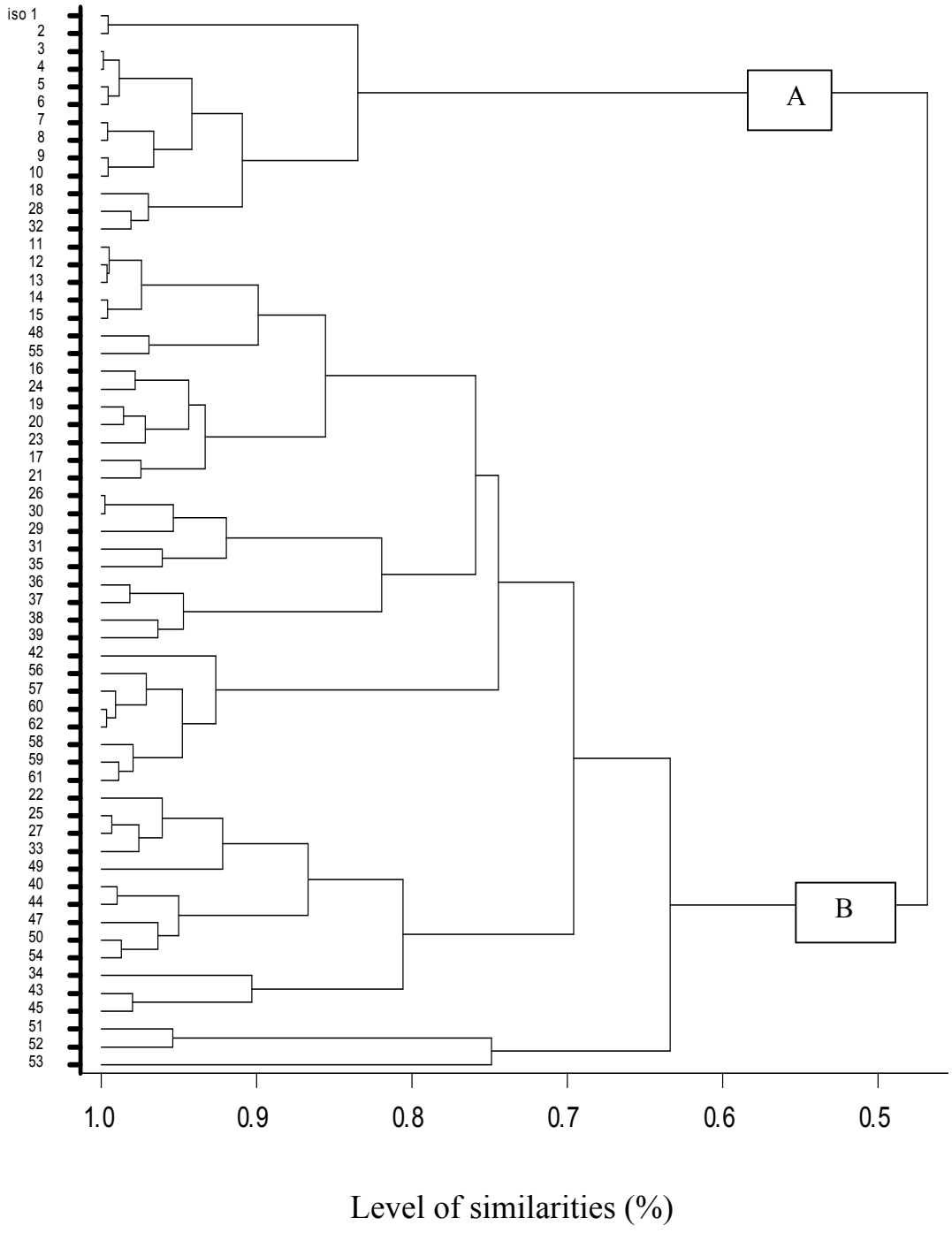

Fig. 17 Dendrogram of indigenous tree legume rhizobia based on combined Hae III, Rsa I, Hpa I, Hpa II and Alu I restriction patterns of amplified 16S rRNA . The data were clusted by using UPGMA in the Bionumeric program.

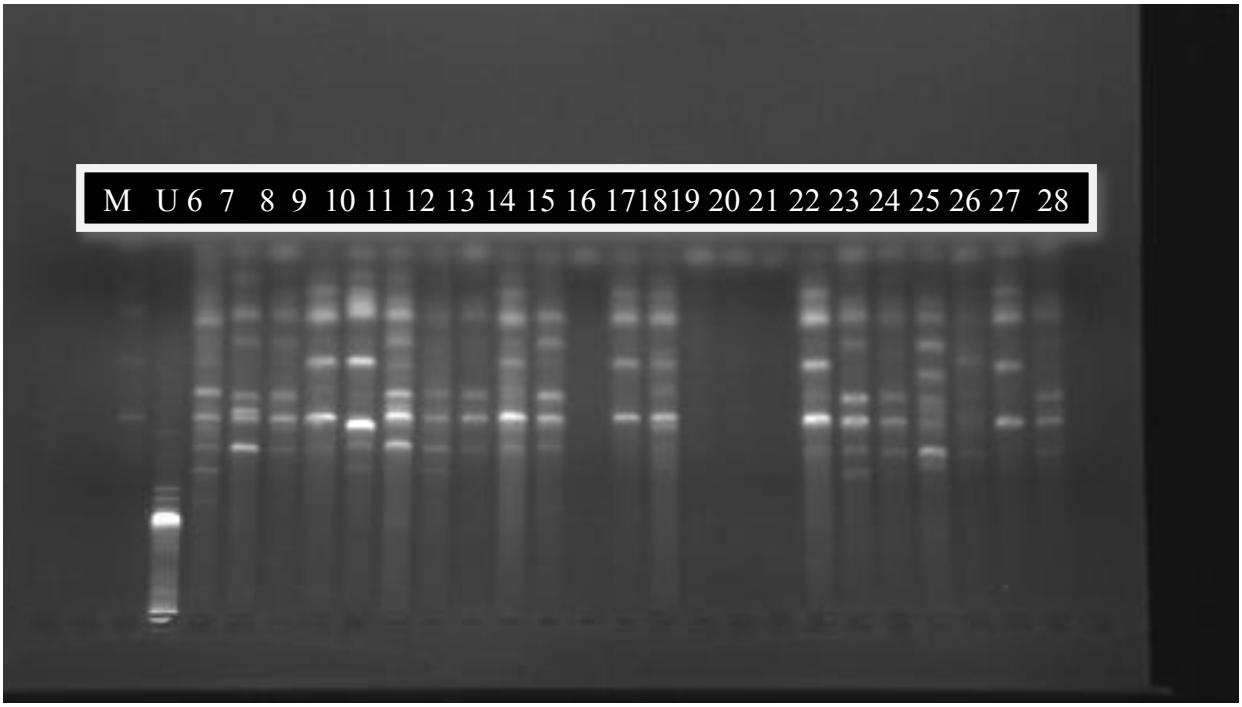

Fig. 18. Restriction patterns of PCR-amplified 16S rRNA digested with Hpa II. Molecular size marker (M) : 2k bp ladder, with U, the control representing uncut 16S rRNA gene.

4.14.2. PCR amplification and RFLP analysis of the 16S and 23S rRNA intergenic spacer (ITS) of some indigenous tree legume isolates.

Polymerase chain reaction (PCR) amplification of the intergenic spacer (ITS) between the 16S and 23S rRNA genes of all the isolates produced multiple polymorphic bands with size ranging from 800 bp to 1.4 kbp (Fig.20). Cluster analysis of undigested PCR product of the isolates ITS shows that, the isolates could be grouped into two main clusters, namely A and B at about 10% similarity level (Fig.21). Cluster A consisted of isolates that were predominantly obtained from *Albizia lebbek, A. mangium* and *A. auricloformis*, whiles cluster B consisted of isolates that were predominantly

obtained from *Leucaena leucocephela* and *Melletia thonningi*. Each cluster was further separated into sub-groups with each sub-group containing a mixture of isolates obtained from all the tree legumes

Restriction of the ITS with the three enzymes Hae III, Alu I and Hpa II did not produce much further differences among the isolates.

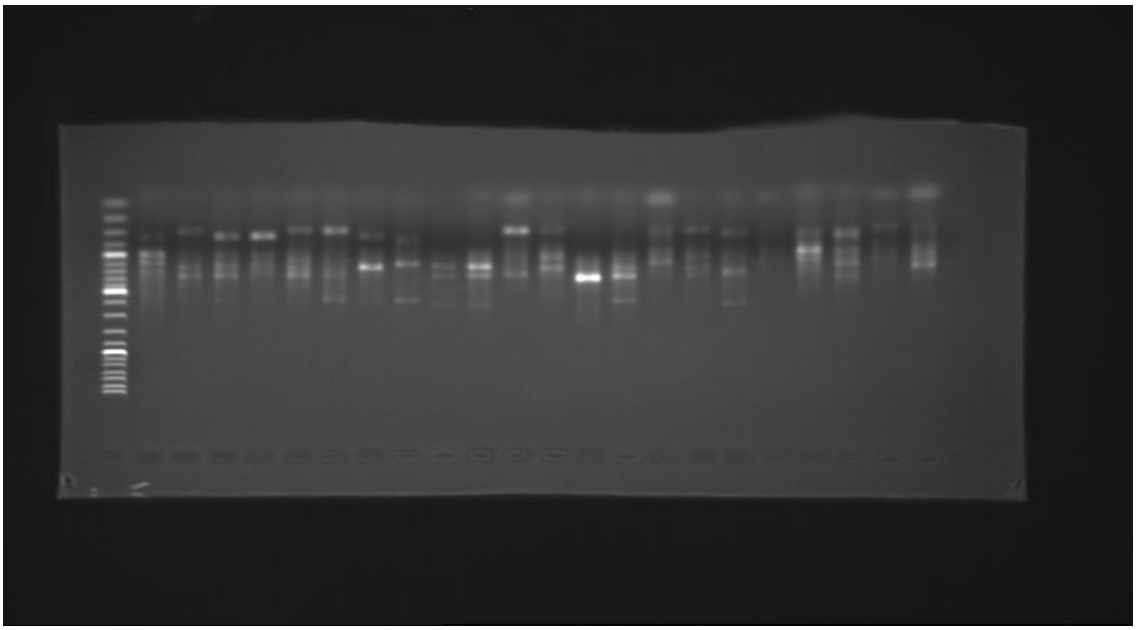

Fig. 19 Example of patterns obtained with undigested ITS genes of 22 tree legume isolates

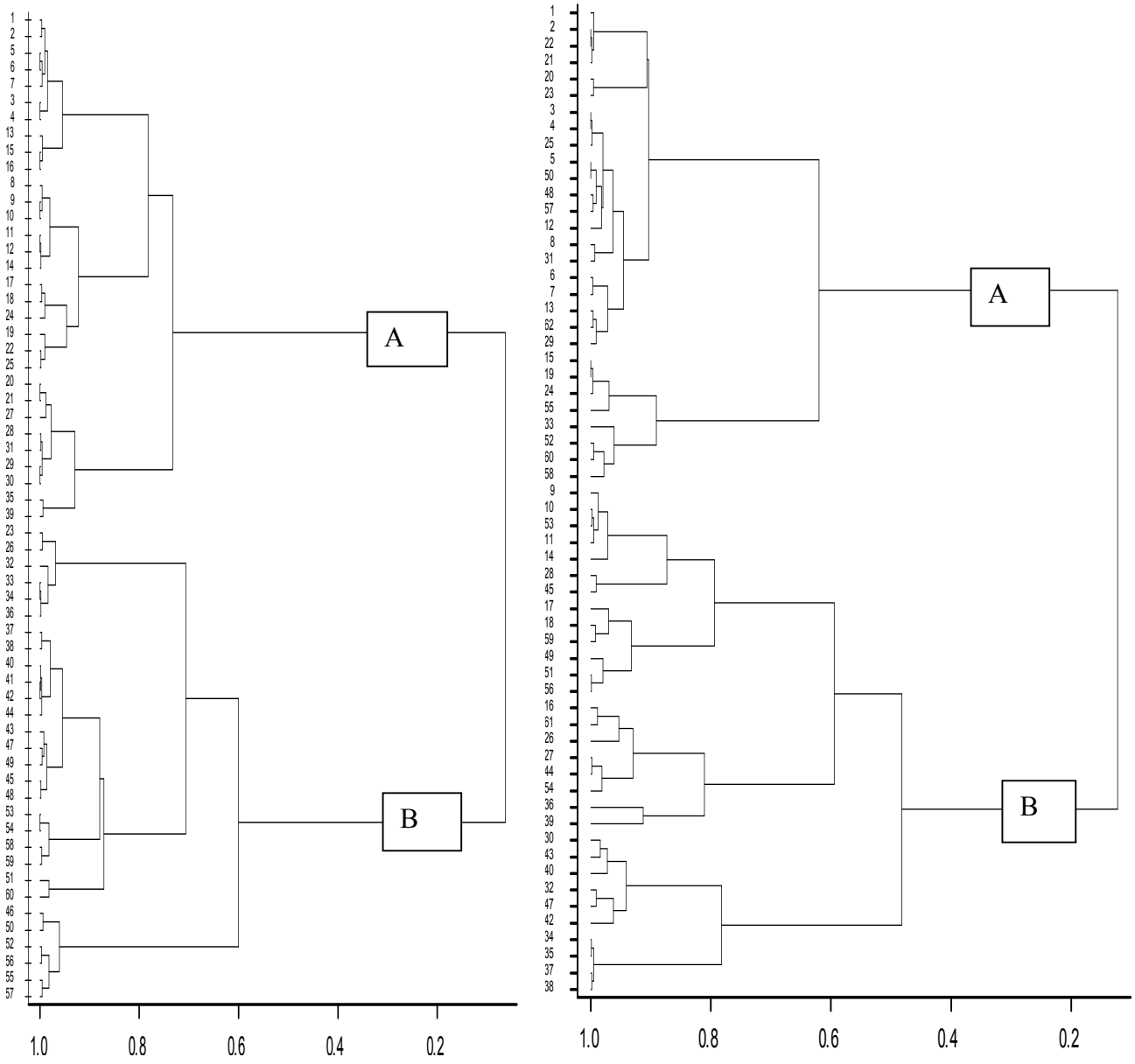

Fig. 20. Dendrogram of five indigenous tree legume rhizobia isolates based on (I) undigested ITS patterns and (II) restriction patterns of ITS with Hae III, Alu I and Hpa II.

CHAPTER FIVE

5.0 Discussion

5.1 Nodulation ability of 18 tree legumes in three Ghanaian soils.

Fifty six percent of the 18 tree legumes examined nodulated in all the three soils whiles 44% did not nodulate in any of the three soils. The majority of the 10 tree legumes examined and that nodulated (70%) in the three soils belonged to the sub-family *Mimosoideae,* while 20% and 10%, belonged to the sub families *Papilionoideae* and *Caesalpinoideae,* respectively. Over 60% of the tree legumes that did not nodulate in any of the three soils belonged to the sub-family *Caesalpinoideae.* This finding is in accordance with the evidence in the literature that, the majority of legumes that have been found not to form nodules belong to the sub-family *Caesalpinoideae (*Allen and Allen, 1981). Boakye (2001), working on the nodulation status of some tree legumes reported lack of nodulation in *Senna occidentalis*, *Tamarindus indica, Tetrapleura tetraptera* and *Parkia biglobosa* in three soils examined. Based on these findings and the lack of any report on nodules found on these trees in the literature, it is most likely that these trees could be non-nodulating. However, other tree legumes like *Pithecelobium spp, A. mangium, A. auricloformis and L. leucocephela* which have been reported by several researchers (Sanginga *et al.* 1989a; Domingo, 1983; Galiana *et al.* 1990; Turk and Keyser, 1992; Allen and Allen, 1981; Anon, 1984 and MacDicken, 1994) to nodulate extensively in most tropical soils, were also found to nodulate in the three soils examined, and additionally suggest that, rhizobia that nodulate these tree legumes (*Pithecelobium spp, A. mangium, A. auricloformis and Leucaena leucocephela*) may be common in many tropical soils. The same argument cannot be made for *A. farresiana* and *Albizia adianthifolia* which although reported to be nodulated (Sanginga *et al.* 1989a) did not form any nodules in the three soils used in this study. This observation plus the poor

nodulation observed for *Albizia zygia* in all the three soils might be due to unfavourable environmental conditions for the rhizobia that induce nodulation in these tree legumes. There are many reports that indicate that unfavourable environmental factors often result in the complete absence or low populations of the rhizobia that nodulate some legumes in certain soils (Day *et al.,* 1978; Graham, 1981; Eaglesham and Ayanaba, 1984; Dudeja and Khurana, 1989). The trend of nodulation observed is an indication of the diversity in abilities of different tree legumes to nodulate and fix N_2 . On the practical side, it shows the need to select for trees that are capable of not only growth, but in addition, are capable of supporting nodulation and N_2 fixation. The formation of many nodules on most of the tree legumes in Hatso and Toje soils is in conformity with the observed presence of high numbers of homologous indigenous rhizobia in these soils. The high rhizobia population observed in Hatso soil might be due to more favourable soil properties or the likelihood that some legumes belonging to the same host group as the tree legumes might have been grown previously on the soil. Thies *et al*. (1991) used the rhizobial population density of a given soil as an indirect means of predicting whether or not a legume will respond to inoculation. It is thus most likely that provided the rhizobia occurring in these soils are highly effective, either these soils may not need to be inoculated, or that, inoculation responses may not be as high as those without rhizobia. In general, the interior of most of the nodules were observed to be pink, indicating the presence of leghaemoglobin in the nodules and signifying that they were effective in N_2 fixation. Also most of the nodules occurred on the lateral roots especially those legumes grown in Alajo soil, indicating sequential nodulation.

5.2 Symbiotic effectiveness and specificity of *Rhizobium* isolates of the tree legumes examined.

The symbiotic effectiveness of indigenous rhizobial population is an important criterion in the selection of strains for inoculant production. It is also a primary factor that influences the magnitude of legume response to inoculation (Singleton and Tavares, 1986, Thies *et al.,* 1991). Thus, the first criterion for a *Rhizobium* strain to be used in legume inoculant production is that it must be highly effective in fixing nitrogen (Matos and Schrooler, 1989). In the present study majority of the isolates were found to be ineffective, and with the pattern of effectiveness distribution of the isolates following a normal distribution curve, with majority (63%) of the isolate being ineffective, and a few being either moderately effective (18%) or highly effective (18%). This finding is in agreement with Fening (1999) and Thies *et al.* (1991) who observed that the effectiveness patterns of indigenous cowpea rhizobia followed a normal distribution curve. The high proportion of ineffective isolates compared to the relatively low proportion of effective or moderately effective isolates calls for the need for inoculation of the seed or soil with effective and competitive strains for maximum nodulation and nitrogen fixation.

Despite the generally high ineffectiveness among the isolates, some of them were outstanding, and of higher symbiotic effectiveness compared to an application rate as high as 200 kg N/ha. This finding gives an indication that a potential exists for the development of high quality tree legume inoculants from indigenous strains provided further work is done to quantify their competitive abilities.

Rhizobium strains have long been described as specific when they are restricted in their legume host range or promiscuous when they have a very broad host range (Burton, 1972). In this regards, the results of the study indicate that *Rhizobium* isolates obtained from *Pithecelobium spp* and *Melletia thonningi* were specific as they nodulated only the respective host legumes from which they were

isolated. Despite not being promiscuous the populations of rhizobia that nodulated these tree legumes were found to be very high in the soils used for the study. This could be due to the fact that, these trees might have grown for many years in the areas from which the soils were sampled.

In contrast, *Rhizobium* isolates obtained from *A. mangium, Albizia lebbek* and *A. auricloformis* were considered to be slightly promiscuous. Interestingly, *Rhizobium* isolates obtained from *A. mangium,* could nodulate *A. auricloformis,* those of *A. auricloformis* could not nodulate *A. mangium*. Similarly, isolates obtained from *A. auricloformis* could nodulate *Albizia lebbek* but isolates obtained from *Albizia lebbek* could not nodulate *A. auricloformis* . These findings indicate the nature of diversity of the rhizobia that nodulate these tree legumes and the complexity or confusion associated with the cross-inoculation concept. *Rhizobium* isolates obtained from *Leucaena leucocephela, Crotalaria ochloleuca, Cajanus cajan* and cowpea were considered to be moderately promiscuous and were nodulated by isolates obtained from one another. This finding is in support of work by Thies *et al.* (1991) who indicated that, *.L. leucocephela, C. ochloleuca, Cajanus cajan* and cowpea could be grouped into one in terms of the rhizobia that nodulate them. However, of all the strains examined, NGR 234 is considered to be most promiscuous, nodulating six of the selected legumes, but not two common Acacias (*A. auricloformis* and *A. mangium*) growing in Ghana and also *Melletia thonningi*. It even nodulated *Pithecelobium spp* that was highly specific. This finding is in support of work done by Trinick (1980) and Pueppke and Broughton, (1999) who showed that NGR 234 was promiscuous and could nodulate over 110 genera of legumes including members of different sub-families such as *Lablab purpureus* and *Leucaena leucocephela.*

5.3 Response of six tree legumes to inoculation, phosphorus and nitrogen fertilizer

It is evident from the present study that, application of phosphorus especially up to 90 kg P/ha to six tree legumes, namely, *L. leucocephela, A. auricloformis M. thonningi, A. mangium, A. lebbek and A. zygia* resulted in significant increases in nodule numbers as well as in shoot and root biomass of all the tree legumes. This finding is in accordance with other reports indicating that high phosphorus levels in soil are necessary for maximum nodulation and nitrogen fixation (Gate and Wilson, 1974; Jakobsen, 1987; Olofintoye, 1986; Pereira and Bliss, 1987). Danso *et al.* (1992) also reported the positive effect of phosphorus on nodulation, root proliferation and its overall beneficial effect on legumes. Sanginga *et al.* (1989) and Bhatnagar (1978) also reported on the acceleration of growth parameters with the application of P- fertilizer, particularly on *Casuarina spp*., while Totey, (1992) and Bhuiyan *et al.* (2000) found a significant positive effect of P – fertilizer on *Tectona grandis*. The significant effects of P fertilizers on plant height of *A. auricloformis*, collar diameter and root length were also evident from the study of Uddin *et al*. (2007) and Verma *et al.* (1996). It can be concluded from all the above reports that P application stimulates both the N_2 fixing parameters of the legume, as well as its overall growth (Uddin *et al*. 2007). Results of the present study further revealed that, although, increasing the amount of P applied to the soil up to 90 kg P/ha resulted in significant increase in number of nodules, shoot and root dry matter; further increase in application to 120 kg P/ha resulted in significant decreases in all parameters measured in most of the tree legumes. Thus apart from *A. auricloformis,* for which application of 120 kg P/ha resulted in significant increase in number of nodules and total biomass in the two soils relative to the control, all the other tree legumes, recorded significant decreases in nodule numbers ranging from 4% in *M. thonningi* to 55% in *A. mangium* in Hatso soil and 33% in *M. thonningi* to 100% in *L. leucocephela*, *A. mangium* and *A. zygia, respectively,* in Toje soil. This finding is in agreement with results by Tsvetakora *et al.* (2003)

who also reported decrease in number of nodules of soya bean by up to 35%, nodule fresh weight and dry weight by almost 50% compared with the control with an excess of P or what could be considered as an over- supply of P. A similar observation was also reported for *A. lebbek* by Uddin *et al*. (2007) following the application of P fertilizer. Negative effects of commercial fertilizers on seedling growth were also observed by Kadeba (1978), who reported that, the addition of excess P fertilizer on *Pinus caribaea* depressed growth and increased mortality of the seedlings. The findings of Van den Driessche (1980) who reviewed both the positive and negative effects of nursery fertilizer application on subsequent seedling growth and survival provide further support.

A popular hypothesis to explain the negative effect of excess P on nodulation and growth of legumes is P toxicity. Results from the present work show that in all cases, despite the negative effect of P on the growth of the tree legumes beyond 90 kg P/ha, uptake of P per se increased between the 90 and 120 kg P/ha rate. Though further work needs to be done to ascertain the P toxicity hypothesis, it however gives an indication that, for better growth and nodulation of tree legumes, excess P must be avoided. An additional observation that warrants discussion is the fact that there was genetic diversity in terms of the toxic effect at 120 kg P/ha on the tree legumes, with *A. auricloformis* tree showing increase in growth up to 120 kg P/ha. Similar genetic differences in P toxicity effect have been reported for soybean (Tsvetakora *et al.* 2003) and *A. lebbek* by Uddin *et al*. (2007).

The high nodulation observed for *A. lebbek* as against the poor nodulation in *A. mangium* in the two soils could be attributed the fact that, compatible rhizobia capable of nodulating *A. lebbek* was higher compared with that of *A. mangium* in the two soils. The data further support the findings of Allen and Allen (1981) for *A. lebbek* and of Galiana *et al.* (1990) for *A. mangium,* but in disagreement with report that, *A. lebbek* is specific in its rhizobial requirements (Duhoux and Dommergues, 1985). The

generally lower phosphorus response by the tree legumes to nodulation and shoot biomass in Toje soil compared to Hatso soil might be due to the existence of already high numbers of compatible rhizobia in Hatso soil coupled with the relatively higher available phosphorus content of the Toje soil (Table 2).

The results of the tree legumes response to nitrogen fertilizer indicate the sensitivity of the symbiotic properties of these tree legumes to inorganic nitrogen. Nitrogen fertilizer even at the lowest rate (40 kg N/ha) was found to inhibit significantly nodulation of the tree legumes. This finding is in agreement with the findings of MacDicken (1994), Davidson and Robson (1986), Eaglesham (1989), Zahran (1999) and Sanginga *et al*, (1985). This observation is also consistent with the findings of Arreseigor *et al.* (1997) and Solaiman (1999) who found the suppression of nodulation in agroforestry crops due to nitrogen fertilizer application. The result is however not consistent with findings by Afza *et al.* (1987) who reported that small doses / levels (20 kg N/ha) of nitrogen fertilizer promoted nodulation of some crops. Zahran, (1999) also reported the beneficial effect of small doses (20 kg N/ha) of nitrogen fertilizer on nodulation of agroforestry crops. The tree legumes' nodulation was significantly more sensitive to nitrogen fertilizer in Toje soil than in Hatso soil, which could due to the relatively lower available nitrogen in Hatso soil than Toje soil.

The finding that, nitrogen application resulted in significant increase in shoot dry matter yield of most of the tree legumes at all N application indicates the lack of adequate N for the growth of these tree legumes. The increase in yield with increasing level of nitrogen also indicates the inability of these nodulated tree legumes to satisfy all their N requirements from the combined N_2 fixed from the atmosphere and the N present in the soil during the growing period, and that, reducing the supply of BNF through the nodules did not result in drop in yields, on the contrary, yields increased. Relative to

the control, application of low amount of N (40 kg N/ha) resulted in the highest increase in dry matter yield as relative to the higher N application rates, this being in accordance with findings by Hardarson *et al.* (1984).

Application of 90 kg P/ha in the nitrogen response studies resulted in the mitigation of the negative nitrogen effect on nodulation in the two soils. This might be due to the better growth of the P-fertilized legume, greater depletion of soil N (and therefore a reduction in the negative effect of available N on BNF), by a bigger plant and the resultant higher N needs to support the growth of a bigger plant.

The observed significant increase in number of nodules and total biomass when trees were inoculated suggests that either the numbers of compatible native rhizobia were insufficient to effect maximum nodulation, or / and that the inoculated strains were more competitive than the indigenous strains, majority of which were found to be ineffective on the tree legumes. This agrees with the report of Singleton and Tavares (1986) that statistically significant responses to inoculation could be eliminated in the presence of as few as 20 indigenous rhizobia/g of soil, as long as this rhizobial population contained some effective strains. Thies *et al.* (1991) also showed that yield enhancement with inoculation decreases dramatically with increasing numbers of indigenous rhizobia containing highly effective strains.

5.4 Response of % and total N fixed in tree legumes to phosphorus, inoculation and harvesting periods in two soil types

In general, nitrogen fixed in terms of both % and total N fixed in the tree legumes increased significantly with increasing phosphorus application rate for all the tree legumes. The significant increase in % N fixed by the tree legumes due to phosphorus was in contrast with findings by

Sanginga *et al.* (1990) who observed that % N fixed per se as aginst total N fixed (a yield dependent parameter) was little affected by increased levels of P. Our finding of the very high increase in total N fixed for *Acacia mangium* and *Albizia lebbek* with phosphorus application is therefore in line with result of Sanginga *et al.* (1990) for some agroforestry tree legumes like *L leucocephela* and *A. lebbek.* This finding was attributed mainly to the increased total dry matter yield when P was supplied to the tree legumes. The results obtained suggest that P is an essential nutrient which needs to be added to most soils in Ghana for maximum benefits from nitrogen fixation. Thus, the results gave an indication for P requirement for growing *Acacia mangium* and *Albizia lebbek* especially on P- limiting soils since this could also affect nodulation, nitrogen fixation and yield. This agrees with previous findings that low level of P is among the main chemical constraints for establishing tree legumes on tropical soils (Sanginga *et al.,* 1985) and that the availability of P is closely associated with yield and nitrogen fixation. Our results showing that total N fixed was higher in Toje soil than in Hatso soil might be attributed to a relatively higher available P content in Toje soil relative to Hatso soil.

The high indigenous rhizobia population observed in the soils indicates that, it is most likely that, most Ghanaian soils may be harbouring a sizeable number of tree legume rhizobia and that even without inoculation, one could expect much benefit from BNF especially in agroforestry and forestry.

The responses of the tree legumes to harvesting periods in terms of both % and total N fixed were statistically different. Increasing harvesting period improved both % and total N fixed by the tree legumes.

The response of the tree legumes to inoculation in terms of both % and total N fixed at 2 months harvest was generally not statistically significant. This could be attributed to the fact that, the tree legumes at this stage were very young and that N_2 fixation system had just been established and

functional. At this time therefore, the plant had to rely a lot on available soil N for growth, during which time symbiotic system is establishing itself. This may explain why starter N sometimes offers advantage. Inoculation in this case helps to establish more of the symbiotic system and increases BNF (Sanginga *et al.,* 1990). Furthermore, the need for N by the young plant at this stage possibly was not beyond what could be provided by the soil, making the need for extra P for more N from N_2 fixation less crucial for the growth of the young plants than at later stages when much more N than could be supplied by the soil was available.

Without inoculation, none of the tree legumes derived up to 50% of its N from fixation especially in the first 2 months of growth. The implication is that, without inoculation and at the first 2 months, the soil rather than atmospheric nitrogen was the major source of N for the growth of all these tree legumes. Within the 2 months of growth, *Rhizobium* inoculation alone had variable effects on the reliance of the trees on atmoshoeric N_2 fixation. in the two soils showing that not only genetic differences, but also soils contribute towards differences in the extent of dependence of N_2 fixing trees as to their sources of N. Thus while sole *Rhizobium* addition to the Toje soil resulted in A.mangium and A.auriculoformis trees sequestering over 55% of their N from fixation, for the Hatso soil, both *A. auriculoformis* and A. *lebbek* still derived less than a third of their N from fixation even when *Rhizobium* was inoculated. Even though P had greater effect on %N fixed within the first 2 months of growth, the soil effect was still evident. While in the Toje soil sole P addition resulted in the three trees deriving over half of their N from fixation, only *A. mangium* derived more than 50% of its N from fixation with *A. auriculoformis* and *A. lebbek* deriving barely a third and sixth respectively, of their N from fixation, in contrast to the corresponding treatments in the Toje soil in which the sole addition of P enabled each of the three trees to capture more than half of its N from symbiotic

fixation. These results further suggest that P addition may be more benefitial to ensuring the early onset of BNF than sole *Rhizobium* inoculation for these three trees. Adding both P and *Rhizobium* however was better. The high increase of % N and also total N fixed by the tree legumes after 4 months and 6 months due to inoculation and or phosphorus are similar to values reported by Sanginga *et al.* (1989). With more plant growth occuring with time, it is expected that the bigger plant will require more nutrients, creating a greater demand for a nurtrient like N, and once the demand is more than the soil can furnish, this will stimulate greater N_2 fixation.

5.5 Phenotypic characteristic of 200 rhizobia isolates of five tree legumes.

Bacteria belonging to the family *Rhizobiaceae* are classified into two on the basis of their growth on yeast extract mannitol (YEM) medium containing bromothymol blue (BTB). These are: the fast growing rhizobia (*Rhizobium)* and the slow growing rhizobia (*Bradyrhizobium*).

The results show that all the tree legumes were nodulated by all the two types of rhizobia, although each legume was predominantly nodulated by either *Rhizobium* or *Bradyrhizobium*. The results confirm earlier reports that some legumes are nodulated by both fast and slow growing rhizobia (Sanginga *et al.,* 1986; 1989, Dreyfus and Dommergues, 1981). Similar findings have also been reported by other researchers (Moreira, *et al*., 1998; Trinick, 1980; Turk and Keyser, 1992 and Zhang, *et al.,* 1991) and indicate that it may not be right to characterize *Rhizobium* isolates from any one legume as belonging to any one of these two broad groups.

In the present study, the tree legumes were predominantly nodulated by 54% and 46% of *Rhizobium* and *Bradyrhizobium* respectively, of a total of 200 rhizobia isolates. Sanginga *et al*. (1989) reported that *L. leucocephela* though nodulated by both *Rhizobium* and *Bradyrhizobium*, only forms effective nitrogen fixing nodules with the fast growing rhizobia. The results of the present study do not agree

with this, as both the fast and slow growing rhizobia formed either effective or ineffective nodules on all the tree legumes examined (Tables 5.1 to 5.5).

The results of the pH tolerance of the tree legumes (brady) rhizobia indicate that, most of the isolates were acid tolerant, with 70% of the total isolates able to grow at a pH of 3.5. If tolerance to acidity is used as the main classifying parameter, then it may be suggested that up to 70% of these strains could be classified as bradyhizobia (Noris, 1959). This tolerance to acid conditions by the strains could possibly be attributed to the fact that majority of the strains were isolated from slightly to moderately acid soil (Table 2). However, further results obtained in this study may contradict such an interpretation and that it may not be totally right to use only ability for growth at low pH to group rhizobia into *Rhizobium* or *Bradyrhizobium.* This is because a great majority (more than 66%) of the isolates that could not grow at pH 3.5 were identified as *Rhizobium*, and still a good proportion of these isolates (34%) that could not grow under such acid conditions were considered as *Bradyrhizobium.* Similarly, a very high proportion (71% to 77%) of the isolates that could not grow at pH 4.5 were found to belong to *Bradyrhizobium spp.* Increasing pH from pH 4.5 to 7.5 resulted in considerable increase in optical density of the isolates, beyond which it decreased. Rodrigues *et al.* (2006) reported that the optimal pH for the growth of root nodulating bacteria was between pH 6.5 – 7.5.

Morphologically, the majority of isolates (85%) were considered to be wet and gummy which confirms that most of the isolates were fast growers (*Rhizobium*). This contradicts earlier belief that, *Rhizobium spp.* is endemic to temperate alkaline soils while *Bradyrhizobium spp*. is considered endemic in acid tropical soils (Norris, 1959).

The phenotypic characteristics of the isolates clearly indicate a large heterogeneity of the tree legume rhizobia isolates. It is likely that genotypic characterization may further reveal additional heterogeneity among the tree legume isolates.

5.6 Molecular characteristics and diversity of 60 indigenous tree legume isolates.

The polymerase chain reaction (PCR) results of the 16S rRNA gene of almost all the rhizobia isolates of the tree legume gave a single band of size 1.5 kb, corresponding to the expected band size reported for rhizobia by earlier workers (Weisburg *et al.,* 1991 and Terefework *et al.,* 1998). The observed single band size of 16S rRNA gene for all the isolates indicates the conservative nature of the 16S rRNA gene of rhizobia.

Combined restriction of the 16S rRNA genes of the 60 rhizobia isolates with five enzymes distinguished clearly 60 different combinations of patterns or fingerprints, which represent 60 distinct 16S rRNA genotypes among the isolates. This finding indicates great variations among the test isolates, and suggest that, the soils harbour large proportions of diverse strains that can nodulate the tree legumes. This finding is in agreement with the results obtained by other researchers who studied diversity among natural rhizobial populations in different parts of the world (Madrzak, *et al.,* 1995; Ando *et al*., 1999 and Niemann *et al.,* 1997).

Characterization of the test rhizobia isolates based on simple PCR of the intergenic spacer (ITS) gene was most resolute as it provided several distinct band sizes indicating great variation among the isolates ITS. The high level of ITS size heterogeneity is consistent with the findings by Bouchaib *et al.* (1998), who in an extensive study of 52 phenotypic characteristics of rhizobia, concluded that the rhizobia studied seemed to belong to several different clusters. Such length variability of the ITS was also recorded by Laguerre *et al.* (1996), between genotypes distantly or closely related within *R.*

leguminosarum. However, restriction of the ITS with three restriction enzymes Hae 111, Alu 1 and Hpa 11 did not give much further distinctions among the test strains. This finding is similar to results obtained by Bouchaib *et al.* (1998), when investigating genetic diversity and phylogeny of several rhizobia that nodulate *Acacia* species using PCR with RFLP. Lack of major difference in the restriction patterns of the ITS relative to their unrestricted counterparts suggests that, there is little sequence variability in the ITS among the tree nodulating rhizobia that cannot be revealed by RFLP analysis.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Trees in general and most especially nitrogen fixing trees are considered as integral part of the traditional farming systems in Ghana due to their added advantage of the latter fixing atmospheric nitrogen gas into the soil. However, majority of the trees which are used in forestry and agroforestry programmes in Ghana have not been assessed for their nodulation and nitrogen fixing potential and the need to inoculate with rhizobia for enhanced nodule formation and nitrogen fixation. Till date, the propensity, diversity and nature of rhizobia that nodulate these tree legumes in Ghanaian soils are still not known. It is to fill this gap in knowledge as well as towards improving and sustaining soil fertility and productivity in Ghana that this study was proposed.

In this study, 18 species of some common indigenous tree legumes of potential use in forestry and agroforestry were initially assessed for their nodulation status as well as ways of enhancing their growth, nodulation and nitrogen fixing ability on three different soil types that occur in the coastal savannah zone of Ghana. Representative isolates were obtained from the nodules of these tree legumes and characterized culturally, metabolically and phenotypically. The isolates were also characterized genotypically using RFLP analysis and their relatedness or diversity determined.

Not all the leguminous trees formed nodules, and the majority of the tree legumes (60%) that did not nodulate in any of the soils belonged to the Caesalpinoideae family whiles those that nodulated were found to belong to Mimosoideae (70%), Papilionoideae (20%) and Caesalpinoideae (10%) families.

The soils were found to harbour diverse strains of rhizobia. Even though majority of the isolates were capable of forming nodules on the tree legumes, most of them were ineffective in fixing N_2 on their

respective host legumes. The rhizobial isolates from each tree belonged to both the slow (*Bradyrhizobium*) and fast-growing (*Rhizobium*) genotypes, with each tree capable of nodulating with any one of them, although each tree legume was nodulated predominantly by one type. Some cross inoculation groups appeared to exist among the tree legumes. However, rhizobia that nodulated *Pithecelobium spp* and *Melletia thonningi* appeared to be specific as these trees were not nodulated by rhizobia from any other tree legume. The other tree legumes were considered to be slightly or moderately promiscuous as they were nodulated by rhizobia from, one, two or tree different tree legumes genera.

Phosphorus fertilizer application generally resulted in significant increases in number of nodules and total biomass of the tree legumes. However, increasing phosphorus levels beyond 90 kg P/ha resulted in significant decrease in nodule numbers and total biomass of the tree legumes in the soils. Also, in general, nitrogen fixed in terms of both % and total N fixed for the tree legumes were increased significantly with phosphorus application for all the tree legumes. However, most of these parameters were reduced when P rate was increased to 120 kg P/ha. This effect however occurred despite the increased uptake of P at the 120 kg P/ha level. Further research to explain why P at 120 kg P/ha had these adverse effects will be needed. Until then, it is however tempting to attribute these to physiological effects or nutritional imbalances.

Nitrogen fertilizer application even at the minimum level (40 kg N/ha) resulted in significant reduction in nodule numbers but increased total biomass of the tree legumes significantly. However, great diversity was observed among the tree genotypes on the detrimental effect of inorganic N on N_2 fixation. Besides, the application of phosphorus mitigated the depressive effect of N on nodulation

Inoculation of the tree legumes resulted in many significant reponses such as inhigher nodule formation and in total biomass of the tree legumes in the soils with more pronounced response observed when both inoculation and P were added together. The outcome of the tree legumes to inoculation in terms of both % and total N fixed at 2 months harvest was generally poor and not statistically significant among the different trees. However, harvesting at 4 months and 6 months after inoculation produced significant differences in both % and total N fixed within the tree legumes. Increasing harvesting periods significantly improved both % and total N fixed by the tree legumes.

In general, for all the tree legumes and for all treatments, *Acacia mangium* fixed the highest proportion of its N requirement followed by *Acacia auricloformis* and the least by *Albizia lebbek*.

Majority of the rhizobial isolates from the tree legumes were fast growers and were classified as rhizobia. Genotypically, the isolates were very divesre and grouped into 60 different types of genotypes based on PCR of RFLP of their 16S rRNA genes with five enzymes. The isolates were also very diverse on the basis of the size of their ITS although RFLP of the ITS did not result in much further diversity.

From the above, it can be concluded that, the rhizobia that nodulate tree legumes in Ghanaian soils are very diverse both phenotypically and genotypically and that to enhance growth, nodulation and nitrogen fixation of the tree legumes, consideration must be given to selecting from among the diverse array, with the most effective strains for inoculation. This should be combined with the addition of moderate amounts of P to the tree legumes at planting.

It is highly recommended that future studies must confirm the observed adverse effect of excess P on the growth and symbiotic abilities of tree legumes and the rhizobial population, distribution and their

competiveness in soils with or without added P. It is also recommended that future studies must include sequencing of the rhizobial isolates and comparing them with already known rhizobial sequences with the view to classifying the indigenous rhizobia into known species and possibly novel species.

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