# DIVERSITY AND SYMBIOTIC CHARACTERISTICS OF COWPEA *BRADYRHIZOBIUM* STRAINS IN GHANAIAN SOILS

**BY**

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## DEDICATION

This work is dedicated to Mrs. Juliana Twum, my brother's wife, who supported and encouraged me during the period of my academic pursuits. In particular, when I decided to return to Legon for my Doctorate Programme, Mama invited me to share their East Legon home and ensured that I had my special diet regularly and promptly. This, of course, relieved me of the chores of campus life and contributed substantially to the completion of the programme on time.

## DECLARATION

I hereby declare that, except for references to works of other researchers, which have been duly cited, this work is the result of my own original research and that this thesis has neither in whole nor in part been presented to any other University for the award of a degree.

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## ABSTRACT

This study reports investigation of the biodiversity of bradyrhizobia isolates that nodulate cowpea in Ghanaian soils. As a prelude, some components of nitrogen fixation of cowpea in the various soils were examined through: (1) assessment of the natural nodulation of 45 cowpea cultivars in 20 soils sampled from 5 ecozones (coastal savanna, rain forest, semi deciduous forest, forest savanna transition and guinea savanna), (2) determination of the numbers of bradyrhizobial isolates in the soils and (3) determination of the response of cowpea to nitrogen fertilization.

The results of the ability of 45 cowpea cultivars to nodulate naturally in different soil types showed large variability among the cultivars. Counts of the indigenous bradyrhizobia population in the soils showed that most of the soils in Ghana harbour large populations of bradyrhizobia (in the range of 0.6 x 10 to 31 x $10^3$) capable of nodulating cowpea. Response of cowpea to nitrogen fertilizer differed in the different soils. In general all the cultivars showed significant responses to increasing levels of nitrogen, an indication that nitrogen fixation was not supplying the plants with all the external nitrogen required for maximum yield.

A combination of morpho-physiological and molecular analysis was used to assess the diversity of the bradyrhizobia isolates. A total of 100 isolates were assessed. The results of the morpho-physiological analysis indicated that cowpea is nodulated by both fast and slow growing rhizobia. The results also showed that the isolates were versatile and could survive under different soil conditions particularly acidity and salt stress. A cross inoculation study of the isolates with nine legume species produced seven major groupings with 28 subgroups based on

distinct nodulation patterns. Results of the serology (ELISA) assay indicated that only a small fraction of the isolates reacted strongly with antisera of each other. The greater proportion showed no cross reactivity. Analysis of the 16S rRNA gene of the isolates by PCR-RFLP identified 20 different composite genotypes. Diversity among the genomic species identified was very high, reaching 80% diversity. The various methods used indicated large diversity among the isolates, but the groupings of the isolates by the various methods were inconsistent, due to the different levels of resolution by the various methods.

Diversity of the isolates in symbiotic effectiveness showed that some of the isolates had high nitrogen fixing capabilities that were comparable to plants fertilized with inorganic fertilizer nitrogen. Some of the isolates even showed superiority in symbiotic effectiveness relative to the standard strain TAL 169, suggesting that the native isolates may be useful strains for cowpea inoculation. The Gus A marker gene technique was used to assess the competitive abilities of the effective and ineffective isolates. Competition between the isolates was examined at different population ratios. The results obtained indicated that competitive ability was not directly related to effectiveness of strains.

Inoculation of cowpea with indigenous bradyrhizobia isolates increased the number of nodules, shoot dry weight and total nitrogen of plants. The method of inoculation was observed to influence these parameters. The results indicated that response of cowpea to inoculation in the presence of native rhizobia in some soils is possible.

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# CHAPTER ONE

# INTRODUCTION

## 1.1. Background

Legumes belonging to the family *Fabaceae* form symbiotic associations with the soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. This symbiosis, often leads to the development of a new plant organ, the nodule, which provides the ecological niche required for the biological fixation of atmospheric nitrogen (Elkan and Bunn, 1991). This process is carried out by rhizobia that reside in the nodule and is represented as:

$$N_2 + 8H^+ + 8e^- = 2NH_3 + H_2$$

The ammonium generated is assimilated as the ion, $NH_4^+$ and is subsequently converted into glutamate, which is further assimilated and utilised by the plant (Giller and Wilson, 1993). By virtue of this symbiotic association, the growth of the plant can be rendered to a large extent, independent of soil nitrogen (Pueppke, 1986; Long, 1989).

The economic significance of the legume - *Rhizobium* association has led to intensive research to improve the efficiency of the symbiosis over the past one hundred years since it was discovered (Gordon *et al.*, 1995). One very important finding made so far is that:

> Judging from the magnitude of biological nitrogen fixation in different cropping systems, it is considered agronomically significant, providing an alternative or at least a supplement to the use of energy – expensive, artificial nitrogenous fertilizers.

It has been estimated that approximately 120 million tonnes of atmospheric nitrogen is reduced by biological nitrogen fixation to ammonium each year (Freiberg *et al.*, 1997).

## 1.2 Problem specification

One major problem facing farmers in Africa is that either the inherent fertility of the soil is low, or the capacities of these soils to supply nitrogen decline rapidly once agricultural activities commence (Buresh, 1997). The annual rate of decline in soil nitrogen stocks has been estimated to vary from 22-112 Kg N $ha^{-1}$ (Stoorvogel *et al.*, 1993). Although the application of fertilizer nitrogen is recognised as the convenient way for rapid correction of nitrogen deficiency in soils, its high cost limits its wide application by farmers (Peoples *et al.*, 1995). Ghana's case is a good example; during the past decade, domestic inflation and removal of fertilizer subsidies in Ghana, have contributed to a rapid increase in fertilizer prices to over 29,000% (Bump, 1994). Consequently, there has been a sharp fall in the purchase and use of fertilizer in Ghana (Bump, 1994), with the attendant lowering in crop yields.

The use of legumes as renewable sources of nitrogen and soil organic matter amendment have long been a major component of many farming systems in Africa, with cowpea as the most prominent legume (Awonaike *et al.*, 1990). As a food legume, the impact of fixed nitrogen to the soil following harvest depends upon the balance between soil nitrogen in the crop and that which was fixed. This is determined by the difference between the amounts of nitrogen fixed and nitrogen removed with the seed. When effectively nodulated, cowpea can produce as much as 90% of its nitrogen requirements from biological nitrogen fixation for maximum yields (Eaglesham *et al.*, 1977). Should this high rate of biological nitrogen fixation be attained, then

even when grown in soils deficient in available nitrogen, (as is the case in many tropical soils), cowpea should be able to give optimum yields without requiring nitrogen fertilizer. However, at the farm level grain yields of cowpea are often low and inconsistent (Summerfield *et al.*, 1974; FAO, 1998). One of the possible hypotheses is that this is due to inadequate infectivity or efficacy of the indigenous rhizobia to supply all the nitrogen required for optimum yields (Singleton and Taveres, 1986). This will suggest a potential for improvement in cowpea yields through the use of microbial inoculants. Although several studies have reported that nodulation of cowpea in tropical soils could not be improved by inoculation (Doku, 1969; Kang *et al.*, 1977; Rhodes and Nangju, 1979; Awonaike *et al.*, 1990), in some cases increased yields were obtained (Danso and Owiredu, 1988; Rajput, 1994). The need for cowpea inoculation is therefore still controversial. In order to overcome and settle this, some well-defined studies are necessary, and include studies on the genetic variability of the indigenous rhizobial strains that have to be evaluated thoroughly (Richardson *et al.*, 1995; Mpepereki *et al.*, 1997; Martins *et al.*, 1997).

Indigenous rhizobia that are able to nodulate cowpea have been described as a heterogenous group, characterised by a high degree of symbiotic promiscuity (Hada and Loynachan 1986; Singleton *et al.*, 1992). Cowpea rhizobia were thought to be slow growing and alkaline producing (Fred *et al.*, 1932). However, several fast-growing strains have now been isolated (Zablotowicz and Foct, 1981; Dakora and Vincent 1984; Mpepereki *et al.*, 1997). These findings indicate that there is possibly more to be examined on the taxonomic relationships of rhizobia that fix nitrogen in symbiosis with cowpea (Jordan, 1984). Several reasons may contribute to the difference in results, one of which may be some reported cases of negative response of cowpea to inoculation (Ezedinma, 1963; Kang *et al.*, 1977; Rhodes and Nangju,

1979; Awonaike *et al.*, 1990). Additionally, most of the agriculturally important tropical legumes are members of the cowpea cross inoculation group, and therefore they are often assumed to be less selective than other non-tropical legumes in the choice of the rhizobial microsymbiont. Thus rhizobial diversity of tropical soils is not considered as important (Dobereiner 1978; Halliday, 1985).

An array of different methods including host range analysis, serology, antibiotic resistance and biochemical analysis have been used to describe cowpea rhizobia (Eaglesham *et al.*, 1987; Ahmad *et al.*, 1981; Mpepereki *et al.*, 1997). Even though these methods are important and play significant roles in the characterisation of rhizobial strains, for species identification and taxonomic purposes molecular analysis has become an effective method (Graham *et al.*, 1991; Ludwig and Schleifer, 1994; Young and Haukka, 1996; Sessitsch *et al.*, 1997; de Lajudie *et al.*, 1998). Moreover, molecular biology techniques have become indispensable for the analysis of biodiversity (Laguerre *et al.*, 1994; Richardson *et al.*, 1995; Laguerre *et al.*, 1996; Sessitsch *et al*, 1997; Vinuesa *et al.*, 1998). The advantage of molecular tools is that because they are more precise, they are able to reveal the existence of marked genetic differences within a group of organisms even when other methods are not sensitive enough to detect differences (Martinez-Romero, 1994).

This study aims at finding out the genetic diversity of native cowpea (brady)rhizobia isolated from soils in different agroecological zones in Ghana. A combination of morpho-physiological analyses and molecular methods was used to characterise the various isolates and in addition their nitrogen fixation effectiveness as well as competitive abilities were determined. This

investigation is providing fundamental information that could pave the way for the improvement of the cowpea-*Rhizobium* symbiosis.

## 1.3. Objectives of the study

(i) To assess the potential to improve nitrogen fixation of cowpea in Ghanaian soils.

(ii) To isolate, identify and analyse biodiversity of the indigenous rhizobia that nodulate cowpea

(iii) To determine the symbiotic properties of the isolates.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1.1 The nodule bacteria

Bacteria capable of nodulating legumes belong to the family *Rhizobiaceae*. They are Gram-negative, rod-shaped, aerobic and mobile saprophytes (Schlegel, 1996). Five separate genera of the rhizobia have been proposed to date. These are *Rhizobium* (Buchanan, 1926; Jordan, 1984), *Bradyrhizobium* (Jordan, 1982), *Azorhizobium* (Dreyfus *et al.*, 1988), *Sinorhizobium* (Chen *et al.*, 1988) and *Mesorhizobium* Chen *et al.*, 1995).

Some of the phenotypic characteristics used to differentiate between the different rhizobial genera are growth rate, flagella type, and carbohydrate metabolism. Some of these traits appear to have been propagated in the literature even when they later have proven not to give definite inter or intra generic differences (Giller and Wilson, 1993). Growth rate shows a gradation of generation times and the conventional terminology of fast or slow growing can only be interpreted according to experience. The concept of fast-growing species being acid-producing and slow-growing being alkaline-producing (Norris, 1965), is also not absolute, and may strongly be affected by the growth medium (Hernandez and Focht, 1984; Ahmad and Smith, 1985; Dreyfus *et al.*, 1988). Phenotypic features such as host range or carbon substrate metabolised seem rarely to be a reliable guide to species identification within a genus. Perhaps the most direct way to assign a new rhizobial strain is to gain some information about its host

range and to carry out some form of analysis that compares the genomic structure of the new strain to that of other named species with a similar host range (Giller and Wilson, 1993).

Nowadays, bacterial taxonomy is to a greater extent build on DNA-based methods. The 16S rRNA gene has been considered as a useful parameter for phylogenetic analysis 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). It also constitutes a significant component of the cellular mass and is readily recovered from all types of organisms, providing adequate sequence information to permit statistically significant comparisons. Furthermore, a ribosomal database has been established (Maidak *et al.*, 1994). In addition, DNA:DNA relatedness has been considered as an important criterion as it has been shown that the 16S rRNA gene sequence similarity among bacteria can be high although the DNA relatedness may indicate different species (Oyaizu *et al.*, 1992; van Berkum *et al.*, 1996).

### 2.1.2 *Rhizobium* taxonomy

The taxonomy of the nodule bacteria has undergone considerable revision in the past two decades and is still in a state of transition (Graham *et al.*, 1991; Elkan, 1992). When nodule bacteria were first isolated, they were called *Bacillus radicola* (Beijerinck, 1888). The following year, Frank (1889), published the name *Rhizobium leguminosarum*, which is still in use today. The taxonomy of the nodule bacteria was reviewed by Fred *et al.* (1932), and was based on the nodulation host range. Over time, overlapping host ranges have been reported (Wilson, 1944), and rhizobia were found to be diversed in symbiotic, physiological and other properties. Consequently, Jordan (1982), described two genera, *Rhizobium* and *Bradyrhizobium*. *Rhizobium*

strains were said to be fast growing whereas *Bradyrhizobium* strains were considered as slow growing. Since 1984, three additional genera, *Azorhizobium* (Dreyfus *et al.*, 1988), *Sinorhizobium* (de Lajudie *et al.*, 1994) and *Mesorhizobium* (Lindstrom *et al.*, 1995) have been described and accepted. All *Bradyrhizobium* strains that nodulate soybean effectively were until recently known as *B. japonicum* (Jordan, 1984). Kuykendal *et al.*(1992), proposed the name *B.elkanii* for group ii strains, a fairly clear subgroup of soybean bradyrhizobia. Another species, *B.liaoningense*, has recently been proposed (Xu *et al.*, 1995), which is distinct by the criterion of DNA-DNA hybridization, besides having an exceptionally slow growth rate. All other slow growing strains were assigned to *Bradyrhizobium* spp, the so called cowpea miscellany rhizobia or tropical bradyrhizobia. There is only one species, *Azorhizobium caulinodans*, in the genus *Azorhizobium*, although a second species has been recognised by DNA-DNA hybridization (Rinaudo *et al.*, 1991). *A.caulinodans* nodulates both stems and roots of *Sesbania rostrata* (Dreyfus *et al.*, 1988). The DNA sequences of the small sub-unit ribosomal RNA genes suggest that the genus *Rhizobium* may comprise several species. The species *R.leguminosarum*, *R. tropici*, *R. etli* and *R.galegae* are clearly separated groups and their taxonomy is also supported by similarities and differences in other properties (Lindstrom, 1989; Segovia *et al.*, 1993; Martinez-Romero *et al.*, 1991). De Lajudie *et al.* (1994) have proposed that the branch that includes *R.meliloti* and *R.fredii* should be transferred to the genus *Sinorhizobium*. This genus was originally proposed by Chen *et al.*(1988), to emphasise differences between *R.fredii* and other rhizobia including *R.meliloti*, but subsequent work has shown that these two species are rather similar (Jarvis *et al.*, 1992), and the new definition of *Sinorhizobium* includes both of them, as well as two new species *S.teranga* and *S.saheli* (de Lajudie *et al.*, 1994, Young and Haukka, 1996). The name *Mesorhizobium* has been suggested to refer to the growth rate of

strains, which are intermediate between typical growth rates for *Rhizobium* and *Bradyrhizobium*. This new genus comprises *M. loti* (Jarvis *et al.*, 1992), *M. huakuii* (Chen *et al.*, 1991), *M. cicer* (Nour *et al.*, 1995), *M. thianshanense* (Chen *et al.*, 1995) and *M. mediterraneum* (Nour *et al.*, 1995). The 16S rDNA sequence information of *R. galegae* which nodulates *Galega* species does not allow it to fit into any of the above mentioned generic groups (Lindstrom, 1989). The sequence shows close similarity to *Agrobacterium* and therefore it has been proposed to be transferred to a different genus (Young and Haukka, 1996).

### 2.1.3 Abundance in soil

The usual source of nodule bacteria is the soil. Nevertheless, many soils remain devoid of rhizobial strains capable of nodulating particular crops; for instance, soybean rhizobia do not occur naturally in Australia (Diatloff and Brockwell, 1976). For this reason a large number of surveys have been conducted to establish the levels of rhizobia in soil. Surveys of rhizobia encounter certain technical problems as no defined culture medium exits for their selective isolation and reliance has to be placed on legume infection. Most probable number (MPN) assays are conducted whereby the soil is progressively diluted with sterile water, saline or medium, and the various dilutions inoculated onto seedlings raised from surface-sterilised seeds (Somasegaran and Hoben, 1985). In a soil dilution containing at least one *Rhizobium* cell, the assumption is that the microorganisms can multiply in the rhizosphere and infect the plant, which is then examined for nodules.

Great variation occurs in the population size of field rhizobia. The presence of an appropriate host and its rhizosphere are major determinants (Hiltbold *et al.*, 1985; Rupela *et al.*, 1987;

Woomer *et al.*, 1988). Other factors are soil acidity (Rice *et al.*, 1977), seasonal effects (Rupela *et al.*, 1987), and soil texture (Woomer *et al.*, 1988). Additionally, many biological factors could also affect the rhizobial population size in the soil including antagonist that produce antibacterial metabolites and effects such as predation by protozoa or attack by bacteriophages (Danso *et al.*, 1975; Barnet, 1980; Roughley, 1985).

The size of indigenous rhizobial populations could present a competition barrier to the establishment of inoculant strains, possibly leading to inoculation failures in some cases (Thies *et al.*, 1991). Singleton and Tavares (1986) found that substantial nodule occupancy by an inoculant strain could only be predicted if the indigenous population was less than 100 cells g soil $^{-1}$ Similarly, Weaver and Frederick (1974) found that the number of nodules formed on soybean increased with increasing amounts of *Bradyrhizobium japonicum* inoculant, but this increase was not observed in soils containing more than 1000 rhizobia g $^{-1}$

Some of the surveys on indigenous cowpea rhizobial populations in soils in Africa have not been very encouraging, for example, numbers ranging from undetectable to about 100 cells g soil$^{-1}$ have been recently reported in some Zimbabwean soils (Mpepereki and Makonese, 1995). Similar population counts were detected in some Egyptian soils (Moawad *et al.*, 1994) Contrary to these low population counts, higher counts ranging from 610 to 4500 g soil$^{-1}$ were detected in three Ghanaian soils (Danso and Owiredu, 1988). However, a comprehensive survey of the population sizes of cowpea rhizobia in Ghanaian soils is required in order to predict the success of rhizobial inoculation over a wider range of soils.

### 2.2.1 Nodule development

Root nodule formation can be divided into three stages: 1. pre-infection, 2. infection and nodule organogenesis, and 3. nodule function and maintenance (Vincent, 1980). The pre-infection starts when rhizobia are attracted by chemostaxis to the organic compounds excreted by root hairs (Turgeon and Bauer, 1985; Nap and Bisseling, 1990; Gerahty *et al.*, 1992), followed by the attachment of the bacteria to the root hairs leading to root hair deformation and root hair curling (Bauer, 1981). Several hypotheses have been suggested to explain the mechanisms involved in the latter process. Hirsch (1992) proposed that the *Nod* factor, embedded in the rhizobial membrane by the lipid tail, causes root hair deformation and cortical cell division by the reaction of the N - glucosamine residue of the *Nod* factor with a sugar - binding site of the plant receptor, presumably a lectin. The strength of this interaction, regulating early events in nodulation, depends on several properties: the length of the glucosamine backbone and the presence or absence of various subtituents like sulphate (Dazzo and Hubbell, 1982; Chrispeels and Raikhel, 1991). Dazzo *et al.* (1984) suggested that the adhesion of the bacterial symbiont to the root surface is the critical step prior to successful infection and development. There is substantial experimental evidence that support this hypothesis, for example, the work of Diaz *et al.* (1989) showed that the introduction of a pea lectin into white clover root, resulted in the production of a hairy clover root. However, other workers have not been able to demonstrate specific attachment (Smit *et al.*, 1986; Smit *et al.*, 1992). Another model explaining the attachment of rhizobial cells to root hairs is that the microbes bind weakly to a plant receptor with a calcium - binding protein on the bacterial surface known as rhicadesin (Smit *et al.*, 1991; Swart *et al.*, 1994). Tighter adherence then occurs by means of bacterial extracellular cellulose fibrils (Smit *et al.*, 1986).

Attachment of rhizobia to the surface of root hairs produce many deformations including the characteristic shepherd crook (Bauer, 1981). At the centre of the crook, disruption of the plant cell wall occurs which enables the rhizobia to enter the root hair (Bauer, 1981). As they do so, a new structure, the infection thread forms within the plant cell and encloses the rhizobia. The rhizobia themselves proliferate in these cells until they have almost filled them. These proliferating cells remain within the root endodermis and form the nodule (Newcomb, 1981).

### 2.2.2 Types of nodules

The type of nodule that develops depends on the host plant, not on the rhizobial strain (Dart, 1977). There are two main nodule types; the determinate and the indeterminate. In general, temperate legumes such as *Pisum*, *Vicia*, *Trifolium*, and *Medicago* develop indeterminate nodules, while tropical legumes such as *Vigna*, *Phaseolus* and *Glycine* develop determinate nodules (Corby *et al.*, 1983). Both types are composed of similar tissues, all formed from the nodule meristem (van de Wiel *et al.*, 1990). Indeterminate nodules are characterised by a persistent apical meristem whilst determinate nodules do not have a persistent meristem (Newcomb, 1976). Determinate nodules grow for a fixed period, all parts of the nodule essentially differentiating at the same time and have a finite life span. In contrast, indeterminate nodules have an apical meristem that continues to be active throughout the lifetime of the nodule, producing zones of new infection, and so giving rise to a gradient of differentiation progressing back towards the root.

Nodules differ in shape and size, partly as a response to soil conditions and partly as a characteristic of the particular bacterial strain-plant variety interaction (Lynch and Wood, 1989).

They may be spherical, cylindrical, flattened and often bidentate or with coralloid branching, or may have an entirely irregular shape. Their size may vary from that of a pinhead to over 1cm. The larger nodules are never spherical but have shapes giving a high ratio of surface area to volume, possibly to ensure an adequate supply of nitrogen gas to the active nodule cells and an adequate means of disposal of carbon dioxide produced in the nodule. In general, a nodule will only contain a single strain of *Rhizobium*, although dual occupancy of nodules is well documented (Lindemann *et al.*, 1974; Johnston and Beringer, 1976; May and Bohlool, 1983; Moawad *et al.*, 1984; Sessitsch *et al.*, 1997).

Once the bacteria have filled a proliferating cell, they change their form into bacteriods. Only bacteriods produce the enzymes required for nitrogen fixation. However, the nodule bacteriods may be effective or ineffective in fixing nitrogen. Lack of bacteriod persistence appears to be the most common cause of nodules being ineffective (Lynch and Wood, 1989). The ineffective nodules are often short lived, and they are also much smaller than effective ones. Although, they are typically far more numerous than the effective nodules, the total volume of ineffective bacteriod tissue per plant is much smaller and the colour is less pink (Lynch and Wood, 1989), indicating a lower leghaemoglobin content. Usually, the legume root can carry only a limited number of nodules per unit length, hence if root growth ceases early in the season, the root system can become saturated with nodules. Once this has happened no further bacteria of any other strain can produce additional nodules on the root. Therefore, nodules already present on the root can affect the numbers of new nodules produced. Initial nodulation is as rapid with effective and ineffective strains, but the effective nodules inhibit further nodulation as soon as nodule growth has started (Kosslak and Bohlool, 1984).

### 2.2.3 Factors affecting legume nodulation

The most obvious requirement for a legume to form an effective nitrogen fixing symbiosis is the ability of the legume to nodulate (Giller and Wilson, 1993). Different *Rhizobium* species have various degrees of specificity or host preference (Quispel, 1983). Therefore, if legumes are introduced into soils in which the appropriate *Rhizobium* species is absent, no nodulation will occur (Rupela *et al.*, 1987). Certainly, it cannot be assumed that a legume can nodulate under all conditions just because it is a legume (Giller and Wilson, 1993). Apart from the need for the presence of the appropriate rhizobial species, nodule formation may also fail as a result of any of the following incompatibilities: (i) bacterial defect, (ii) hereditary defect in the legume and (iii) specific interaction between the bacterial and the legume (Lynch and Wood, 1989).

Hostile conditions such as high temperature, drought, acidity and nutrient deficiencies can at several different levels of the nodulation process prevent nodulation (Day *et al.*, 1978). Excessive soil temperatures can kill the majority of the bacteria in the surface layers of soil, although some rhizobia are able to survive at 70$^{o}$C in dry soil (Marshall, 1964; Danso, 1977). The Survival of bacteria in soil at high temperature appears to be improved by the presence of clay particles and soil organic matter (Day *et al.*, 1978). Differences in the effect of temperature on the ability of various *Rhizobium* strains to nodulate have been reported (Kvien and Ham, 1985; Piha and Munns, 1987). Nodules formed by an effective strain at high temperatures (35 and 38$^{o}$C) were observed to be ineffective (Hungria and Franco, 1993). Furthermore, deeper-placed nodules have been observed to be more active in nitrogen fixation when top soil temperatures are high (Piha and Munns 1987). Several studies have assessed the effects of temperature on the growth of various legumes (Dart and Day, 1971; Date, 1989; Schomberg and

Weaver, 1992). The work of Montanez *et al.* (1995) indicated that while the growth of the legume was not very sensitive to high temperatures, the symbiosis was seriously affected. Elevated temperatures may delay nodule initiation and development and interfere with nodule structure and functioning especially in temperate legumes, whereas in tropical legumes, nitrogen fixation efficiency is mainly affected. Moderate ($30 - 20^0$C) day – night temperatures give early nodulation and high nitrogen fixation rates. However, duration of active nitrogen fixation is shortened because of rapid degeneration of nodules (Graham, 1979). Low temperatures delay root hair infection, and decrease nodulation and nitrogenase activity (Waughman, 1977).

Legumes are intolerant to shortage and excess of water. This is primarily due to the ultrasensitivity of the symbiosis to water stress (Sprent, 1984). Infection is restricted in dry soils, because of absence of normal root hairs; short stubby root hairs appear, instead, which are inadequate for infection by rhizobia (Lie, 1981). Water stress reduces both nitrogen fixation and respiration of nodules, and within certain limits this reduction is proportional to the degree of water loss of the nodules (Guerin *et al.*, 1990). Excess water is particularly detrimental to nitrogen fixation. A thin layer of water on the nodule surface reduces nitrogen fixation considerably presumably due to the low diffusion of oxygen. Build up of carbon dioxide may occur under waterlogged conditions, which at high concentration inhibit nodule formation (Guerin *et al.*, 1990). Another gas known to be produced in anaerobic soils is ethylene, which, at low concentrations, can also restrict nodulation (Eaglesham and Ayanaba, 1984).

Legumes are generally more sensitive to salinity than bacteria (Singleton *et al.*, 1982). This is perhaps not surprising in view of the fact that in the symbiotic state rhizobia live within cells

which have much greater solute concentrations than those generally experienced in soils (Sprent, 1984). The process of root hair infection of legumes in particular is sensitive to saline stress, perhaps due to the common cessation of root hair growth in these conditions (Sprent, 1984). It may also be caused by the bacterial partner as different strains of rhizobia were found to show marked differences in their ability to infect and form nodules on pigeon pea under saline conditions (SubbaRao *et al.*, 1990).

Among the grain legumes, cowpea and groundnut are more tolerant of soil acidity than are soybean or common bean (Munns, 1978). Large differences in sensitivity to the toxic effects of acid soils have also been found between different species of tropical pasture legumes (Andrew, 1976; de Carvalho *et al.*, 1982). Acid soils usually have some inherent adverse concentrations of certain elements coupled with related nutrient deficiencies. The principal effects of soil acidity may be resolved into hydrogen ion concentration, deficiencies of calcium, phosphorus and molybdenum, and excessive quantities of aluminium and manganese. The presence of available aluminium in acid soils inhibits nodulation directly and indirectly by stunting root growth, and also inhibiting calcium uptake (Bell and Edwards, 1987). Nodule number decreases with decreasing calcium availability and with increasing aluminium level in the soil. Aluminium is a potent stress to the growth of free-living rhizobia. Fast-growing rhizobia appear to be less tolerant to aluminium than slow-growing rhizobia (Munns and Keyser, 1981). Aluminium not only prevents some plants from nodulating, but also delays and depresses nodulation.

Effects of high pH on rhizobial growth, nodulation and legume growth have been reported to some extent in the literature. Yadav and Vyas (1971), in two surveys of 23 rhizobial isolates

from eight legume species, reported that all grew well at pH values up to 10. By contrast, none of the 17 strains of *Bradyrhizobium japonicum* tested showed significant growth at pH 8.5 (Diatloff, 1970). However, beneficial effects on root hair infection and nodulation on alfafa were reported on high extremes of soil pH (Lakshi-Kumari *et al.*, 1974), whereas pH above 6.0 reduced nodulation in lupins (Tang and Robson, 1993).

Several of the nutrients essential for the growth of plants or bacteria play specific roles in the nodulation process and acute deficiency can hinder nodulation (O'Hara *et al.*, 1988). Nutrients such as phosphorus and sulphur are required for nodule metabolism. When legumes dependent on symbiotic nitrogen fixation receive an inadequate supply of phosphorus, they also suffer from nitrogen deficiency. Under these conditions, nitrogen deficiency symptoms are dominant and can be alleviated by the application of phosphorus fertilizers (Dadson and Acquaah, 1984). Nitrogen has the most prominent influence on nodulation of legumes (Horst, 1986). This influence can be stimulating or depressing, depending on the level of available soil nitrogen and the legume. Except for the stimulatory effect of "starter" doses where nitrogen deficiency or hunger occurs in young seedlings, high levels of inorganic nitrogen are generally inhibitory to biological nitrogen fixation (Giller and Wilson, 1993). The effect of nitrogen on the different stages of the nodulation process has been examined, and all the various steps, from the induction of rhizobial nodulation genes, through root hair curling, penetration and infection thread formation, are inhibited to a greater or lesser extent by the presence of inorganic nitrogen (Carrol and Mathews, 1990). The result is that, the actual number of nodules formed is reduced, leading eventually to complete suppression of nodulation if concentrations exceed a certain threshold value. This may vary from plant species to species (Harper and Gibson, 1984) and cultivar to cultivar (Hardarson *et al.*, 1984; Senaratne *et al.*, 1987). At intermediate

concentrations the effect of nitrogen is manifested in the developing nodules being smaller, such that the nodule mass per plant is reduced while the total number of nodules remains almost unaltered (Streeter, 1988). Another effect of nitrogen is the actual inhibition of fixation in active nodules. This has been demonstrated both in greenhouse (Streeter, 1985) and in field-grown plants (Eardly *et al.*, 1984). The requirement for molybdenum, the metal component of nitrogenase, explains the occurrence of nitrogen deficiency symptoms in legumes growing on soils low in available molybdenum (Parker and Harris, 1977). Cobalt is also required for the synthesis of leghaemoglobin. Therefore, in legumes, there is a close correlation between cobalt supply, nitrogen fixation and leghaemoglobin content of nodules (Chatel *et al.*, 1978).

Exploitation of the potential to increase nitrogen fixation in a legume requires a good knowledge of its nodulation capabilities. Studies on the genetics of legume nodulation have indicated that some legume genotypes have lost the ability to form effective nodules, whilst others have lost the ability to nodulate completely (Gibson, 1988; Vance *et al.*, 1988). The presence of large genotypic variability for nodulation in cowpea has long been known (Zari *et al.*, 1978). Variations in nodulation within a species may be due to cultivar effects and duration of growth (Awonaike *et al.*, 1990; Armstrong *et al.*, 1994; Bell *et al.*, 1994). Large genotypic variability for nitrogen fixation traits like nodule number, nodule mass and acetylene reduction activity per plant has been known since the early eighties for groundnut and pigeon pea (Nambiar *et al.*, 1988) soybean (Wacek and Brill, 1976), cowpea (Zari *et al.*, 1978), common bean (Graham and Rosas, 1977). These studies have shown that low as well as high nodulating lines occur among cultivars and that even non-nodulating plants occur among normal cultivars or races.

Late maturing cultivars have been found to fix more nitrogen than early maturing cultivars (Rennie *et al.*, 1982). However, within maturing groups only small differences in nitrogen fixation were observed (Paterson and La Rue, 1983). *Rhizobium* strains also differ in their ability to form nodules and to support nitrogen fixation and yield of legumes (Bezdicek *et al.*, 1978; Rennie and Dubertz, 1984). The interaction between cultivar and strain has been reported to significantly influence plant dry weight, nitrogen yield, percent nitrogen derived from the atmosphere, and amount of nitrogen fixed in soybean (Senaratne *et al.*, 1987; Rennie and Dubetz, 1984).

Nitrogen fixation in some nodulated legume cultivars can be maximised by inoculating with effective *Rhizobium* strains. The need to inoculate is a question that is established in several ways (Allen and Allen, 1961; Roughley and Brockwell, 1987; Thies *et al.*, 1991c). Field experiments have been designed to determine the need for inoculation (Bell and Nutman, 1971; Brockwell, 1971; Date, 1977; Thies *et al.*, 1991b), but take several months to complete. Bonishy (1979), using dilutions of soil samples to inoculate clover seedlings growing aseptically in test tubes demonstrated a laboratory means for characterising simultaneously the size and nitrogen fixing capacity of soil-borne populations of rhizobia. Brockwell *et al.* (1988), developed this method into an expeditious assay which could be combined with a serial dilution plant infection technique for the enumeration of rhizobia. A related procedure (Thies *et al.*, 1991c), makes it possible to forecast the likely success of introducing inoculant rhizobia into the soil, by considering indices of size of resident rhizobial population and the nitrogen status of the soil. A unique proposal for predicting the need for inoculation on a regional basis using a Geographical Information System has also been advanced by Thies *et al.* (1994).

### 2.2.4 Longevity of nodule

The amount of nitrogen fixed by a leguminous plant depends largely on the longevity of the nodules formed. Four factors affect nodule longevity or persistence: (1) the physiological condition of the plant, (2) the moisture content of the soil, (3) parasites in the nodule, and (4) the bacterial strain inhabiting the nodule (Lynch and Wood, 1989). The effect of the physiological condition applies particularly to annual plants whose nodules tend to die at flowering and seed set (Lynch and Wood, 1989). This is presumably because at this time the flowers and developing seeds are drawing on the carbohydrate reserves of the plant very heavily, and the young seeds may also be drawing on the nitrogen compounds in the nodules and leaves. Perennial legumes differ appreciably in the longevity of their nodules. Leguminous shrubs and trees may carry nodules for several years. Nodules seem to remain on the roots of many leguminous plants if soil is kept moist (Albrecht *et al.*, 1984). The first effect at the onset of drought is for the plant to shed its nodules (Lynch and Wood, 1989), though unfortunately no systematic work has been done on the moisture deficiency, or the water tension in soil, at which shedding is severe.

## 2.3. Inoculation of legumes with rhizobia

An important aspect of the successful exploitation of the *Rhizobium* - legume symbiosis has been the opportunity of introducing effective rhizobial strains into the soil. This is usually carried out at seed planting time with the aim of ensuring adequate nitrogen fixing rhizobia. Successful inoculation depends on the selection of the appropriate rhizobial strains and their provision in high enough numbers to colonise the developing root system. Inoculation is usually adopted for two main reasons. The first instance is when a legume is grown and the appropriate rhizobia are absent or present in numbers too low to form adequate nodules. A different and

more difficult problem exits for many legume crops grown in the tropics. Here, the soil often contains abundant rhizobia capable of nodulating the crop but the indigenous rhizobia are often of low nitrogen fixing effectiveness (Nambiar and Dart, 1982).

The most important point to consider is whether inoculation is needed Information regarding the nodulation of the legume species to be grown must be available, other important aspects are: the presence or absence of compatible rhizobia in the soil, their efficiency in fixing nitrogen and the previous cropping history of the soil. Where native rhizobia are ineffective in fixing nitrogen and the numbers fluctuate during the year, knowledge of their population shifts as well as their saprophytic competence is also important when legumes are to be inoculated (Obaton, 1975). Although information on the nodulation responses of legumes in tropical soils is scanty, a moderate amount of information on nodulation of cowpea is available (Giller and Wilson, 1993) Inoculation of cowpea in most of the humid tropics is considered to be unnecessary, due to the occurrence of large populations of highly competitive indigenous cowpea bradyrhizobia (Sellschop, 1962; Ezedinma, 1963; Doku, 1969; Ayanaba and Nangju, 1973), though in some cases inoculation has increased yields (Rotini, 1972; Danso and Owiredu, 1988; Rajput, 1994)

Application of inoculants to the seed surface prior to sowing is the traditional and most commonly means of inoculation, although viability of the rhizobial inoculant strain is subject to the hazards of drying (Salema *et al.*, 1982), fertilizer contact (Kremer *et al.*, 1982), seed coat toxicity (Materon and Weaver, 1984), incompatible pesticides and mineral additives (Gault and Brockwell, 1980) and inimical soil factors (Kremer and Peterson, 1983). Proposals to extend the life expectancy of rhizobia on seed, including curing inoculants before use (Burton, 1976; Materon and Weaver, 1985), and suspending cultures in alginate gel rather than sucrose before

their application to the seed (Rawsthorne and Summerfield, 1984), have been adopted. There are some situations where seed application of rhizobia may be an inefficient means of inoculation. For example, for seeds dressed with a pesticide incompatible with rhizobia, for inoculation of broad acre sowing of crop legumes with high seeding rates, and for seeds such as peanut which are too fragile for seed-surface inoculation (Brockwell, 1982). Preparations and procedures for inoculant application directly into the seed bed have been developed, either as solid inoculant (Barkdoll *et al.*, 1983; Hegde and Brahmaprakash, 1992) or liquid inoculant (Hely *et al.*, 1980). These methods have proved to be more effective in most cases than conventional seed inoculation for initiating nodulation and nitrogen fixation (Danso *et al.*, 1990; Rice and Olson, 1992; Rice *et al.*, 1998), but are often more labourious.

The normal criteria for rhizobial strain selection for inoculant production have been summarised by Keyser *et al.* (1992). Where there are large indigenous populations of rhizobia with poor effectiveness, it is necessary to add a further criterion to strain selection, that of competitiveness (Keyser *et al.*, 1992). The latter characteristic is very difficult to define and may vary from soil to soil. There is no correlation between effectiveness and ability to compete with other strains. Beattie *et al.*, (1989), stated that competitiveness reflects the behaviour of a particular strain in a particular soil with a particular plant and in a particular season. If any other factor is altered the competitiveness may be altered. Simply increasing the number of inoculant rhizobia is not always a guarantee of success as a good competitive strain, however, ineffective, may be able to overcome a numerical disadvantage of as high as 1:1000 as observed by Lynch and Wood, (1989). At least part of the competition phenomenon is related to the pre-infection stage of rapid rhizobial growth in the rhizosphere (Keyser *et al.*, 1992).

Very useful information can be gained from laboratory and greenhouse trials, but the eventual performance of an inoculant must be evaluated from field trials in a variety of soils. Some insurance against poor rhizobial performance in specific situations can be gained by using inoculants that comprise a mixture of strains. Here the precaution needed is that the strains are not mutually antagonistic.

## 2.4. Diversity of rhizobial isolates

Successful management of symbiotic associations between plants and their bacterial endosymbionts requires that specific strains of the bacteria can be readily identified. (Richardson *et al.*, 1995; Mpepereke *et al.*, 1997; Di Cello *et al.*, 1997). Any microbial utilisation in agriculture requires an evaluation of the environmental risks that are associated with the introduction of indigenous or non indigenous microorganisms into the rhizosphere (Di Cello *et al.*, 1997). It also requires assessment of the most suitable conditions for the effective and successful establishment of the inoculum in the rhizosphere of the host plant (de Leij *et al.*, 1994; 1995). Analysis of the structure of the microbial population therefore has practical importance; the results can be used to assess the fate of released strains and their impact on resident microbial communities.

African soils may harbour a large diversity of rhizobial populations, however, only little information is available. Cowpea rhizobia indigenous to Nigerian soils are probably the only group that has been studied (Ahmad *et al.*, 1981; Eaglesham *et al.*, 1987; Sinclair and Eaglesham, 1984). Assessing the diversity of West African cowpea bradyrhizobia based on physiological and biochemical characteristics, Eaglesham *et al.* (1987), found some traits

common to all or most of the isolates, some related to geographical origin and some colony morphology. The indigenous cowpea rhizobia strains in Zimbabwean soils showed considerable cultural and physiological diversity that included unique types belonging to several, as yet undefined species (Mpepereki *et al.*, 1997). Recent studies on fast growing rhizobia of *Sesbania* and *Acacia* species obtained from soils in Senegal, has led to the description of two fast growing species, *Sinorhizobium saheli* and *S. teranga* (de Lajudie *et al.*, 1994). Slow growing rhizobia nodulating soybean in Zimbabwean soils have also been reported by Davis and Mpepereki, (1994). In Ghana, the diversity of rhizobial populations of different legumes has not been examined.

Several classical and/or molecular techniques are available for the identification and analysis of the biodiversity of bacterial strains of a natural population. They include intrinsic antibiotic resistance (Muller *et al.*, 1988), serology (Ayanaba *et al.*, 1986), biolog automated analysis (Klinger *et al.*, 1992), multilocus enzyme electrophoresis techniques (Wise *et al.*, 1996), PCR ribotyping (Kostman *et al.*, 1992), and the random amplified polymorphic DNA (RAPD) method (Fani *et al.*, 1993). Analysis of the diversity of the cowpea rhizobia in this thesis was carried out by a combination of classical and molecular methods which had previously been successfully applied to studies of rhizobial populations isolated from different environments (Sessitsch *et al.*, 1997; Santamaria *et al.*, 1997; Tyler *et al.*, 1997; Zewdu *et al.*, 1998; Vinuesa *et al.*, 1998).

### 2.4.1. Methods for analysing diversity of rhizobia

#### 2.4. 1.1 Cross inoculation

The cross inoculation group concept is based on the ability of *Rhizobium* strains to specifically nodulate a group of legume host species (Fred *et al.*, 1932). Based on this concept, rhizobial strains have long been described as specific for strains apparently restricted in their host range or promiscuous for strains with a very broad host range (Burton, 1972). However, examination of a wide range of species has shown that many legumes are nodulated by rhizobia outside their own groups (Thies *et al.*, 1991). Consequently, the integrity of the cross inoculation concept as a system for determining relatedness among rhizobia strains has been questioned (Wilson, 1944; Bromfield and Barran, 1990), and is now in general disrepute.

Many *Rhizobium* strains have also been found to be so promiscuous that their host ranges do not even consist of closely related legumes, but may include legume plants that are so distantly related as to be placed in different sub-families within the leguminosaese. For example, the fast growing *Rhizobium* strain NGR234 has been shown to elicit nodules on over 37 genera of legumes including members of different sub families such as *Lablab purpureus* and *Leucaena leucocephala* (Trinick, 1980). The cross inoculation concept like the other methods for the characterisation of *Rhizobium* strains pays no attention to nitrogen fixation abilities. It is common for instance, to find strains of rhizobia that can elicit nodules on say ten 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). In view of these negative features of the cross inoculation concept, its continued usage in systematics has been justified on the basis of convenience and agronomic significance (Graham *et al.*, 1991). 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.*, 1996).

Using the cross inoculation classification system, Habish and Khairi (1968), showed that most grain legumes including cowpea could be grouped accordingly, even though the cowpea group showed some inconsistencies. Thies *et al.*, (1991), also found that only 18% of cowpea-derived isolates formed effective nodules on peanut, although peanut nodulates heavily in most African soils. Similar results were obtained by Mpepereki *et al.*, (1996), who concluded that the indigenous cowpea rhizobia of Zimbabwean soils had relatively narrow host ranges. These reports contradict the statement that cowpea rhizobia indigenous to African soils are promiscuous, and nodulates a wide range of legumes (Singleton *et al.*, 1992). These bring to the fore that very little is actually known about the symbiotic characteristic of the cowpea rhizobia indigenous to African soils. The cross inoculation concept was therefore used in this study to (i) determine the extent of symbiotic specificity of indigenous cowpea rhizobia isolated from a range of physiographic environments in Ghana and (ii) characterise the indigenous rhizobia.

#### 2.4.1.2 Cultural and metabolic characterisation

Cultural and metabolic characteristics have been described as useful guides for the recognition of rhizobial groups at the species level (Vincent, 1970). Rhizobial strains may be recognised as such by a combination of a large number of traits such as growth characteristics, carbon source utilisation, 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 characterised was suggested by Lange (1961), as a way to resolving the taxonomic

difficulties within the genus *Rhizobium*. Meanwhile, many researchers consider the above mentioned methodology as impractical (Graham and Parker, 1964). However, cultural and metabolic parameters are used for a phenotypic characterisation that is 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 so-called slow-growing strains that nodulate tropical legumes and the fast-growing strains that nodulate temperate legumes (Jordan, 1984). Evidence has however, accumulated through a variety of cultural and physiological tests which show that some tropical legumes are nodulated by both fast and slow-growing rhizobial strains (Lim and Ng, 1977, 1979; Pankhurst, 1977; Keyser *et al.*, 1982; Lawrie, 1983; Broughton *et al.*, 1984; Padmanabhan *et al.*, 1990; Mpepereki *et al.*, 1997). The results of these reports point to the possible existence of several unique but unidentified *Rhizobium* species that nodulate tropical legumes.

Currently, no data exist on the cultural and physiological characteristics of the indigenous rhizobial populations of Ghanaian soils. However, it is important to describe the cultural and physiological characteristics of the native rhizobia since it serves as a guide to species identification (Vincent, 1970). It also provides information about the performance of rhizobial strains under stress conditions, which gives additional criteria for the selection of rhizobial strains for inoculant production.

### 2.4.1.3. Serology

Serological analysis characterises rhizobia according to their reactions with antisera produced against strains having some agronomic or particular interest. The most common serological methods currently used are agglutination (Means *et al.*, 1964; Date and Decker, 1965; Wollum, 1987), flourescent antibody techniques (Bohlool, 1987) and various forms of the enzyme-linked immunosorbent assay (Asanuma *et al.*, 1985; Ayanaba *et al.*, 1986; Fuhrmann and Wollum, 1985; Kishinevsky and Jones, 1987). Polyclonal antisera have been used in most cases, although monoclonal antibodies have been used successfully with soybean bradyrhizobia (Vellez *et al.*, 1988). Serological studies of indigenous rhizobia have revealed considerable diversity within and among rhizobial from different geographysical 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 (Damirgi *et al.*, 1967; Ham *et al.*, 1971) or total nitrogen content (Bezdicek, 1972).

One common goal of serological characterisation of bacterial strains is to identify groups that have practical importance to the management of a particular symbiosis. Y*et al*though many studies have documented serological diversity within rhizobial populations, relatively few have assessed the value of the resulting groupings in predicting symbiotic performance. One problem with using serology to characterise rhizobia is the presence of strains that do not react with all antisera tested and the frequency of non-reactive strains is often significant (Fuhrmann, 1990; Mpepereki and Wollum, 1991). Furthermore, serological analyses commonly reveal strains that cross - react with antisera derived from two or more reference strains. 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 (Gibson *et al.*, 1971; Hickey *et al.*, 1987; Sadowsky *et al.*, 1987).

Numerous studies have reported that the incidence of serogroups of *Bradyrizobium japonicum* in root nodules of soybean can be affected by the plant genotype (Caldwell and Vest, 1968; Caldwell and Hartwig, 1970; Kvien *et al.*, 1981; Cregan and Keyser, 1986). However, the results of Fuhrmann (1989), suggested that the influence of plant genotype is minimal. The incidence of different serogroups have also been attributed to the sampling location or soil type (Ham *et al.*, 1971; Keyser *et al.*, 1984; Kamicker and Brill, 1986.). Caldwell and Hartwig (1970), examined the effect of the location as well of the plant genotype on the serological distribution of *B. japonicum* in field sampled nodules. The study revealed high significant effects of similar magnitude for both variables. An analysis of cowpea miscellany rhizobia isolated from three West African sites showed that only 50% of the 53 isolates examined were serologically typed; those with dry colony morphology were found to be serologically reactive and diverse, whereas wet strains were found to be unreactive (Ahmad *et al.*, 1981). Similarly, in a study of rhizobial isolates from groundnut grown in Sudan, high cross reactivity of surface antigens were observed and strains with no cross-reactivity were found to have different colony morphologies (Hadad and Loynachan, 1986).

### 2.4.1.4. Molecular analysis

***The polymerase chain reaction (PCR)***

The polymerase chain reaction was developed about a decade ago (Mullis and Faloona, 1987). Yet PCR-based techniques have been shown to be one of the most effective means of differentiating complex genomes (Williams *et al.*, 1990; Welsh and McClelland, 1990; Caetano-Anolles *et al.*, 1991). Various PCR-based techniques have been used to differentiate the genomes of a wide range of diverse organisms including *Rhizobium* (Harrison *et al.*, 1992; Richardson *et al.*, 1995; Sessitsch *et al.*, 1997). PCR is basically a biochemical amplification process where a single target DNA segment can be amplified a million-fold or more in several hours (Mullis and Faloona, 1987). The main feature of PCR is that, if the sequences of DNA flanking an unknown region of a DNA molecule are known, the unknown 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 positions of 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, magnesium chloride and a heat resistant DNA polymerase. A thermocycler is used to provide a strict regime of temperature cycling, and each cycle consists of a denaturation, an annealing and an extension step. For denaturation, the temperature is raised to 94 – 95$^{o}$C in order to melt the target DNA and subsequently lowered to 30 – 55$^{o}$C to allow the primer to anneal with the

corresponding sequence of the template. The annealing temperature depends on the melting point of the primer – template complex. Finally, a temperature of usually 72°C is used for the DNA synthesis of the new DNA molecule. The PCR consists typically 30 to 35 cycles.

The utilisation 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. Potential applications of PCR-based methods include: (i) the authentication of specific inoculant strains, (ii) the determination of nodule occupancy in competition trials where mixed inocula are used (iii) the analysis of the recovery and persistence of inoculant 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 rhizobial field populations (Richardson *et al.*, 1995).

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 of the limitations of this methodology are that its huge amplification 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; Hill *et al.*, 1991; Wimpec *et al.*, 1991).

The development of the PCR has led to a battery of new fingerprinting methods. For instance, it had 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; de Bruijn, 1992; Sessitsch *et al.*, 1997). The REP and ERIC sequences contain highly conserved central inverted repeats, do not show significant homology to each other, and are normally found in intergenic transcribed, but not translated, regions (Versalovic *et al.*, 1991; Lupski and Weinstock, 1992). REP and ERIC PCR have been demonstrated to be a powerful tool for community analysis at the strain level (Judd *et al.*, 1993; Laguerre *et al.*, 1996; Sessitsch *et al.*, 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 RPO1-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 the genetic characterisation of rhizobial strains (Laguerre *et al.*, 1996). However, the ribosomal operon has been looked at most frequently by PCR-RFLP analysis (Nour *et al.*, 1994; Massol-Deya *et al.*, 1995; Sessitsch *et al.*, 1997). The sequence of the small sub-unit of ribosomal, the 16S rRNA gene, is a highly suitable phylogenetic marker (Woese, 1987; Schleifer and Ludwig, 1989), and genetic variation within this molecule give useful information on microbial taxonomy. Therefore RFLP analysis of PCR-amplified 16S rRNA genes has 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 (IGS) between the 16S and the 23S rRNA genes and has been applied to examine chromosomally encoded genetic variations at the strain level (Barry *et al.*, 1991; Sessitsch *et al.*, 1997).

Differentiation of rhizobial isolates indigenous to tropical soils using molecular methods is yet to be exploited. However, the realisation that genetic characterisation 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 cowpea rhizobia.

## 2.5. Symbiotic effectiveness

Determination of the population size of indigenous rhizobia and their diversity do not provide information on symbiotic effectiveness. However, it is important to know to what extent the native rhizobial strains nodulate cowpea effectively, because a low rate of nitrogen fixation can occur with normal nodule appearance (Eaglesham, 1985). Singleton and Tavares (1986), found that within a soil sample, the range of effectiveness of indigenous rhizobial strains differed significantly. This indicates that considerable diversity in the relative effectiveness of the

indigenous rhizobia forming symbiotic association with cowpea may exist. Such population diversity may be reflected in differences in the sizes of indigenous rhizobial populations (Singleton and Tavares, 1986; Thies *et al.*, 1991).

The results of screening 23 cowpea rhizobia in two field sites in Nigeria showed that only 30% of the isolates were effective (Ahmad *et al.*, 1981). 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. Similar findings were reported by Fredericks *et al*,. (1990), with clover isolates obtained from Ethiopian soils. In contrast to these results, Gibson *et al.*, (1975), observed only small differences in effectiveness among *Rhizobium trifolii* populations from eight regions in Australia. Lie and Goktan (1984) as well as Lie *et al.* (1987) also verified that the symbiotic variation among the European *Rhizobium* population nodulating pea was relatively small. Differences in effectiveness among cultivars of legume species may occur. This hypothesis was supported by Robinson (1969), who reported that rhizobia isolated from *Trifolium pratense* were ineffective on *T. subterraneum*. Similar findings were reported by Ferreira and Marques (1992).

Several environmental factors that may affect the symbiotic effectiveness of a rhizobial strain have been suggested. Holding and King (1963) found that the mean effectiveness of *Rhizobium trifolii* was significantly correlated with the base saturation, pH, as well as the exchangeable calcium and exchangeable magnesium content of a soil. Similar results were obtained by Hagedorn (1978), who correlated the poor effectiveness of *Rhizobium trifolii* populations with soil base status and phosphorus levels. These results however contradict with that of Ferreira and Marques (1992). They showed that soil type and plant origin did not influence general

effectiveness of natural populations of *Rhizobium leguminosarum* bv. *trifolii*. Brockwell and Katznelson (1976) also found that soil type did not affect the general effectiveness of natural populations. Rhizobia are well adapted to life 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 *Rhizobium 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 and Palacios, 1990; Brom *et al.*, 1991), which cause recombination and lead to rearrangements and deletions (Hahn and Hennecke, 1987).

## 2.6. Competition for nodule occupancy

Competition in broad terms refers to interactions between two or more organisms struggling in order to gain advantage over limited resources such as nutrients, water, light and space, that are present in the environment in an amount insufficient to meet the biological demand (Alexander, 1971). In the case of rhizobia, competition is most commonly used to refer to struggle for supremacy in nodule occupancy. Rhizobial strains differ greatly in competitiveness, a fact that underlies the observed differences in percentage of nodules formed by individual strains present in the same root environment of legumes. A number of reviews are available on rhizobial competition (Date and Brockwell, 1978; Amarger, 1984; Dowling and Broughton, 1986; Triplett, 1990; Bottomley, 1992). Each summarises the profuse literature which illustrates the fact that different rhizobial strains may show different abilities to compete for nodule occupancy. Using three streptomycin-resistant cowpea bradyrhizobial strains, Danso and

Owiredu (1988) showed that the three strains differed in their inherent competitive abilities, which was revealed by great differences in nodule occupancy when the strains were equally represented in mixed inocula.

Relative success in achieving nodule occupancy is affected by environmental factors, host plant species and cultivar, initial population size and distribution in the soil and by competition from other organisms (Bottomley, 1992). Soil is the reservoir of *Rhizobium* strains and the intrinsic make up of the soil can affect the outcome of competition (May and Bohlool, 1983; Moawad and Bohlool, 1984). *Rhizobium* strains do not respond equally to the application of nitrogen. McNeil (1982), found that one strain of *B. japonicum* out-competed another for nodulation sites in the presence of nitrate. Correspondingly, this strain would have an advantage in soils with relatively high nitrogen levels. A study on competition of two serogroups of *R. trifolii* in a tropical soil demonstrated that when the soil was limed (to increase available phosphates) the dominant serogroup was replaced by a minor one (Almendras and Bottomley, 1985). The addition of phosphate alone had little effect on the outcome of competition between the two strains. However, the addition of phosphate and lime restored the dominance of the original serogroup (Almendras and Bottomley, 1985). This suggests that phosphorus limitation is exacerbated by low pH and the combination of pH and phosphorus levels can have a strong influence on competition. The effect of soil pH on rhizobial competition is well documented. Dughri and Bottomley (1983) were able to alter the outcome of competition between indigenous rhizobia in the soil by changing the pH. When Russel and Jones (1975) adjusted soil pH from acid to neutral by liming, they inadvertently favoured nodule formation by the ineffective strain. Soil temperature can also alter the outcome of competition between strains. In mixed inoculants containing *Vigna* rhizobia as well as *B. japonicum* applied to a promiscuously nodulating

cultivar of *Glycine max*, the *Vigna* rhizobia were found to be more competitive at higher temperature (36°C) whereas *B. japonicum* competed better at lower temperature (24-30°C)(Roughley *et al.*, 1980). Drought conditions, which are often found in conjunction with salt stress, also affect *Rhizobium* competition (Joshi *et al.*, 1981; Osa-Afiana and Alexander, 1982). *Bradyrhizobium* strains isolated from arid areas were found to be more tolerant of desiccation than strains isolated from cooler, wetter regions (Hartel and Alexander, 1984). Rhizobial strains differ in their motility in soil and it has been suggested that motile strains may be able to occupy lateral roots resulting in late stage nodulation and increased higher nitrogen fixation (Wadisirisuk *et al.*, 1989).

Rhizobia must multiply in the rhizosphere and attach to the host plant in order to initiate nodulation. The macrosymbiont therefore plays an important role in competition (Hardarson *et al.*, 1982; Cregan and Keyser, 1989). In a study by Moawad *et al.* (1984), competition among three serogroups of *B. japonicum* was examined in the rhizosphere and in non-rhizosphere soil. They observed that the *Bradyrhizobium* populations increased gradually from $10^4 g^{-1}$ to $10^6 g^{-1}$ in the rhizosphere soil during the first weeks after planting *Glycine* max, while the numbers in fallow or non-rhizosphere soil remained at $10^4 g^{-1}$. Furthermore, Moawad *et al.*, (1984) reported that there were no significant differences between the abundance of the three serogroups, and serogroup 123, which formed 60 - 100% of the nodules, and thus showed no obvious dominance in the rhizosphere. Other authors have shown that the proportion of nodules formed by a strain could be correlated with its relative representation on the root surface (Marques *et al.*, 1974; Franco and Vincent 1976).

The soil population density of indigenous rhizobia is a major factor in determining competition for nodule occupancy. In field trials Weaver and Frederick (1974), demonstrated that, nodule occupancy of greater than 50% by an inoculant strain was achieved by applying it at a rate of at least 1000 times greater than the estimated number of indigenous rhizobia. Several authors have actually demonstrated that there is little chance of inoculated strains forming many nodules if there is a significant indigenous population of compatible rhizobia (Carter *et al.*, 1995). The negative response of cowpea to rhizobial inoculation in tropical soils for instance, has been attributed to the occurrence of large populations of highly competitive indigenous cowpea rhizobia (Sellschop, 1962; Kang *et al.*, 1977).

Numerous efforts have been made to bias the outcome of competition between rhizobial strains. These include the improvement of the inoculant formulation (Zdor and Pueppeke, 1990; Zablotowick *et al.*, 1991), the application of extremely high inoculation rates (McLoughlin *et al.*, 1990a and b) and variation in the inoculum placement (McDermott and Graham, 1989). Breeding programmes with this as a major objective have also been carried out. For example, experiments have been directed towards the selection of highly effective combinations of host plant cultivar and bacterial strains (Alwi *et al.*, 1989) or the development of host lines with restricted nodulation and thus are able to bypass the native soil rhizobia (Cregan and Keyser, 1986; Montealegre and Kipe-Nolt, 1994). The other approach involves screening for plants that are nodulated by the most effective strains present in a natural soil population (Kueneman *et al.*, 1984; Herridge and Rose, 1994).

Bacteria are almost always haploid; they possess only one single set of genes, but they also form zygotes. These are never the result of fusion of whole cells. Usually a part of the genetic material of a donor cell is transferred to a recipient (acceptor) cell, so that a partial zygote (Merozygote) is produced. The subsequent chromosomal and cell division results in a cell that contains only the recombined chromosome (Schlegel, 1996). Three types of transfer of genetic character are known in bacteria: conjugation, transduction and transformation. In the course of all three processes, DNA is transferred from a donor bacterium to a recipient bacterium. The process differs only in the manner in which the DNA is transported. The transfer process is immediately followed by DNA recombination in the recipient cell (Schlegel, 1996).

Transfer of genetic material between *Rhizobium* strains in the legume rhizosphere has been observed to occur most frequently by conjugation (Broughton *et al.*, 1987; Sullivan *et al.*, 1995; Bo Normander *et al.*, 1998), although transformation by circular plasmid DNA and bacteriophage-mediated transduction also occurs (Kiss and Kalman, 1982; Finan *et al.*, 1984; Martin and Long, 1984). Most fast-growing strains of *Rhizobium* harbour symbiotic genes in large plasmids (Hombrecher *et al.*, 1981; O'Connel *et al.*, 1984), and some of these plasmids are self transmissible (Johnston *et al.*, 1978). In addition, *Rhizobium* plasmids are capable of genetic recombination, producing novel plasmids (Djordjevic *et al.*, 1983). Thus, genetic exchange among rhizobia and genomic instability of *Rhizobium* (Flores *et al.*, 1988; Brom *et al.*, 1991) can lead to altered competitiveness and nodulation properties.

### 2.6.1. Molecular gene markers in competition studies

Low symbiotic nitrogen fixation in plants is in many cases a result of competition between effective and ineffective rhizobia for nodule occupancy (May and Bohlool, 1983). In such cases, solving the rhizobial competition problem is essential in order to improve the symbiotic interaction between bacteria and plants. Apparently, the lack of suitable methodology to properly identify rhizobial strains has been the greatest barrier (Wilson, 1995). Evaluation of the competitive ability of rhizobial strains has been done by employing intrinsic (Broughton *et al.*, 1987) or induced (Bushby, 1981; Danso and Owiredu, 1988) antibiotic resistances as identifying markers. Other markers used include the enzyme linked immunosorbent assay (ELISA) (Berger *et al.*, 1979). Analysis of plasmid profiles has also been used in rhizobial competition studies (Broughton *et al.*, 1987; Pepper *et al.*, 1989; Shishido and Pepper, 1990). Techniques also have been developed by which a specific marker gene can be introduced into the genome of the organism to be studied. The marker gene codes for an enzyme that gives rise to a coloured product following incubation with a histochemical substrate. The marker gene thus allows the visual detection of the marked organism. Such a marker gene in current use in ecological studies of rhizobia is the gus A gene encoding the enzyme ß -glucuronidase (GUS) (Jefferson *et al.*, 1987; Wilson *et al.*, 1991; Wilson *et al*, 1995). GUS is a hydrolase that cleaves a wide range of substrates - almost any aglycone conjugated to D - glucuronic acid in the configuration. Frequently used substrates are X-gluc and Magenta-gluc giving rise to a blue or red colour. Nodules occupied by *gusA*-marked rhizobia are detected by virtue of a simple colour change (Wilson, 1995). The greatest advantage of GUS is the nearly complete lack of endogenous activity in plants and most agriculturally important bacteria. The assay has so far been used with bradyrhizobia that nodulate cowpea (Wilson *et al.*, 1991) and *Rhizobium* sp. inoculated onto

siratro and pigeon pea (Wilson *et al.*, 1992; Wilson, 1995). Furthermore, the gusA marker gene has been used for competition studies of *R. etli* (Streit *et al.*, 1995) and *R. tropici* (Sessitsch *et al.*, 1997). Another marker gene that has been used for the detection of bacterial strains and that can be used in combination with *gusA*, is the *celB* gene (Sessitsch *et al.*, 1996). It encodes the enzyme ß-glucosidase with a high galactosidase activity that is thermostable and thermoactive and has a half time of 85 hours at 100°C (Voorhorst *et al.*, 1995). Assays for the detection of *celB* activity within a nodule or on plant sample are simple. The washed legume root is incubated in phosphate buffer at 70°C in order to destroy endogenous enzymes. The roots are then incubated in the presence of a chromogenic substrate for *celB* product such as X-gal (5-bromo-4-chloro-3-ß-D-galactoside) giving rise to a blue colour (Sessitsch *et al.*, 1996).

To date only a limited amount of data has been collected on the impact of inserted genes on the ecological fitness of the host organism (Doyle *et al.*, 1995). Initially it was widely assumed that genetically engineered organisms (GEMs) would be impaired in fitness compared to their parental strains, due to the additional metabolic load imposed by expression of the inserted DNA. In practice, this has proven not to be the case, with a number of studies demonstrating equal survival of GEMs and their parents. Sessitsch *et al.* (1997) measured the competitiveness index of various *gusA*-marked derivatives of *R. tropici* strain CIAT899. In that study, no effect on the competitive ability due to the presence of the marker gene was found indicating that the *gusA* cassette used is a suitable marker for rhizobial competition studies. Likewise, a strain of *Erwinia carotovora*, engineered to contain a chromosomal kanamycin resistance gene, showed equivalent survival capabilities as its parental strain in soil (Orvos *et al.*, 1990). *Pseudomonas aeroginosa* and *P putida* growth rates were unaffected by introduced plasmids, although

survival capabilities may have declined slightly (Yeung *et al.*, 1989). However in some other examples, fitness was found to be compromised. For example, strains of *P. fluorescens* marked with a *Bacillus*-endotoxin gene had slightly decreased growth and survival capabilities compared to the parental strain (van Elsas *et al.*, 1991). Further complications derive from the observation that effects on fitness may be dependent on the nature of the host strain rather than the nature of the foreign DNA (Devanas *et al.*, 1986) and that the host genome may even evolve to become adapted to the introduced DNA such that loss of the DNA subsequently reduces fitness (Bouma and Lenski, 1988). There are few examples where fitness parameters other than growth or survival have been measured. Lam *et al.*, (1990) analysed over 1200 mutants of *P. putida* containing a promoterless *lacZ* gene on a transposon Tn*5* derivative, for their ability to colonize roots and found isolates with both increased and decreased colonisation ability. The majority of the isolates, however, showed a colonisation ability that differed little from the wild type strain. A few studies have looked at the effect on competition of marking rhizobia with the intact transposon Tn5 element. Sharma *et al.* (1991) found that the competitive ability as well as the nitrogen fixation ability of two strains of chickpea rhizobia tagged with Tn5 was not affected. Similar findings were reported by Sessitsch *et al.* (1997) where *R. tropici* derivatives carrying *gusA* minitransposons showed symbiotic traits as well as growth characteristics similar to their parent strain.

Aspects of the legume-*Rhizobium* symbiosis relevant to this study have been reviewed in the foregoing literature analysis. While it is quite clear that the legume-*Rhizobium* symbiosis has and continues to receive research attention, information from tropical Africa is scanty. For the purposes of improving nitrogen fixation and yield of cowpea to enhance its contribution in

farming systems in Ghana, research on the population structure of the native rhizobia that nodulate cowpea is needed. This will not only help to reveal the numerous yet undiscovered strains of rhizobia, but would lay firm foundations on which agronomic strategies can be developed.

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Soil sampling

Soil samples representing 20 soil series (Table 3.1) were collected (from the 0-15 cm layer) from the five ecological zones of Ghana (Fig 3.1). Each soil type was air-dried, undecomposed plant materials were removed by hand from the dried soil, and the soil aggregates were gently crushed in a mortar to pass through a 2mm sieve. Subsamples were used to analyse for soil physiochemical properties (Table 3.2).

### 3.1.1 Soil analysis

#### 3.1.1.1 Soil pH

Soil pH was determined in both distilled water and in 0.01M $CaCl_2$. Soil pH was measured in 1:2 soil solution ratio using a Pracitronic pH meter. Twenty grammes of soil was weighed into a beaker and 40ml of distilled water or 0.01 M $CaCl_2$ added to give a soil to solution ratio of 1:2. The mixture was stirred several times for 30 min and left for about 1 hr to allow most of the suspended clay to settle. The pH meter electrodes were then immersed into the partly settled suspension and the reading on the pH meter recorded. There were three replicates of each soil type for the pH measurement.

Table 3.1 Classification of the soils studied

| SOIL SERIES | CLASSIFICATION | | PARENT MATERIAL |
|---|---|---|---|
| | LOCAL | USDA | |
| Adenta | Savanna Ochrosols | Typic Paleustalf | Quartzite schist |
| Agawtaw | Tropical Grey Earths | Typic Natrustalf | Colluvium from acidic gneiss |
| Akuse | Tropical Black Earths | Typic Calciustert | Colluvium from basic hornblende gneiss |
| Aveime | NA* | Rhodustalf | Alluvium |
| Abenia | Forest Oxisols | Ultisols | In situ weathered from biotite granite |
| Ankasa | Forest Oxisols | Ultisols | Colluvium derived from biotite granite |
| Boi | Forest Oxisols | Ultisols | Tertiary deposits |
| Tikobo | Forest Oxisols | Ultisols | Tertiary deposits |
| Akumadan | Forest Ochrosols | Rhodic Paleudult | Phyllite |
| Bekwai | Red Forest Ochrosols | Rhodic Paleudult | Phyllite |
| Nzima | Brown Forest Ochrosols | Rhodic Paleudult | Colluvium from phyllite |
| Wacri | Brown Forest Ochrosols | Rhodic Paleudult | Colluvium from granite |
| Amantin | Savanna Ochrosols | Ustochrepts | Colluvium from Voltaian sandstone |
| Bediesi | Red Forest Ochrosols | Rhodic Paleudult | In situ weathered from sandstone |
| Denteso | Savanna Gleisols | Aquents | Colluvio-Alluvium from sandstone |
| Ejura | Savanna Ochrosols | Eutrustox | In situ weathered from Voltaian sandstone |
| Lima | Savanna Gleisols | Aquepts | Alluvium |
| Nyankpala | Savanna Ochrosols | Lithic Paleustults | In situ weathered from mudstone |
| Siare | Alluviosols | Fluvents | Alluvium |
| Tafali | Savanna Ochrosols | Typic Haplustalfs | Colluvium derived from granite |

* Not available

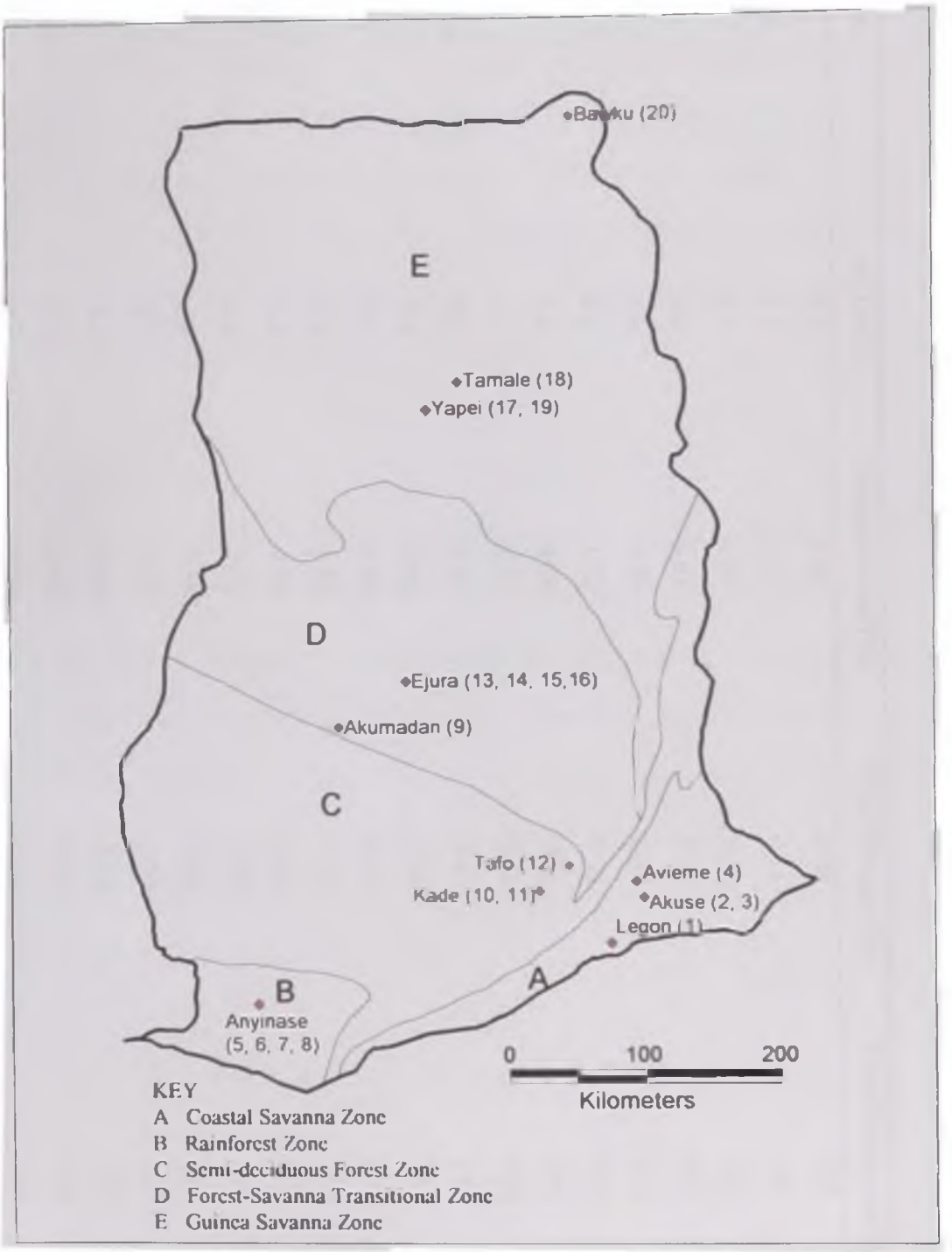


Fig. 3.1 Map of Ghana showing the ecological zones and sites where the soils (showing by numbers) were sampled

Table 3.2 Some chemical characteristics of the soils

| Soil Series | pH ($H_2O$) | Organic carbon (%) | Total Nitrogen (%) | Total Phosphorus (mg/kg) |
|---|---|---|---|---|
| Adenta | 4.6 | 0.84 | 0.58 | 120 |
| Agawtaw | 5.9 | 1.24 | 0.11 | 86 |
| Akuse | 6.1 | 0.62 | 0.16 | 103 |
| Aveime | 6.4 | 0.61 | 0.05 | 127 |
| Abenia | 3.9 | 4.24 | 0.38 | 150 |
| Ankasa | 4.0 | 1.52 | 0.12 | 141 |
| Boi | 3.8 | 4.09 | 0.34 | 178 |
| Tikobo | 4.3 | 2.11 | 0.19 | 164 |
| Akumadan | 6.2 | 2.18 | 0.17 | 148 |
| Bekwai | 5.6 | 2.86 | 0.21 | 173 |
| Nzima | 5.5 | 4.80 | 0.42 | 161 |
| Wacri | 5.9 | 1.59 | 0.17 | 203 |
| Amantin | 6.0 | 0.53 | 0.04 | 93 |
| Bediesi | 6.3 | 2.50 | 0.16 | 227 |
| Denteso | 5.1 | 0.78 | 0.04 | 78 |
| Ejura | 6.5 | 0.59 | 0.04 | 121 |
| Lima | 6.4 | 0.99 | 0.12 | 61 |
| Nyankpala | 6.2 | 0.69 | 0.05 | 129 |
| Siare | 6.6 | 0.51 | 0.08 | 166 |
| Tafali | 6.2 | 0.58 | 0.06 | 138 |

#### 3.1.1.2 Total phosphorus

Total phosphorus was determined by digesting 2 g soil with 30 mls of 60% perchloric/nitric acid mixture for 40 mins. The soil sample was pre-digested with the nitric acid for 20 mins and the sample allowed to cool before adding the perchloric acid, which was allowed to digest for 30 min. The digest was filtered into a 250ml volumetric flask and made to volume. Phosphorus in the digest was measured with the Phillips PU 8620 spectrophotometer using the molybdate ascorbic acid method of Watanabe and Olson (1962)

#### 3.1.1.3 Total nitrogen

A tablet of selenium catalyst was added onto 0.5 g of soil in a 250 ml Kjeldahl flask. This was followed by digestion with 5 ml concentrated $H_2SO_4$ until the digest became clear. The contents of the flask were transferred into a 100 ml volumetric flask and brought to volume with distilled water. An aliquot of 5 ml of the digest was taken into a Markham distillation apparatus. Five milliliters of NaOH was added to the aliquot and the mixture distilled. The distillate was collected in 5 ml of 2% boric acid to which 3 drops of mixed methyl red and methylene blue indicator had been added. This was then titrated against 0.01M HCl. Total nitrogen was calculated by the method of Bremner (1960).

### 3.2. Nodulation capabilities of cowpea

#### 3.2.1 Planting materials

Seeds of 45 cowpea cultivars were obtained from the Plant Genetic Resources Centre of the Council for Scientific and Industrial Research (CSIR), Bunso, Ghana. All the cultivars were of determinate growth habit and mature between 60 to 80 days.

### 3.2.2 Pot experiment

The abilities of the 45 cowpea cultivars to nodulate in the various soils were evaluated in black polythene seedling bags (15 x 23 cm) containing 1.5 Kg of each soil type. The bags were perforated at the bottom to allow free drainage of excess water. Four surface sterilised seeds of each of the 45 cultivars were planted in each bag which were thinned to two plants per bag after germination. There were three replicates for each soil for each cultivar. The plants were grown in the greenhouse and watered regularly. The plants were harvested 5 weeks after planting and assessed for nodulation. At harvest the seedling bag was removed from the soil-root matrix and a gentle stream of water from a hose was used to wash off the soil to expose the nodules. Nodulation was scored as positive when a plant bore at least a single nodule.

## 3.3. Enumeration of rhizobia

Rhizobial populations capable of nodulating cowpea in all the 20 soils were enumerated by the most probable number (MPN) method (Vincent, 1970) using plastic growth pouches (17cm x 15cm sizes) (Weaver and Frederick, 1972). Cowpea var. *Asontem* which gave the highest nodulation score was the host used to enumerate rhizobia. Clean seeds free of damage and of uniform size and good viability (99%) were surface sterillised in 70% alcohol for 4 minutes and 0.1% mercuric chloride for 3 minutes. The seeds were then rinsed in several changes of sterile distilled water (Somasegaran and Hoben, 1994). Seeds were germinated on 1% water agar until cotyledons were about 2 cm long. Seedlings were planted two per pouch. The pouches contained N- free nutrient solution (Somasegaran and Hoben, 1994). Ten-fold dilutions of each soil sample with four replicates per dilution were used to inoculate the pouches, at 1 ml per pouch. The pouches were randomly arranged on wooden racks and kept in the greenhouse with temperature of 30/23 $^{0}$C day/night and natural light of approximately 12 h photoperiod. The

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plants were supplied with sufficient N- free nutrient solution when required. Nodulation was assessed after 28 days of inoculation and the most probable numbers of rhizobia were calculated (Vincent, 1970).

### 3.4. Response of cowpea to nitrogen fertilization

#### 3.4.1 Pot experiment

Response of cowpea to nitrogen fertilization was evaluated in four cowpea cultivars (*Asontem*, *Benga*, *Sanji* and *Soronko*) that showed variable nodulation abilities from the nodulation experiment. The experiment was conducted in plastic pots(18cm high, 15cm wide at the top and 12cm at the base), using soils collected from Adenta, Akuse, Tafale and Wacri soil series, found to contain variable rhizobial populations. Each pot was filled with 5 kg of soil. Four seeds were planted in each pot and later thinned to two after germination. Treatments were (i) no fertilizer as control, and (ii) six levels of nitrogen fertilizer of 40, 80, 120, 160, 200 and 240 mg/kg (equivalent to 40, 80, 120, 160, 200 and 240 kg/ha). The fertilizer was applied in two splits at 4 days after germination (DAG) and at the onset of flowering, at 28 DAG. The experiment was arranged as a randomised complete block design with three replications. The plants were watered daily until harvest. Plants were harvested 35 days after planting (DAP) by cutting the stem at soil level. Roots were carefully washed from the soil in running tap water. Nodules were then separated from roots and counted. The vegetative parts were dried in the oven at 60$^{o}$C until constant weight was obtained.

### 3.5. Isolation of rhizobia

Representative nodule samples were taken from each of the MPN assays performed. The nodules were surface sterilised with 70% alcohol for 3 min and then with 0.1% mercuric

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chloride for another 3 min 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 glass rod. A loopful of the suspension was then streaked on yeast extract mannitol (YEM) agar plates and incubated at 28$^{o}$C.

### 3.5.1 Authentication of isolates

The isolates were evaluated as pure cultures that could form nodules on cowpea. Surface sterilised cowpea seeds were pre-germinated on 1% (w/v) water agar and planted in growth pouches containing N- free nutrient solution. The pouches were inoculated with 1ml 5 days YEM broth culture of each isolate. Uninoculated pouches served as controls. The pouches were placed on racks and kept at the green house. The plants were harvested after 28 days and the roots were observed for the presence of nodules. One hundred out of the total of 300 isolates obtained were randomly selected for further studies and characterisation.

### 3.5.2 Culture maintenance

Bradyrhizobia isolates were maintained on slopes of YEM and stored at 4$^{0}$C. All the isolates were re-plated on YEM and checked for purity at least every three months.

## 3.6. Physiological and metabolic characterisation of isolates

### 3.6.1 Growth rates and colony morphology

The abilities of the isolates to change the pH of their growth medium were scored on YEM agar plates amended with 0.25mg $l^{-1}$ bromthymol blue. Each isolate was cultured in YEM broth cultures grown to early log phase (population density of approximately $10^{8}$ cells $ml^{-1}$) and

streaked on duplicate YEM plates. Estimation of population density of cells was by the Miles and Misra drop count method (Collins and Lyne, 1985). Streaked plates were incubated at 28°C for 5 days and examined daily to determine the time to first appearance of colonies. Colony appearance was scored as dry where the surface was smooth and firm and wet for those which were watery or slimy.

### 3.6.2 Salt tolerance

Tolerance of the isolates to NaCl was determined by checking for growth on YEM agar plates containing 1, 2, 3, and 5% NaCl (w/v). Each isolate was streaked on duplicate plates and incubated at 28°C for 5 days.

### 3.6.3 Acid tolerance

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. The pH of tubes containing 10 ml of YEM broth with $KH_2PO_4$ omitted (Zablotowicz and Focht, 1981), was adjusted after sterillization by the addition of HCl or NaOH. Tubes were inoculated with 100µl aliquots of each isolate and incubated at 28°C for 5 days before scoring for growth.

### 3.6.4 Carbon utilization

The isolates were tested for their abilities to grow when provided with different carbohydrates as the sole carbon source. The test was carried out in a basal medium with mannitol omitted (Zablotowicz and Focht, 1981). The carbohydrates were added to give 10% (w/v) solution. The carbohydrates were sterilised separately by Millipore membrane filtration and were then added

to the sterilised liquefied medium just before plates were poured. Each isolate was streaked on duplicate plates and after 5 days incubation at 28°C, growth was scored. The following carbohydrate sources were tested: L- arabinose, D- glucose, D- galactose, fructose, lactose, maltose, mannitol and sucrose.

### 3.7. Host range analysis

The ability of the isolates to nodulate eight leguminous crops (*Arachis hypogea* (L), *Calopogonium phaseoloides (L)*, *Clotalaria* Spp, *Glycine max* (L) Merrill, *Leucaena leucocephala* (L) de Wit, *Mimosa ratusa* and *Voandzeia subterranean* (L) Verdc) randomly selected from the cowpea subfamily Papillionoidea and subfamily Mimosoideae were tested. These legumes are commonly found growing in Ghana. Seeds of the legumes were surface sterilised with 0.1% mercuric chloride (Somasegaran and Hoben, 1994). The seeds were pre-germinated on 1% (w/v) water agar and planted in growth pouches containing sterile N- free nutrient solution. Each growth pouch was inoculated with 1ml suspension of late log phase YEM broth culture of one of the isolates. Uninoculated pouches served as controls. The pouches were arranged randomly on wooden racks and plants grown in the greenhouse. After 35 days of growth, the plants were scored for nodulation.

### 3.8. Serological characterisation

#### 3.8.1 Preparation of rhizobia antigens

Fourteen of the isolates were randomly selected to test for the sero-relatedness of the isolates. The isolates were grown in YEM broth until late log phase. The cultures were centrifuged at 15,000 rpm for 10 min to obtain pellets. The pellets were resuspended and washed three times in sterilised 0.85% NaCl and boiled for 30 min.

### 3.8.2 Formulation of antigens for immunization.

Antigens for immunization were prepared with a 1:1 ratio of antigen suspension and Freund's incomplete adjuvant (Nambiar and Anjaiah, 1985). The antigen-adjuvant mixture was prepared by repeated drawing in and expelling in a glass syringe. The right consistency was reached when a drop of the mixture did not disperse when dropped in water (Somasegaran and Hoben, 1985). The antigen adjuvant mixture was used for only the first immunization. Subsequent boosters were done with 1:1 ratio mixture of sterile 0.85% NaCl and antigen suspension.

### 3.8.3 Experimental animals

Inbred BALB/c mice, aged between 12 and 14 weeks and 56 weeks old New Zealand rabbits were used for the production of antibodies. The mice were obtained from the Animals Breeding Unit of the Noguchi Memorial Institute for Medical Research and the rabbits from the Animal Husbandary Division, Ministry of Agriculture, Nungua-Accra.

### 3.8.4 Coating of microtitre plates with antigen

Rhizobial antigens were diluted in carbonate-bicarbonate buffer, pH 9.6 (coating buffer) (Voller *et al.*, 1977) Flat bottom 96-well polysterene microtitre plates (Sumilon type C), were coated with 100 µl per well of antigen coating buffer. The plates were shaken for 1 min and incubated overnight at 37$^{o}$C to enable adsorption of antigen onto polysterene wells.

### 3.8.5 ELISA procedure

Screening of antigens for antibody production, screening of rabbits for background antibodies, estimation of sera and conjugate titres and the detection of antibody response of the rabbits to antigen immunization were all assayed by the indirect ELISA procedure (Voller *et al.*, 1977).

In this assay, micro-ELISA plates coated with antigens were rinsed twice with washing buffer (PBS Tween 80, pH 7.4), flipped empty and banged to remove excess unbound antigen. The plates were incubated with titrated test sera diluted in washing buffer for 1hr at room temperature. The plates were flipped empty, banged and rinsed twice with washing buffer to remove excess unbound antibody. The plates were then incubated with 100 µl of goat anti-mouse or goat anti-rabbit horseradish peroxidase (HRPO) conjugate for 30 min at room temperature. The plates were then washed three times, each for 10 min. with washing buffer to remove excess unbound conjugate. The presence of bound conjugate was revealed or visualised by the addition of substrate ABTS and hydrogen peroxide pH 4.0, and incubated at room temperature for 30 min. The colourless substrate solution changed to green in wells with bound enzyme conjugates. The optical densities (OD) were read at 415 nm wavelength using MTP-32 ELISA reader

### 3.8.6 Evaluation of antigen for antibody production

Inbred BALB/c mice were immunized with the prepared antigens to test the production of antibodies by the antigens. The mice were injected intraperitoneally with 200 µl each of antigen and Freund's incomplete adjuvant mixture. The mice were given booster immunization with antigens only at 2 and 3 weeks. Ten days after the third booster, the mice were bled as indicated in the following section and tested for antibody response to homologous rhizobia antigens by the microplate indirect ELISA method.

### 3.8.7 Bleeding of mice and screening for antibody response

The mice were bled from the tail veins and from the heart by cardiac puncture and the serum tested at 1:50 in PBS. The tip of the tail of each mouse was cut with a pair of scissors and the tail gently squeezed from the base towards the tip. Blood from the severed tail veins was aspirated using a 50µl eppendorf pipette and then transferred into an eppendorf tube containing 500 µl of PBS and mixed thoroughly. Bleeding from the heart was done by killing the mice by anaesthesia using diethyl-ether. The mice were held at their back and blood was extracted from the heart through the sternum with a 5ml syringe. The blood in PBS was microfuged at 10,000 rpm for 2 min. The supernatants were pipetted into different tubes and kept at -20°C. Each antiserum was tested for antibody activity by titration in two-fold dilution on micro-titre plates previously coated with rhizobia antigen.

### 3.8.8 Selection of rabbits for immunization

Blood was extracted from the ear vein of 30 rabbits with a syringe and emptied into screw capped tubes. The blood was allowed to clot at room temperature and centrifuged at 14,000 rpm to separate the serum from the clot. The clear serum was drawn into sample bottles and kept at -20°C. Each serum was tested against all the antigens for background antibody using the ELISA procedure as described previously. Rabbits with no or minimal background antibodies were selected for each antigen.

### 3.8.9 Immunization of rabbits

The rabbits were injected with between 0.5 and 0.8ml of antigen emulsified with Freund's incomplete adjuvant or antigen mixed with 0.85% NaCl. The immunization schedules were as follows:

| Day | Procedure |
|---|---|
| 1 | intraperitonea |
| 7 | intravenous |
| 14 | back |
| 28 | bleeding/back booster |
| 35 | final bleeding |

### 3.8.10 Detection of antibody

Microtitre plates were coated with 100 µl of each antigen in quadruplicate and held overnight at 37$^{o}$C. The plates were washed twice in PBS-Tween, flipped empty and banged to dry. Rabbit serum in PBS-Tween at a predetermined optimal titre of 1:156 was added in 100 µl aliqouts to each well and held at room temperature for 1hr. The plates were washed three times. Goat anti-rabbit globulin conjugated to peroxidase (Sigma) was diluted at a pre-determined concentration of 1:1600 in PBS-Tween and 100 µl added to each well. The plates were incubated at room temperature for 30 min. The plates were washed again 5 times and 100 µl of enzyme substrate solution (standard ABTS in citrate buffer with 35% $H_2O_2$) added to each well. The plates were gently shaken and held at room temperature for 30 min. Absorbances were read at 415 nm with MTP-32 ELISA reader

## 3.9. Molecular characterisation

### 3.9.1 Sample preparation for DNA amplification

The isolates were grown on YEM agar plates at 28°C for 24 and 48 hr respectively for the fast and slow growers. Pelleted cells of each isolate were suspended in 100 µl Tris EDTA (TE) (Ausubel *et al.*, 1994) and the optical densities at 600 nm of all the samples were adjusted to 2.6 with sterile distilled water. The samples were then frozen for 4 min at -70°C. Afterwards, the cells were set on ice for 1min, boiled for 2 min, again set on ice for 1min and then boiled once more for 2 min. Finally, the cells were centrifuged for 2 min at 15,000 rpm and the supernatant used for the PCR assay.

The above procedure did not produce optimal DNA amplification for some of the isolates. In these cases, single colonies of the isolates were grown in YEM medium. The cells were pelleted by centrifugation at 15000 rpm. Genomic DNA was then isolated from the pellets using DNeasy Plant Mini Kit (Qiagen) according to the supplier's instruction. DNA concentration was adjusted spectrophotometrically to 100ng/ml.

### 3.9.2 PCR amplification of the 16S rRNA gene

Primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') described by Weisburg *et al.* (1991) were used for PCR amplification. They are derived from conserved regions of the 16S rRNA genes and amplify nearly full-length 16S rRNA genes (Weisburg *et al.*, 1991). PCR amplification was carried out in total reaction volume of 100µl. DNA was amplified by mixing 100 ng template DNA (pure DNA) or alternatively (cell extract) 5-8 ul, with 1 x PCR buffer (50 mM KCl; 20 mM Tris.HCl, pH 8.0), 200 µM each of dATP;

dCTP; dGTP and dTTP (Boehringer Mannheim), 3 mM $MgCl_2$, 0.2µM of each primer, and 2 U Taq DNA polymerase (Gibco,BRL). All amplifications were carried out in a Perkin-Elmer thermocycler (GeneAmp PCR System 9600). The temperature profile was as follows: an initial denaturation step at 95°C for 5 min followed by 35 cycles of 30 sec. denaturation at 94°C, 1 min annealing at 50°C and 2 min extension at 72°C and a final extension step for 4 min at 72°C (Sessitsch, 1997).

### 3.9.3 Electrophoresis and imaging

Aliquots (5µl) of the amplified DNA were mixed with loading buffer (2 µl) and analysed by horizontal agarose gel electrophoresis in1% agarose gels stained with 0.5µg $ml^{-1}$ ethidium bromide for 30min. The gels were photographed on a UV transilluminator after the electrophoresis.

### 3.9.4 Restriction fragment analysis

Aliquots (10 µl) of PCR products were digested with the tetrameric restriction endonucleases: Dde *I*, Hae *III*, Msp *I*, and Rsa *I* (Gibco BRL) in a total reaction volume of 20µl and incubation at 37°C for 2 hr. The resulting DNA fragments were analysed by horizontal agarose gel electrophoresis in 2.5 % agarose gels stained with ethidium bromide. Molecular weight markers (100 bp) were run a lane of each gel to enable calculation of the sizes of the resulting restriction fragments. Electrophoresis was carried out at 100V for 3 hr and the gels were photographed on a UV transilluminator.

### 3.10. Effectiveness of isolates in fixing nitrogen

The experiment was carried out in Leonard jars (of 250ml volume) containing 200 g acid-washed sand and N-free nutrient solution (Somasegaran and Hoben, 1985), in the bottom compartment. The isolates were grown in 100 ml YEM broth until late log phase (determined by optical density values at 600nm). Cowpea seeds were surface sterilised with 0.1% mercuric chloride (Somasegaran and Hoben, 1994) and germinated on 1% (w/v) water agar. Two of the pre-germinated seeds were planted in each jar that had been sterilised by autoclaving. The seedlings in each jar were inoculated with 1 ml (approximately $10^8$ cell $ml^{-1}$) of the rhizobial culture. There were three replicate jars for each of the *Rhizobium* isolates. Uninoculated seedlings with or without nitrogen (70µg $ml^{-1}$ $KNO_3$) served as controls. The jars were arranged in a randomized complete block design in the green house. The inoculated plants were kept supplied with N-free nutrient solution, while the uninoculated control received nitrogen. (Somasegaran and Hoben, 1994). The plants were harvested 35 days after planting (DAP). Nodulation, shoot dry weight and shoot nitrogen content were recorded. The mean dry weight of shoot (X) was used to calculate an index of effectiveness (E) defined as:

$$E_J = \frac{X_J - X_{TO}}{X_{TN} - X_{TO}} \times 100$$ ( Ferreira and Marques, 1992),

Where, j is the shoot dry weight of inoculated test strain, TO is that of the uninoculated control and TN that of the nitrogen control. Plant dry weight values of each isolate was compared with those of N controls and the LSD at P = 0.05 level was used to delineate isolates significantly different from the N controls (Beck et al., 1994). Classes of effectiveness were defined from comparison with the controls. Symbiotic effectiveness was high when the isolate produced plant yield equal to or greater than N- fertilised plants; moderate when slightly less than N controls

and ineffective when isolates produced yields similar to uninoculated controls (Beck et al., 1994).

### 3.10.1. Relative effectiveness of isolates in fixing nitrogen

Differences in symbiotic effectiveness of the 10 most effective isolates were compared in a separate experiment TAL 169 as a standard strain. Procedures described previously (3.10.1.) were followed. Relative effectiveness compared with strain TAL 169 was calculated by the following expression:

$$\text{Relative Effectiveness} = \frac{\text{Shoot dry wt inoculated test strain}}{\text{Shoot dry wt inoculated standard strain}} \times 100$$ (Gibson, 1980).

### 3.11. Measurement of nodulation competitiveness by glucuronidase (GUS) fusion

### 3.11.1 Gus fusion donor strain

The *E. coli* strain S17-1 λ-pir containing the relevant GUS transposon as donor strain (Jefferson *et al.*, 1986) was used. The strain was obtained from the FAO/IAEA Agricultural and Biotechnology Laboratory, Seibersdorf, Austria.

### 3.11.2 Marking rhizobia with gusA gene

Five millilitre cultures of three of the effective isolates (2, 10 and 14) and the donor strain were prepared in a modified YEM broth (Danso and Alexander, 1974) and Luria Bertani broth (LB) containing spectinomycin (50µg/ml) (Ausubel *et al.*, 1995) respectively. The cultures were microfuged at 4,000 rpm for 10 min. The supernatant was carefully removed and the cells were

washed in YEM medium to remove the antibiotic (spectinomycin) and microfuged again. Plate matings were carried out as follows. The *E. coli* and rhizobia cells were resuspended in 1 and 0.25 ml respectively, of YEM medium. One hundred microlitre of each cell suspension was spotted together on YEM agar plates. The two drops were mixed and spread over the plate until the plates were dry. The plates were incubated overnight at 28°C. After the overnight incubation, 2 ml sterile distilled water was pipetted onto the surface of the mating plate and spread around using a glass spreader. One millilitre of the suspension was then transferred from the plate into 1.5-ml sterile tubes using a micropipette. Three-fold (10x, 100x and 1000x) dilutions of the suspension were made in sterile distilled water. One hundred microlitre of each dilution was subsequently spotted and spread on Brown and Dilworth (B and D) medium (Brown and Dilworth, 1975) containing sucrose. The plates were incubated at 28°C and examined regularly for growth of single colonies. Transconjugants were grown on B and D medium supplemented with spectinomycin (50 μg $ml^{-1}$) to select for the insertion of the transposon.

### 3.11.3. Detection of gusA - marked *Bradyrhizobium* derivatives

One litre YEM agar was prepared, autoclaved and allowed to cool. Fifty milligram of isopropyl-B-D- thiogalactopyranoside (IPTG) and 50 mg of 5-bromo-4-chloro-3-indolyl-B-D-glucuronide (x-gluc) were dissolved separately in 1 ml sterile distilled water and added to the YEM. One millilitre N, N-dimethyl formamide (DMF) was also added and plates poured. Single colonies of the gus A marked derivatives were then streaked on the poured plates and incubated at $28^0$C. Blue colonies appeared on the plates after two to three days of incubation, confirming that the

*Bradyrhizobium* isolates were gusA marked. The gus marked isolates were thereafter represented as G2, G10, and G14.

### 3.11.4. Competition experiment

Competition between the gus-marked (effective) isolates and the unmarked ineffective isolates was studied in jars containing sterile sand and N- free nutrient solution (Somasegaran and Hoben, 1994). Surface sterilised cowpea seeds were pre-germinated on 1% (w/v) water-agar plates before being transplanted into the Leonard jars. Each seedling was inoculated with 1ml suspension of the marked and unmarked isolates in ratios of 1:1, 1:2 and 2:1, to supply approximately $10^8$ cells per seedling. The numbers were verified by the Miles and Misra drop count method (Collins and Lyne, 1985), at the time of inoculation. Jars containing the plants were arranged in a completely randomized block design in the greenhouse. There were three replicates of each treatment. Controls included uninoculated plants and plants supplied regularly with 0 05% (w/v) $KNO_3$ i.e., 70 µg $ml^{-1}$ nitrogen. The plants were harvested at 28 DAP. Shoot dry weight and nodule numbers were determined.

### 3.11.5. Staining of nodules

The roots of the cowpea plants were washed thoroughly with water and immersed in 40 ml GUS extraction buffer containing 50 mM sodium phosphate buffer pH 7.0, 0.1% (w/v) Triton X-100, 0.1% (w/v) Sarkosyl, 0.05% (w/v) SDS, 1 mM EDTA amended with 40 µg IPTG $ml^{-1}$ and100 µg X-GlcA $ml^{-1}$ (Jefferson *et al.*, 1987). A vacuum was applied for 15 min from a water jet pump to facilitate penetration of the substrate into the nodules. Thereafter, the roots were incubated for 24 hr at 37°C in the substrate-containing buffer. The roots were then transferred to fresh buffer with IPTG and X-GlcA and vacuum was again applied for 15 min followed by

incubation at 37°C overnight. All nodules containing the marked isolates turned blue showing that the nodules were formed by the gusA marked effective isolates. The proportion of nodules occupied by the gusA marked isolates were counted for each inoculation ratio and competitive indices calculated by linear regression using the equation of Beattie *et al.* (1989). In this model, competition between two different strains is described by the relationship:

$$\mathbf{Log\ [(Px + Pboth) / (Py + Pboth)] = Cxy + K\ (Ix / Iy)}$$

Where: X and Y are the two competing strains; Px and Py are the proportions of nodules occupied by X and Y in the inoculum. Pboth is the proportion of nodules by both strains; and Ix and Iy represent the concentration of X and Y in the inoculum. The intercept of this equation, Cxy, is defined as the competitiveness index and the slope is K: a statistically significant positive value indicates that strain X is more competitive than strain Y, while a negative value shows that it is less competitive

### 3.11.6. Effect of placement and time of placement of inoculum on competitive ability of isolates

Competition for nodule occupancy among the isolates was evaluated further, by changing the relative times of exposure of the competing isolates on the seed using gusA marked isolates as described above to differentiate between the nodule forming isolates.

### 3.11.7. Speed of infection of host

Cultures of the competing isolates (designated 2, 10, 14, 29, 30, 72 and 94), were grown separately in YEM broth and used to individually inoculate pre-germinated surface sterilised seeds in plastic growth pouches containing nitrogen-free nutrient solution. There were six replicates for each isolate. The plants were maintained in the greenhouse and the seedlings were monitored for the first appearance of nodules every 12 hours.

## 3.12. Inoculation of cowpea with isolated indigenous cowpea bradyrhizobia isolates

### 3.12.1. Soils used

The experiment was conducted in four of the 20 soils, Ankasa, Akuse, Bekwai and Tafali. The soils contained variable numbers of indigenous cowpea bradyrhizobia isolates (Table 4.1) and also come from different ecological zones (Fig 3.1). Plastic pots (18cm high, 15cm wide at the top and 12cm at the base) were filled with 1.5 kg of each of the soils. All the soils were fertilized with essential macro and micro-nutrients except nitrogen as described by Owiredu and Danso (1988). Three of the effective isolates 2, 10 and 14, and a mixture of the three were used for inoculation. The isolates were cultured aseptically at room temperature (28∀ 3°C) on a wrist-action shaker for 5 - 7 days in culture bottles containing YEM broth until cell densities were about $10^8$ cells $ml^{-1}$ as determined by Misra and Miles Drop Count method (Collins and Lyne, 1985).

### 3.12.2. Inoculation procedures

#### 3.12.2.1 Seed inoculation

Seeds of cowpea cultivar (*Asontem*) were surface-sterilised and pre-germinated on 1% (w/v) water agar. Four of the germinated seeds were planted in each pot. One millilitre aliquot of each bradyrhizobial culture ($10^8$ cell $ml^{-1}$) was then inoculated onto and around the seed before covering the seed with soil. In the case of the isolate mixture, 0.33 ml of each of the isolates was combined to achieve this 1ml aliquot.

#### 3.12.2.2 Soil inoculation

One millilitre suspension of each of the isolates was diluted to 50 ml in sterile distilled water and mixed thoroughly with the soil. Each pot was then planted with four of the pre-germinated seeds.

#### 3.12.2.3. Plant growth

The plants in the two different inoculation methods were thinned to two plants 4 days after germination. The treatments were each replicated 4 times and the pots were arranged in a randomised complete block design on raised benches in the open air. The plants were watered daily and harvested at 35 DAP. The roots were carefully washed and the number of nodules (plant$^{-1}$) counted. The shoots were oven-dried at 60-65 °C for 48 hours and weighed. Kjeldahl N analysis was done on ground samples (0.2 mm) of the shoot.

# CHAPTER FOUR

## 4.0 Results

## 4.1 Examination of components of nitrogen fixation in cowpea

### 4.1.1 Nodulation potential of cowpea in Ghanaian soils

Nodulation of 45 cowpea cultivars grown by farmers in various parts of the country were evaluated in 20 different soils to assess their nodulation potential with native bradyrhizobia. The cowpea cultivars tested exhibited variable nodulation potentials in the various soils; in general nodulation in soils of the various ecozones was more than 50% (Fig. 4.1). None of the soils could support nodulation of all the cultivars tested. Generally, nodulation of the cowpea cultivars was relatively low in soils from the high rainforest zone, for the majority of which no nodules were formed. The highest nodulation was observed in soils of the Forest savanna transition zone. Cowpea cultivar, Asontem, recorded the highest nodulation frequency, nodulating in 18 out of the 20 soils.

### 4.1.2 Estimation of indigenous bradyrhizobia numbers in the soils

Soil had a pronounce effect on the abundance of cowpea bradyrhizobia. While no cowpea bradyrhizobia were detected in soils (Ankasa and Tikobo which were sampled from the high rain forest zone with a pH of less than 4.4), at least 60% of soils contained more than $10^3$ bradyrhizobia cells (g $soil^{-1}$ ) with the highest, $3.1x10^4$ being encountered in the Akuse soil (Table 4.1). There was also a clear relationship between the abundance of cowpea bradyrhizobia and the ecological zone under which the soil developed. The lowest number of cowpea bradyrhizobia, between zero and less than 10 cells (g $soil^{-1}$ ) in all four cases examined,

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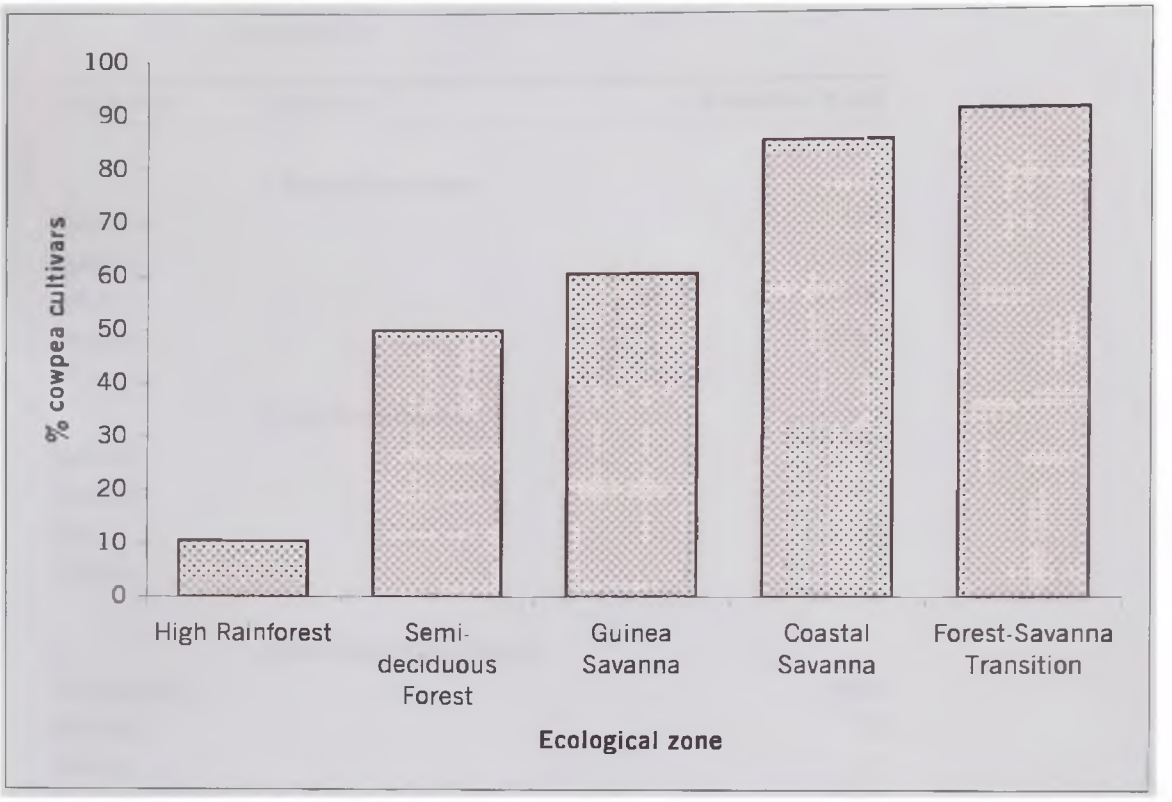


Fig. 4.1 **Proportion of cowpea plants which formed nodules in soils from the different ecological zones.**

Table. 4.1 Most probable number estimates of cowpea bradyrhizobia isolates in the various soils

| Soil Series | Ecozone | Rhizobia/g soil |
|---|---|---|
| | **Coastal Savanna** | |
| Adenta | | 100 |
| Agawtaw | | 17000 |
| Akuse | | 31000 |
| Aveime | | 10000 |
| | **High Rainforest** | |
| Abenia | | 0 |
| Ankasa | | 6 |
| Boi | | 6 |
| Tikobo | | 0 |
| | **Semi-deciduous forest** | |
| Akumadan | | 1000 |
| Bekwai | | 58 |
| Nzema | | 17 |
| Wacri | | 1000 |
| | **Forest-Savanna Transition** | |
| Amantin | | 3100 |
| Bediesi | | 17000 |
| Denteso | | 10000 |
| Ejura | | 10000 |
| | **Guinea Savanna** | |
| Lima | | 5800 |
| Nyankpala | | 1000 |
| Siare | | 3100 |
| Tafale | | 10 |

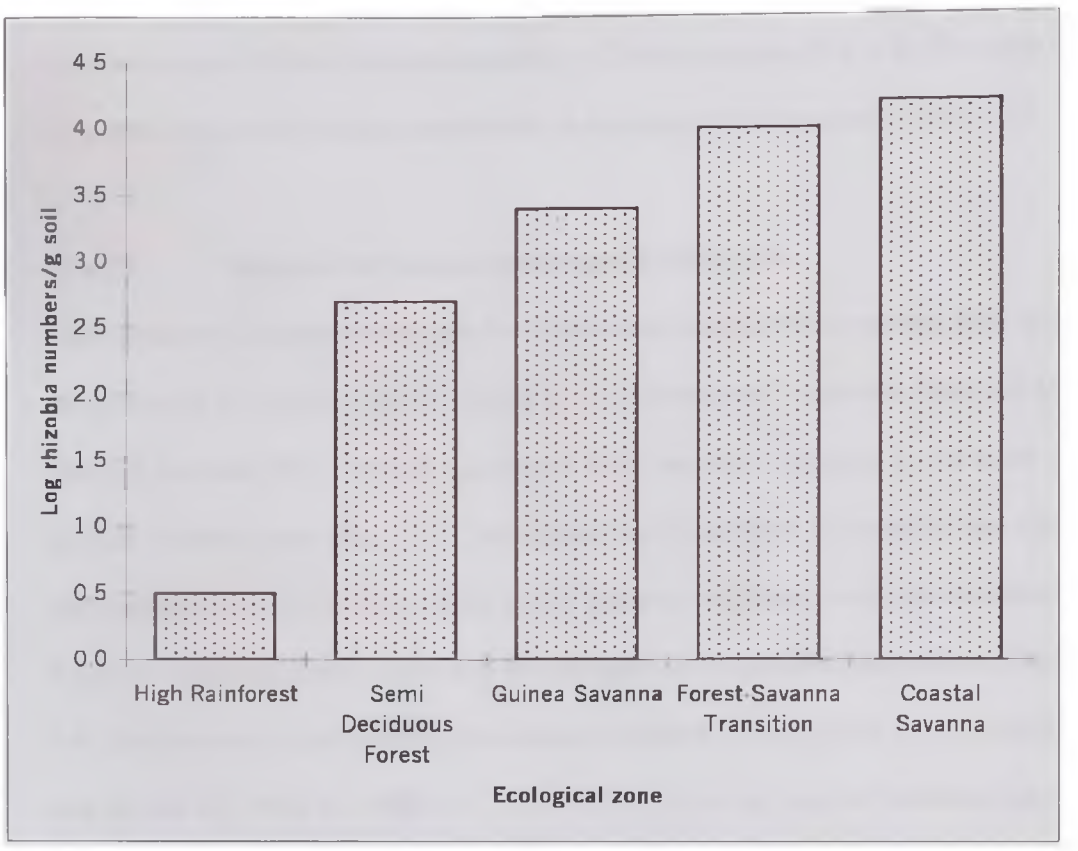


Fig. 4.2 **The populations of cowpea bradyrhizobia in soils from different ecological zones.**

occurred in the High rainforest ecozone. The trend was High rainforest < Semi decidous forest < Guinea savanna < Forest savanna transition < Coastal savanna (Fig. 4.2). The soils from which the counts were made varied considerably in their properties measured (Table 3.2).

### 4.1.3 Response of cowpea to nitrogen fertilization

The response of cowpea to nitrogen fertilization was tested at rates ranging from zero to 240 Kg N/ha. Except for Adenta soil for which no significant yield increases occurred at any of the nitrogen fertilizer rates, variable significant yield increases occurred in the other soils for the cowpea varieties used (Fig. 4.3). The highest yield response of Asontem and Sanji cultivars were recorded at 120 Kg N/ha, whilst that of Soronko and Benga cultivars were recorded at 160 Kg N/ha, when the mean response of the cowpea cultivars were evaluated on all the soils (Fig. 4.4). For Asontem, significant yield increases occurred at 80 Kg N/ha in Akuse and Wacri soils and at 120 Kg N/ha in Tafali soil (Figs 4.5; 4.6). In the case of Soronko significant yield increases occurred at 120 Kg N/ha in Akuse and Wacri soils and at 160 Kg N/ha in Tafali soil. Benga also recorded significant yield increases at 160 Kg N/ha in Akuse soil and at 80 Kg N/ha in the other soils (Figs 4.5; 4.6; 4.7). Sanji on the other hand showed significant yield increase at 120 Kg N/ha in Akuse and Tafali soils and at 40 Kg N/ha in Wacri soil (Figs 4.5; 4.6; 4.7). In all the soils, nodulation of the cowpea cultivars was generally enhanced from between 40 to 80 Kg N/ha except for Adenta soil (Fig. 4.8). Beyond 80 Kg N/ha nodulation was on the average inhibited but not completely (Fig. 4.3).

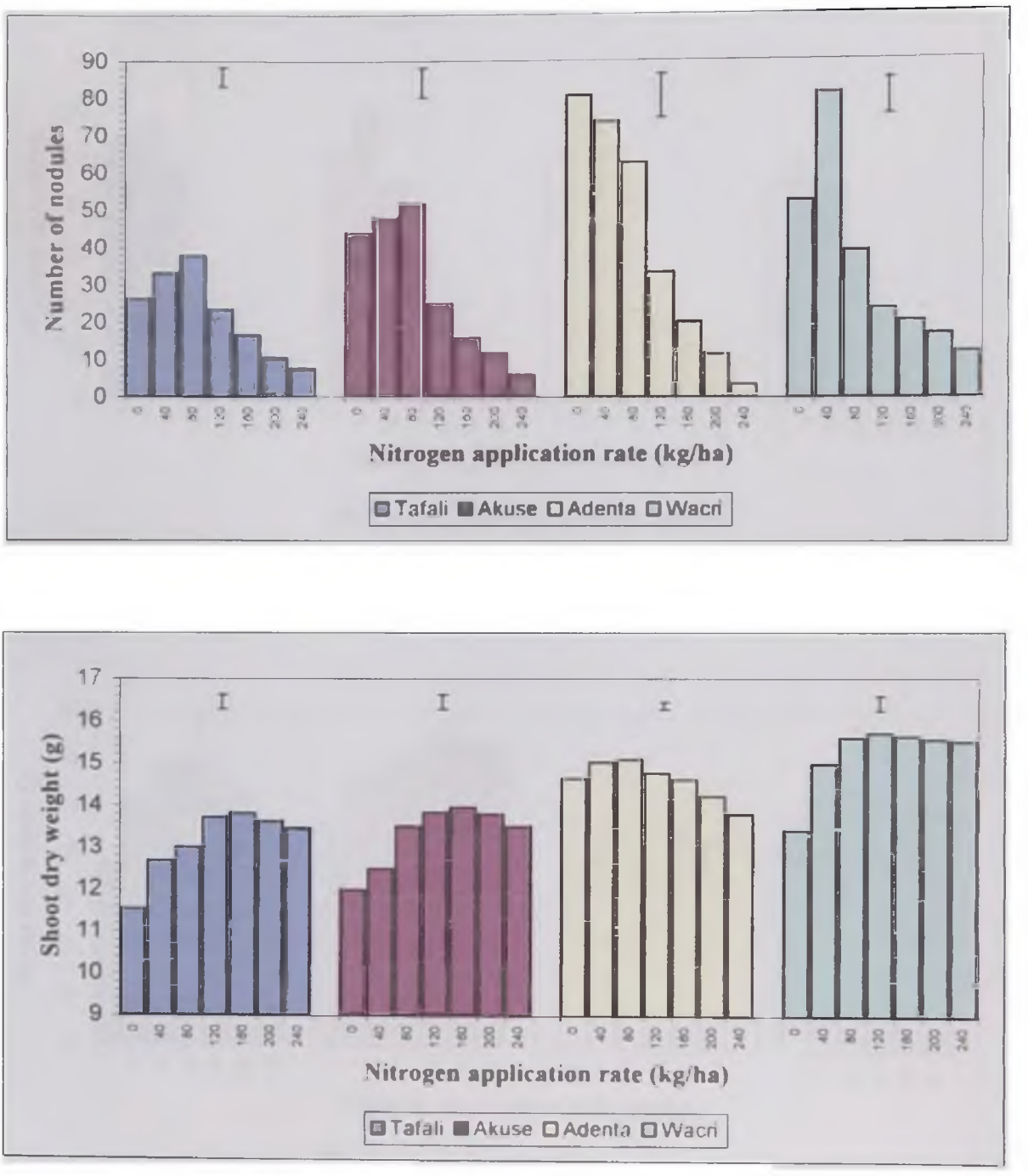


Fig. 4.3 Mean responses of cowpea varieties to nitrogen application on various soil types.
*Error bars represent standard errors of the means*

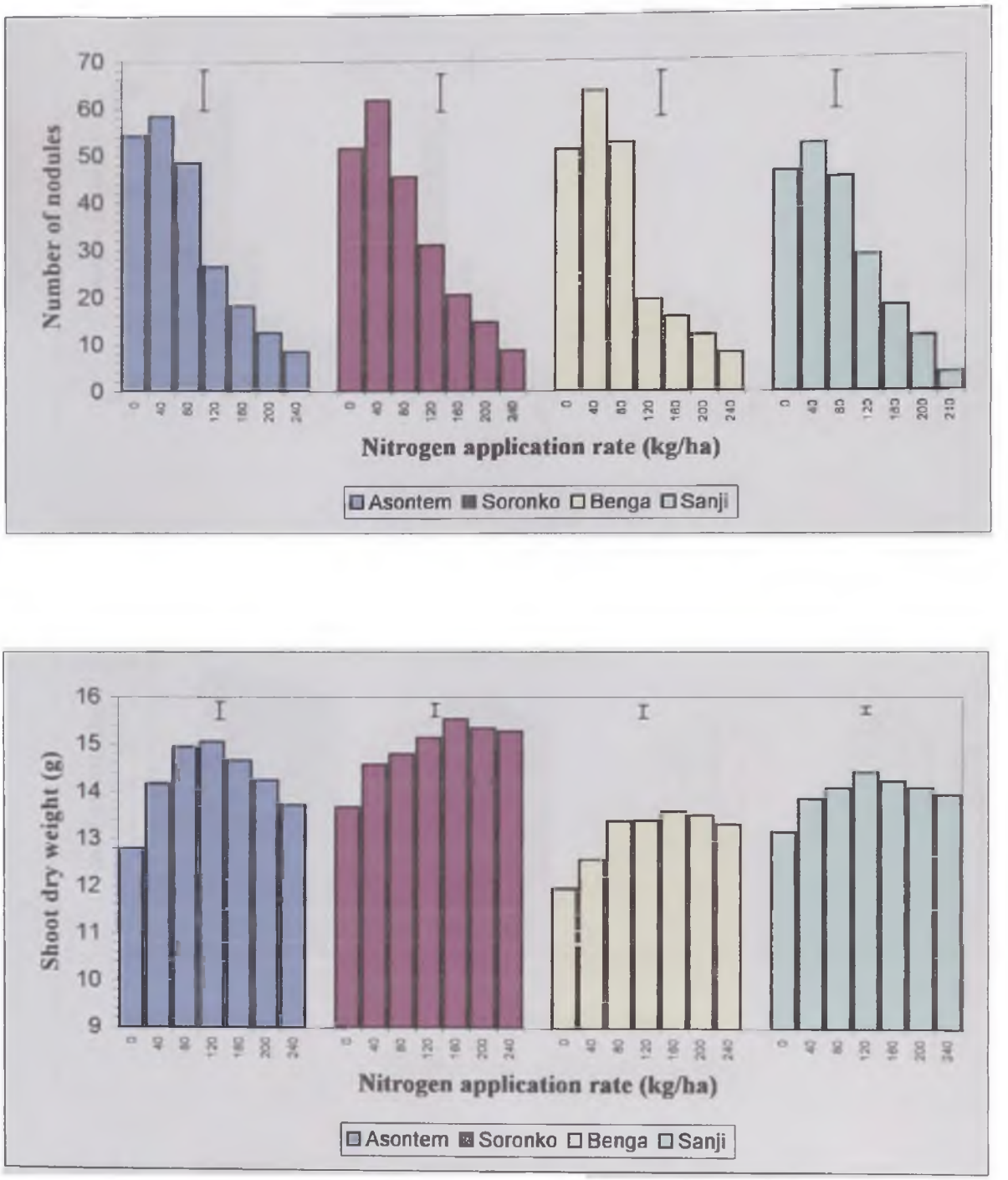


Fig. 4.4 Mean performance of cowpea varieties to nitrogen application.
*Error bars represent standard errors of the means*

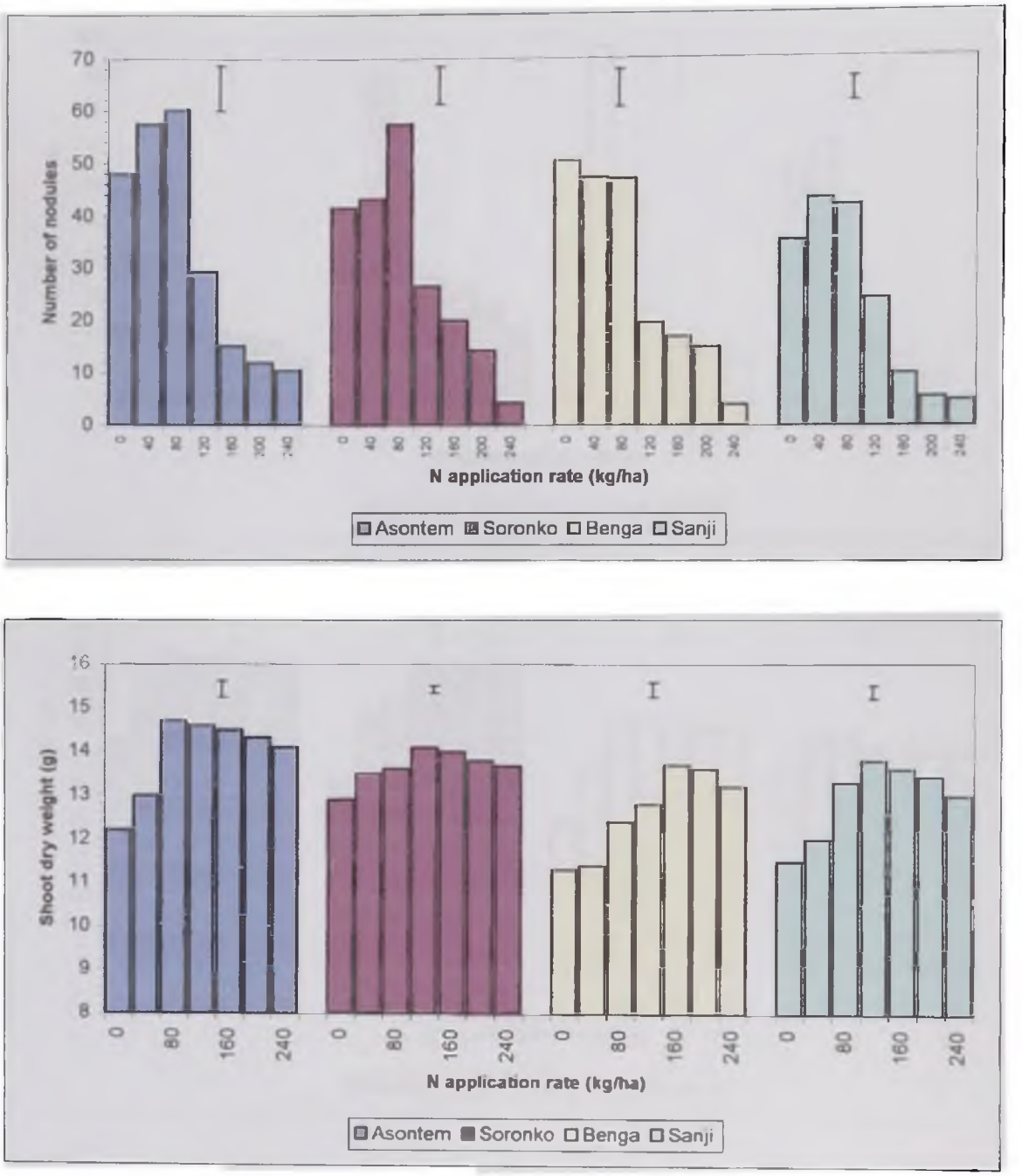


Fig. 4.5 Response of cowpea varieties to nitrogen fertilization on Akuse soil series. *Error bars represent standard errors of the means*

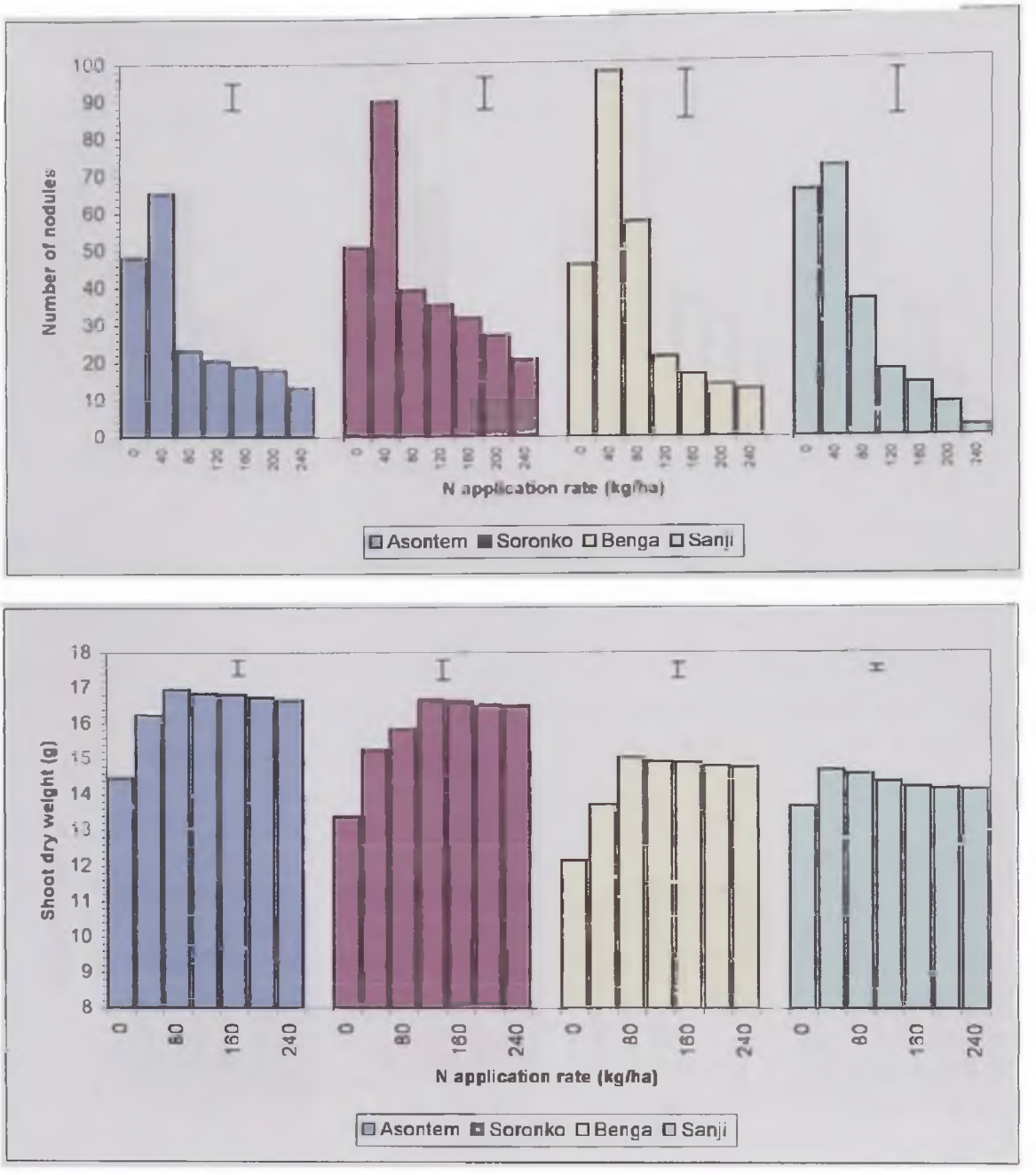


Fig. 4.6 **Response of cowpea varieties to nitrogen fertilization on Wacni soil series.** ***Error bars represent standard errors of the means***

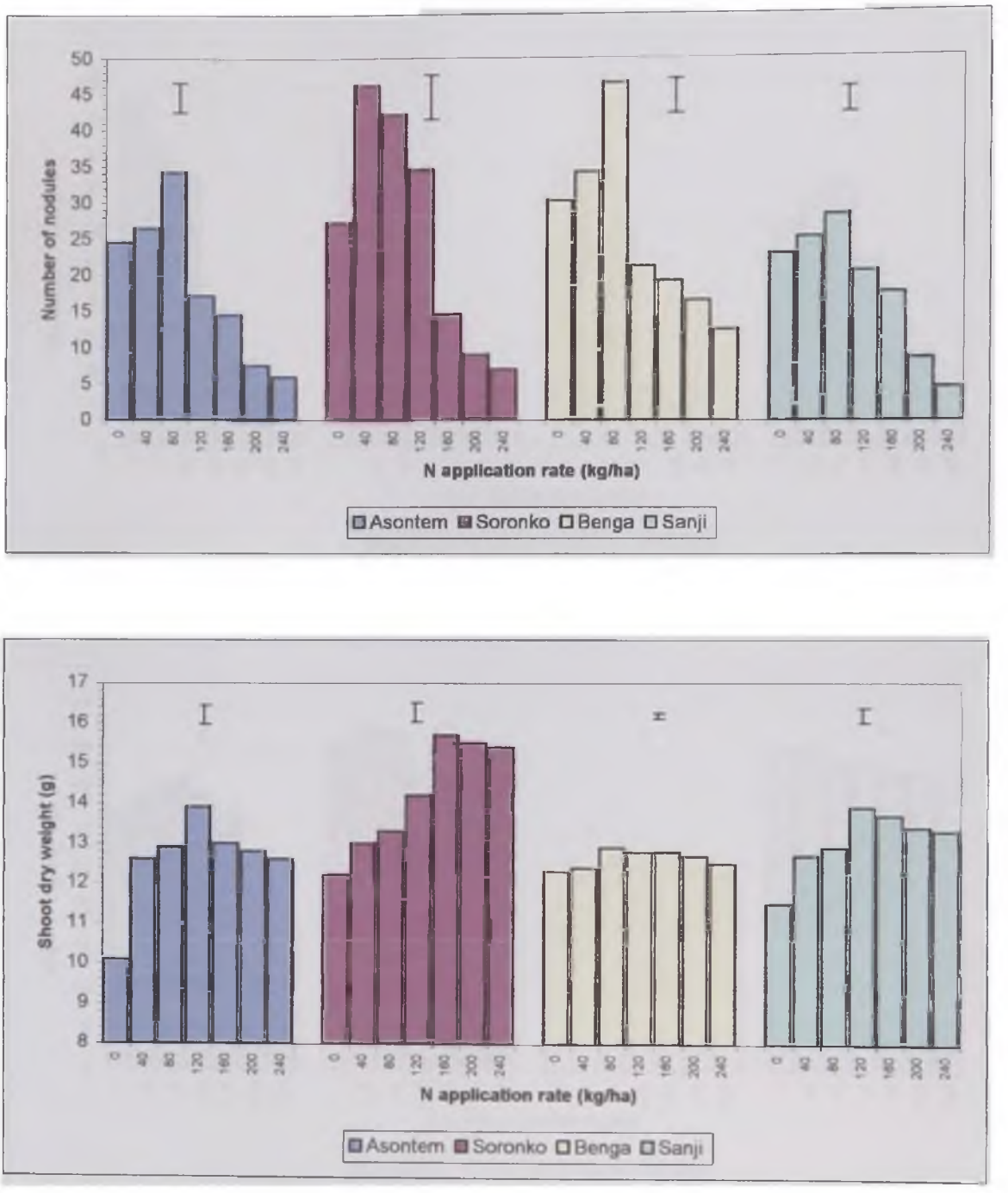


Fig. 4.7 **Response of cowpea varieties to nitrogen fertilization on Tafali soil series**. *Error bars represent standard errors of the means*

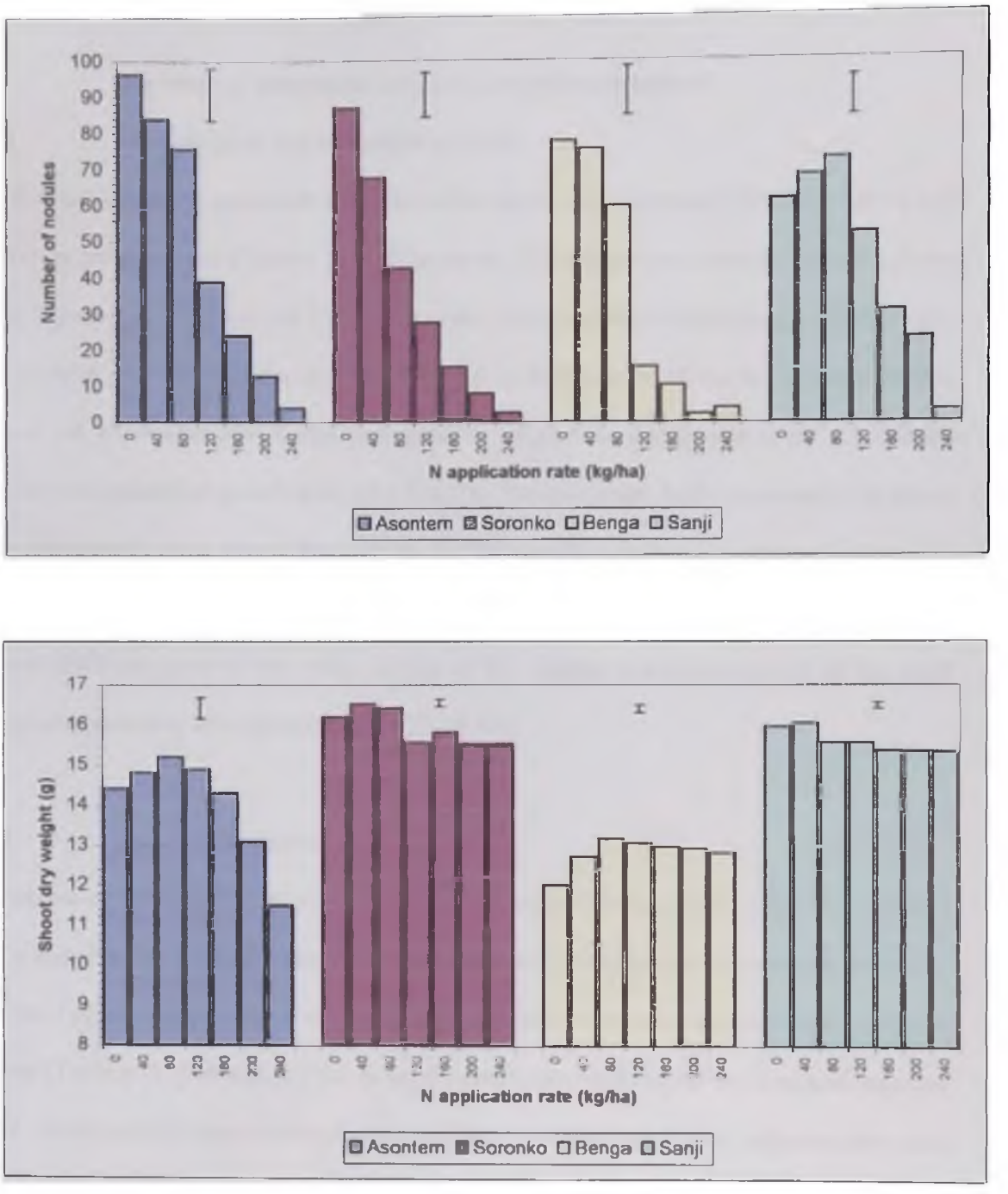


Fig. 4.8 **Response of cowpea varieties to nitrogen fertilization on Adenta soil series.** ***Error bars represent standard errors of the means***

## 4.2 Diversity of indigenous cowpea bradyrhizobia isolates

### 4.2.1 Physiological and metabolic analysis

The 100 indigenous cowpea bradyrhizobia isolates examined comprised 18% fast growing and 82% slow growing types (Table 4.2). Differences in pH tolerance are shown in Table 4.3. None of the isolates could grow at pH 3.0. Twenty nine percent of the isolates could grow at pH 3.5, out of which only 5% were fast growing (Table 4.2). Fifty percent of the fast growing isolates grew at pH 4.5, whilst 53% of the slow growing isolates showed growth at pH 4.5. All the isolates were capable of growth from pH 5.5 to 7.0. The maximum NaCl concentration at which the isolates could grow ranged from 1% to 5% (Table 4.2). All the fast growing isolates and 72% of the slow growing ones tolerated NaCl concentration of 3%. Only 33% of the total isolates could not grow in 5% NaCl. Almost all the isolates tested metabolised all the eight carbon compounds as sole carbon sources (Table 4.2).

### 4.2.2 Host range analysis

The degree of compatibility of indigenous cowpea bradyrhizobia isolates when in symbiosis with some of the host legumes commonly found growing in Ghana was examined to determine their level of promiscuity. There were differences in the type of host nodulated by the different isolates (Table 4.3), with none of the isolates capable of nodulating all the nine host legumes tested. All the isolates when re-inoculated, nodulated the homologous host, indicating they were not contaminants (Fig. 4.9). Fourteen percent of the isolates were specific on cowpea and did not nodulate any other host legume species (Table 4.3). The broadest host range of seven host species was shown by six of the isolates (14, 43, 49, 51, 29 and 87). *Mimosa spp* was the

Table 4.2 Physiological and metabolic characteristics of cowpea bradyrhizobia isolates

| Isolates | Growth | | pH | | | | | | NaCl (%) | | | | Carbon Sources | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fast | Slow | 3.5 | 4.5 | 5.5 | 8.5 | 9.5 | 10.5 | 1 | 2 | 3 | 5 | Ara | Fru | Gal | Glu | Lac | Mal | Man | Suc |
| 1 | | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 2 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 3 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 4 | - | + | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 5 | - | + | - | - | + | + | + | + | + | - | - | - | + | + | + | + | - | + | + | + |
| 6 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 7 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 8 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 9 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 10 | | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 11 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 12 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 13 | - | + | | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 14 | - | + | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 15 | - | + | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 16 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 17 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 18 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 19 | + | - | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 20 | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 21 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 22 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 23 | + | - | - | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 24 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 25 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 26 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 27 | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 28 | - | + | - | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 29 | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 30 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 31 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 32 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 33 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 34 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 35 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 36 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 37 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 38 | + | - | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 39 | - | + | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 40 | - | + | - | + | + | + | + | + | + | - | - | - | - | + | - | + | + | + | + | + |
| 41 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 42 | - | + | - | - | + | + | + | + | + | - | - | - | - | - | + | + | - | - | + | + |
| 43 | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 44 | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 45 | - | + | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 46 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 47 | - | + | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 48 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 49 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 50 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

*Table 4.2 continued*

| Isolates | Growth[1] | | pH | | | | | | NaCl (%) | | | | Carbon Sources[2] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fast | Slow | 3.5 | 4.5 | 5.5 | 8.5 | 9.5 | 10.5 | 1 | 2 | 3 | 5 | Ara | Fru | Gal | Glu | Lac | Mal | Man | Suc |
| 51 | + | - | - | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 52 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 53 | - | + | - | - | + | + | + | + | + | - | - | - | + | + | + | + | + | + | + | + |
| 54 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 55 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 56 | - | + | - | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 57 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 58 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 59 | + | - | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 60 | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 61 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 62 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 63 | - | + | | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 64 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 65 | - | + | | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 66 | - | + | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 67 | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 68 | - | + | | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 69 | + | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 70 | | + | + | + | + | + | + | + | + | + | + | | + | + | + | + | + | + | + | + |
| 71 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 72 | - | + | - | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 73 | - | + | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 74 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 75 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 76 | - | + | - | | + | + | + | + | + | - | - | - | + | + | + | + | + | + | + | + |
| 77 | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 78 | - | + | | - | + | + | + | + | + | - | - | | - | - | | + | + | | + | + |
| 79 | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 80 | - | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 81 | - | + | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 82 | - | + | - | - | + | + | + | + | + | + | + | + | | + | - | + | | + | + | + |
| 83 | - | + | - | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | + |
| 84 | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 85 | - | + | - | | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | + |
| 86 | | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 87 | - | + | - | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 88 | + | - | - | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 89 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 90 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 91 | + | - | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 92 | + | | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 93 | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 94 | - | + | - | - | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 95 | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 96 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 97 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 98 | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 99 | - | + | - | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |
| 100 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + |

1 "+" indicates growth as on control plate; "-" indicates no growth.

2 Ara = Arabinose; Fru = Fructose; Gal = Galactose; Lac = Lactose; Mal = Maltose; Man = Mannito ; Suc =Sucrose

Table 4.3 Diversity of cowpea bradyrhizobia isolates by cross inoculation

| GROUP[1] | SUBGROUP[2] | ISOLATES | LEGUMES[3] |
|---|---|---|---|
| 1 | | 24, 50, 56, 60, 62, 67, 76, 80, 93, 95, 96, 98, 99, 100 | CP |
| 2 | 2a | 57, 72, 79, 97 | CP, BG |
| | 2b | 81 | CP, SB |
| | 2c | 23 | CP, CLG |
| | 2d | 6, 22, 25, 28, 68, 69, 73, 78, 83 | CP, GN |
| | 2e | 92 | CP, PRR |
| 3 | 3a | 1, 3, 5, 16, 64, 65, 70, 71, 77, 87, 94 | CP, SB, GN |
| | 3b | 10 | CP, GN, CLG |
| | 3c | 11, 90 | CP, GN, PRR |
| | 3d | 26, 55, 59, 63 | CP, GN, BG |
| | 3e | 58 | CP, BG, PRR |
| 4 | 4a | 54 | CP, SB, GN, CTL |
| | 4b | 32, 39, 53, 75 | CP, SB, GN, CLG |
| | 4c | 15, 17, 21, 27, 37, 48, 66, 74 | CP, SB, GN, BG |
| | 4d | 86 | CP, BG, PRR, CLG |
| | 4e | 88 | CP, GN, CLG, CTL |
| | 4f | 91 | CP, GN, BG, PRR |
| 5 | 5a | 4, 30, 34, 35, 36, 38, 44, 46, 84 | CP, SB, GN, BG, PRR |
| | 5b | 85 | CP, SB, GN, CLG, CTL |
| | 5c | 7 | CP, SB, GN, CLG, PRR |
| | 5d | 18, 19 | CP, SB, GN, BG, CLG |
| | 5e | 33 | CP, SB, GN, LC, CLG |
| 6 | 6a | 9, 12, 20, 31, 40, 41, 42, 47, 52, 82 | CP, SB, GN, BG, PRR, CLG |
| | 6b | 89 | CP, SB, GN, BG, PRR, CTL |
| | 6c | 2, 13, 45 | CP, SB, GN, BG, CLG, CTL |
| | 6d | 8 | CP, SB, GN, BG, CLG, MM |
| 7 | 7a | 14, 43, 49, 51 | CP, SB, GN, BG, PRR, CLG, CTL |
| | 7b | 29 | CP, SB, GN, BG, LC, PRR, CLG |
| | 7c | 61 | CP, GN, BG, LC, PRR, CLG, CTL |

1 Group number indicates number of legumes nodulated
2 Subgroup indicates different legume combinations
3 Abbreviations for the legumes

LEGEND

| | |
|---|---|
| BG | Bambara Groundnut |
| CLG | Calapogonium |
| CP | Cowpea |
| CTL | Crotalaria |
| GN | Groundnut |
| LC | Leucaena |
| MM | Mimosa |
| PRR | Pueraria |
| SB | Soyabean |

least nodulated host by the isolates, being nodulated by only one of the isolates (Isolate 8), (Fig. 4.9) and (Table 4.3). Nodulation of the legumes in terms of number of isolates capable of inducing nodule formation on a legume species was in the order cowpea > groundnut > soybean > bambara groundnut > calopogonium > pueraria > leucaena > mimosa. Based on the number of legume species nodulated by an isolate, seven major groupings with 28 sub groups were obtained (Table 4.3). Group one contained the greatest number of isolates (14%). These were all the isolates that were specific on cowpea only. The four food legumes, groundnut, soybean and bambara groundnut, were nodulated by over 50% of the isolates whilst the non-food legumes, *mimosa*, *leucaena*, *crotalaria*, *pueraria* and *calopogonium* were nodulated by less than 35% of the isolates (Fig. 4.9).

### 4.2.3. Serology

#### 4.2.3.1 Antibody responses in immunized mice

Response of the BALB/c mice to rhizobial antigen immunization is illustrated in Fig. 4.10. High serum antibody responses, with titres far beyond 1:6000 were obtained against homologous antigens by indirect ELISA. Mean antibody titre against both antigens was quite high, maintaining optical density greater or equal to 0.1 for serum dilutions of up to 1:3000.

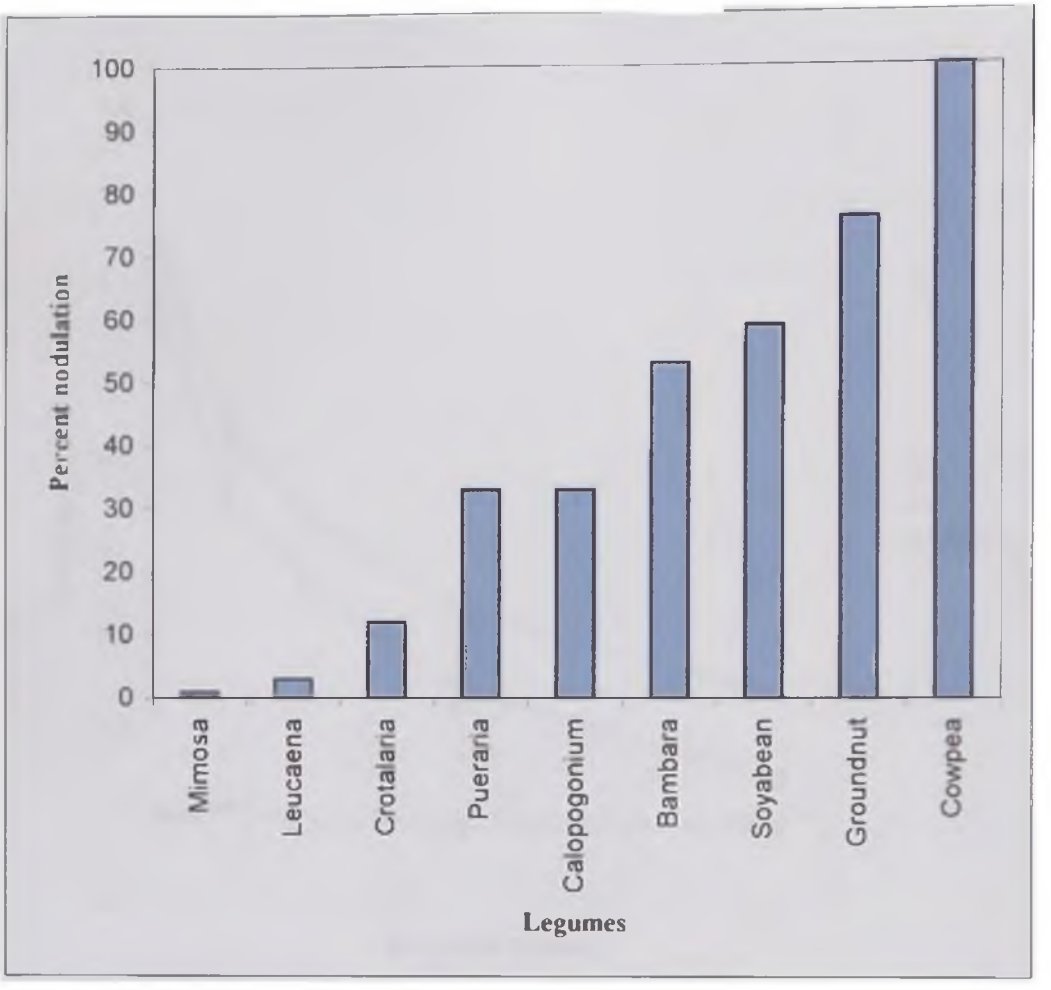


Fig. 4.9 Proportion of cowpea bradyrhizobia isolates that nodulated several legume species.

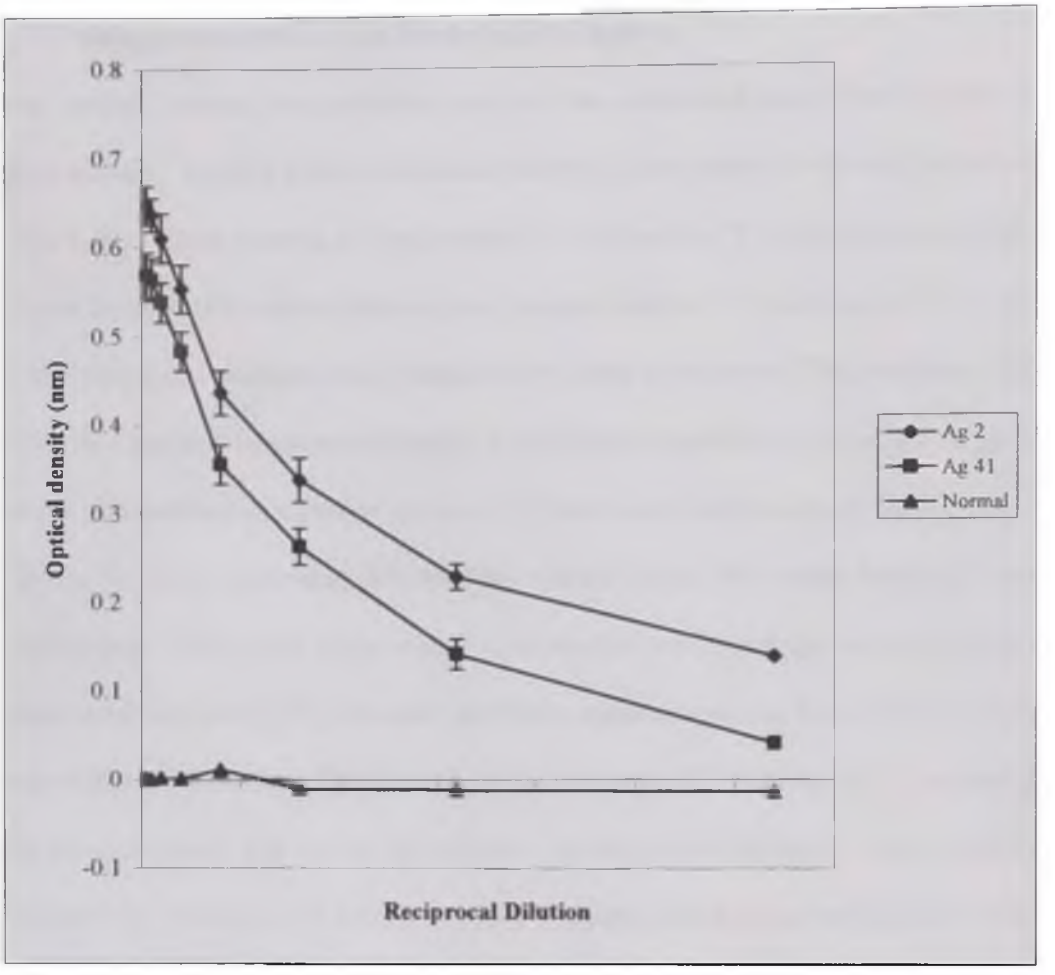


Fig. 4.10 Serum antibody response of BALB/c mice following immunisation with cowpea rhizobial antigen extracts

#### 4.2.3.2 Serogrouping of cowpea bradyrhizobia isolates

Relatedness among cowpea bradyrhizobia isolates was examined using homologous and heterologous antisera. Table 4.4 shows the cross reactivity mean values for the antigen antisera reaction. Each absorbance value (e.g. From isolate "1" x antiserum "2") was normalised, that is expressed as a fraction of the mean homologous reaction (isolate "1" x antiserum "1") value. Based on the results, the reactions were designated positive or negative. The minimum value considered to be a positive reaction was fixed at a normalised absorbance (NA) value of greater or equal to 0.5. Normalised absorbance values of 1.0 were considered as closely related. Those between 0.9 to 0.5 were considered differentially related, whilst NA values below 0.5 were considered unrelated. The results of the overall cross reactivity of the antigen antisera reactions of the isolates enabled them to be grouped into three major categories, those closely related (8.5%) those differentially related (52.5%) and those not related (38.5%) (Fig. 4.11). Isolates 29 and 77 did not cross react with any of the antisera. Normalised absorbance values were less than 0.5 (Table 4.4). Isolates 1, 10, 16, 50, 73 and 75 showed broad spectrum reaction. Isolate 41 on the other hand, was specific and reacted with only one serum (Table 4.4).

### 4.2.4. Molecular analysis

Gel electrophoresis of the PCR products of the 100 isolates revealed that the amplification reaction produced a common single DNA molecule about 1.5 kilobase long for all the isolates which corresponded to the expected size of the 16S rRNA genes among bacteria. Between 2 to 11 distinct restriction patterns were detected with each of the four endonucleases, *DdeI, HaeIII MspI and RsaI* (Table 4.5 and Plate 4.1). The combination of the four patterns identified 20 composite genotypes that were arbitrarily named A-K (Table 4.6). The 18 fast growing isolates produced what could be considered as nine distinct genotypes, each of which consisted of between

Table 4.4 Normalised absorbance values for antigen antibody reactions of cowpea bradyrhizobia isolates

| Isolate | 1 | 2 | 10 | 16 | 20 | 29 | 41 | 49 | 50 | 65 | 73 | 75 | 77 | 80 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.00 | 0.40 | 0.58 | 0.98 | 0.06 | 0.08 | 0.11 | 0.96 | 0.85 | 1.02 | 1.01 | 1.03 | 0.15 | 0.75 |
| 2 | 0.99 | 1.00 | 1.07 | 1.08 | 0.14 | 0.09 | 0.21 | 1.04 | 0.96 | 1.04 | 1.09 | 1.04 | 0.19 | 0.51 |
| 10 | 0.96 | 0.35 | 1.00 | 1.00 | 0.17 | 0.10 | 0.13 | 0.87 | 0.69 | 1.00 | 0.97 | 1.01 | 0.13 | 0.52 |
| 16 | 0.97 | 0.48 | 1.01 | 1.00 | 0.30 | 0.13 | 0.11 | 0.89 | 0.87 | 1.05 | 1.03 | 1.02 | 0.17 | 0.53 |
| 20 | 0.47 | 0.28 | 1.00 | 0.93 | 1.00 | 0.47 | 0.14 | 1.25 | 0.69 | 0.47 | 0.62 | 1.32 | 0.32 | 0.47 |
| 29 | 0.29 | 0.14 | 0.50 | 0.71 | 0.07 | 1.00 | 0.06 | 0.37 | 0.26 | 0.35 | 0.43 | 0.88 | 0.10 | 0.21 |
| 41 | 1.00 | 0.49 | 1.01 | 1.03 | 0.07 | 0.18 | 1.00 | 1.07 | 1.41 | 1.02 | 1.05 | 1.04 | 0.27 | 1.02 |
| 49 | 0.85 | 0.89 | 0.99 | 0.91 | 0.56 | 0.11 | 0.14 | 1.00 | 0.75 | 0.89 | 0.95 | 1.05 | 0.14 | 0.54 |
| 50 | 0.91 | 0.42 | 0.96 | 0.92 | 0.12 | 0.08 | 0.30 | 0.79 | 1.00 | 0.93 | 0.97 | 1.02 | 0.17 | 0.95 |
| 65 | 0.85 | 0.24 | 1.04 | 0.96 | 0.07 | 0.01 | 0.07 | 0.70 | 0.76 | 1.00 | 0.93 | 1.05 | 0.08 | 0.47 |
| 73 | 0.76 | 0.19 | 0.19 | 0.88 | 0.08 | 0.16 | 0.12 | 0.60 | 0.81 | 0.87 | 1.00 | 1.02 | 0.10 | 0.45 |
| 75 | 0.76 | 0.24 | 0.96 | 0.90 | 0.24 | 0.17 | 0.20 | 0.64 | 0.99 | 0.90 | 0.91 | 1.00 | 0.11 | 0.51 |
| 77 | 0.15 | 0.88 | 0.13 | 0.17 | 0.92 | 0.34 | 0.51 | 0.14 | 0.17 | 0.18 | 0.12 | 0.10 | 1.00 | 0.18 |
| 80 | 0.80 | 0.50 | 1.01 | 1.02 | 0.64 | 0.16 | 0.19 | 0.30 | 1.01 | 1.08 | 1.28 | 1.02 | 0.17 | 1.00 |

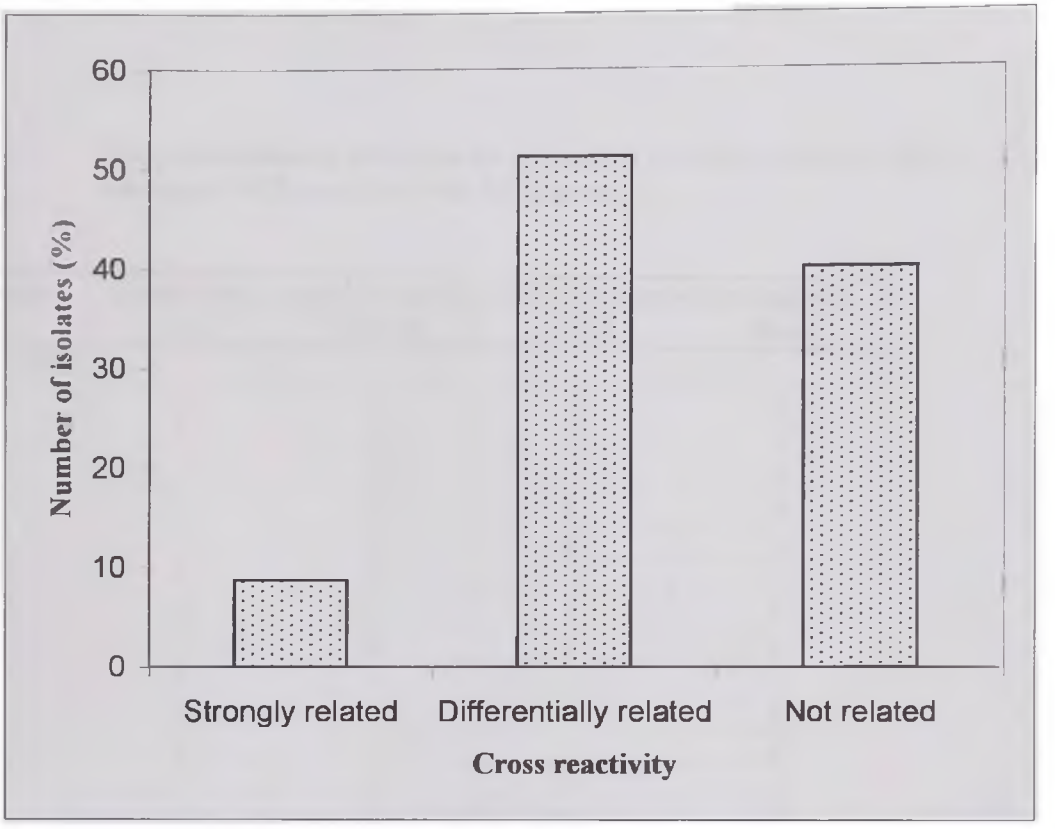


Fig. 4.11 Relationship between cowpea rhizobia isolates as determined by reactivity of homologous and heterologous antisera

Table 4.5 Restriction patterns of cowpea bradyrhizobia isolates revealed by RFLP analysis of PCR-amplified 16s rRNA genes

| Isolates | Restriction pattern[1] of amplified 16s rRNA genes digested with | | | |
|---|---|---|---|---|
| | DdeI | Hae III | Msp I | Rsa I |
| Fast growers | | | | |
| 19 | a | a | a | a |
| 20 | b | b | b | a |
| 23 | c | c | c | b |
| 27 | b | b | b | a |
| 29 | b | b | b | a |
| 38 | d | d | d | c |
| 43 | e | c | e | b |
| 44 | f | e | f | b |
| 51 | b | b | b | a |
| 57 | c | c | c | b |
| 59 | g | f | g | d |
| 60 | b | b | b | a |
| 69 | g | f | g | d |
| 79 | b | b | b | a |
| 88 | a | a | a | a |
| 92 | h | g | h | ? |
| 98 | i | ? | i | e |

1 The different patterns detected with each enzyme among the 100 isolates analysed are designated by the lower case letters.

Table 4.5 *continued*

| Isolates | Restriction pattern of amplified 16s rRNA genes digested with | | | |
|---|---|---|---|---|
| | Dde*I* | Hae *III* | Msp *I* | Rsa *I* |
| **Slow growers** | | | | |
| 1 | a | b | a | a |
| 2 | b | a | b | b |
| 3 | a | a | c | b |
| 4 | b | c | d | a |
| 5 | a | a | c | b |
| 6 | a | d | a | c |
| 7 | a | f | c | d |
| 8 | a | a | e | b |
| 9 | a | f | c | d |
| 10 | a | b | a | a |
| 11 | a | e | f | a |
| 12 | a | b | a | a |
| 13 | c | a | e | b |
| 14 | d | b | d | a |
| 15 | a | a | c | b |
| 16 | a | a | c | b |
| 17 | a | a | a | b |
| 18 | a | a | a | b |
| 21 | e | e | a | b |
| 22 | a | b | a | b |
| 24 | a | b | a | a |
| 25 | f | f | j | b |
| 26 | g | g | c | b |
| 28 | h | k | k | b |
| 30 | a | b | a | b |
| 31 | a | a | a | b |
| 32 | i | l | l | d |
| 33 | a | a | a | b |
| 34 | d | b | d | b |
| 35 | a | b | a | b |
| 36 | a | b | a | a |
| 37 | a | b | a | a |
| 39 | j | j | j | i |
| 40 | k | i | g | f |
| 41 | k | j | g | f |
| 42 | a | a | a | b |
| 45 | a | a | a | b |
| 46 | g | k | g | c |
| 47 | a | a | a | b |

**Table 4.5** *continued*

| Isolates | Restriction pattern of amplified 16s rRNA genes digested with | | | |
|---|---|---|---|---|
| | **Dde*I*** | **Hae *III*** | **Msp *I*** | **Rsa *I*** |
| 48 | a | a | a | i |
| 49 | a | a | a | b |
| 50 | a | a | h | i |
| 52 | g | k | g | b |
| 53 | a | a | b | a |
| 54 | a | b | a | b |
| 55 | g | k | g | b |
| 56 | g | k | g | c |
| 58 | i | l | i | j |
| 61 | a | a | a | b |
| 62 | a | b | a | b |
| 63 | a | a | a | b |
| 64 | a | a | a | b |
| 65 | a | a | a | b |
| 66 | a | a | a | b |
| 67 | a | a | a | b |
| 68 | d | b | d | d |
| 70 | f | f | j | f |
| 71 | f | f | j | f |
| 72 | a | a | a | b |
| 73 | e | e | a | b |
| 74 | a | a | a | b |
| 75 | a | a | a | a |
| 76 | j | j | i | j |
| 77 | c | d | a | a |
| 78 | c | c | a | a |
| 80 | h | k | k | f |
| 81 | h | k | k | e |
| 82 | c | a | a | a |
| 83 | i | l | l | c |
| 84 | h | k | k | b |
| 85 | a | a | a | b |
| 86 | a | a | l | a |
| 87 | c | d | a | a |
| 89 | h | k | k | c |
| 90 | i | l | l | d |
| 91 | j | j | j | j |
| 93 | j | j | l | j |
| 94 | a | b | a | a |
| 95 | a | a | a | b |
| 96 | e | e | a | b |
| 97 | f | f | j | e |
| 99 | c | a | a | a |
| 100 | f | f | f | e |

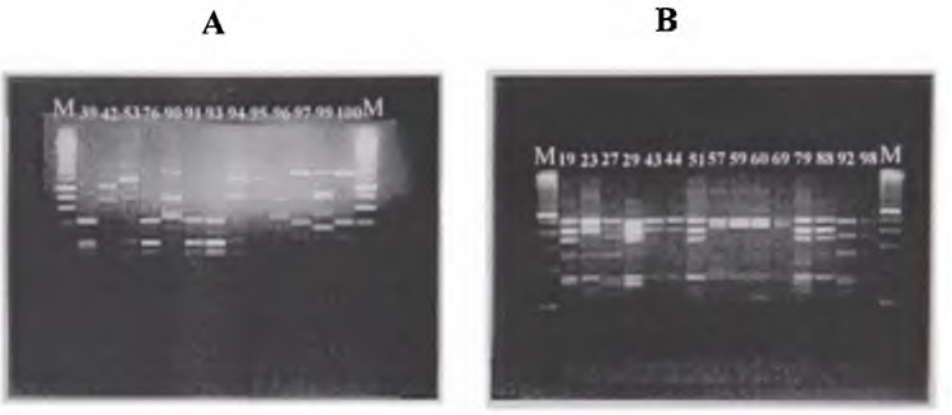


Plate 4.1 Restriction patterns of PCR-amplified fragment of 16S rRNA genes digested with Hae*III* (A) or Msp*I* (B). The lane assignments (numbers) represent *Bradyrhizobium* strains. Lane M = molecular marker.

Table 4.6 Distribution of cowpea bradyrhizobia isolates among 20 genotypes identified by RFLP analysis of PCR-amplified 16s rRNA genes

| 16s rRNA genotype[1] | Isolates |
|---|---|
| **Fast growers** | |
| A | 19, 88 |
| B | 20, 27, 29, 51, 60, 79 |
| C | 23, 57 |
| D | 38 |
| E | 43 |
| F | 44 |
| G | 59, 69 |
| H | 92 |
| I | 98? |
| | |
| **Slow growers** | |
| A | 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17, 18, 22, 24, 30, 31, 35, 37, 42, 45, 46, 49, 50, 53, 61, 62, 63, 64, 65, 66, 67, 74, 75, 85, 86, 94, 95 |
| B | 2, 4 |
| C | 13, 77, 82, 87, 99 |
| D | 14, 34, 68 |
| E | 21, 73, 96 |
| F | 25, 70, 71, 97, 100 |
| G | 26, 46, 52, 55, 56 |
| H | 28, 80, 81, 84, 89 |
| I | 32, 58, 83, 90 |
| J | 39, 76, 91, 93 |
| K | 40, 41 |

1 The 16s rRNA genotypes lettered A to K represent the species group of bradyrhizobia isolates obtained with the four endonucleases used.

one to five isolates (Table 4.6). The ninth genomic species of the fast growing isolates designated I, was not included in the relative similarity analysis due to inconsistent bands it produced. The slow growing isolates produced 11 different composite genotypes (Table 4.6). The distribution of the isolates among the slow growing genotypes was highly unbalanced. The number of species in of the genotypes designated A, were more than half of the total number of the isolates, whilst each of the remaining 10 composite genotypes contained between 1.05% to 3.05% isolates (Table 4.6). Diversity among the genomic species identified in both the fast and slow growing isolates was very high, reaching 80% divergence (Figs 4.12 & 4.13).

### 4.3 Effectiveness of isolates in fixing nitrogen

Symbiotic effectiveness was determined for each isolate from the mean of six plants inoculated with an isolate. Number of nodules formed varied among isolates, as well as symbiotic effectiveness. Estimated values for effectiveness (relative to uninoculated control) ranged from 23.5% to 118% per plant for the 100 isolates (Table 4.7). Effectiveness in fixing nitrogen obtained by five of the isolates was similar to, or higher than that of plants fertilized with 70 kg N/ha (Table 4.7). Based on the index of effectiveness the isolates varied from ineffective to highly effective, but with a predominance (68%) of isolates being ranked as moderately effective (Fig. 4.14). The effectiveness of the isolates in fixing nitrogen followed the trends observed with shoot dry weights (Table 4.7). Shoot dry weight and shoot nitrogen produced by the most effective isolate (isolate 44) were 50% higher than the values for the least effective isolate (isolate 28) (Table 4.7 and Plate 4.2). Correlation between effectiveness and shoot dry weight of plants inoculated with the different isolates was the highest (0.91), and more than correlations between the other parameters (Table 4.8). The effectiveness of the isolates grouped within each of the various ecological

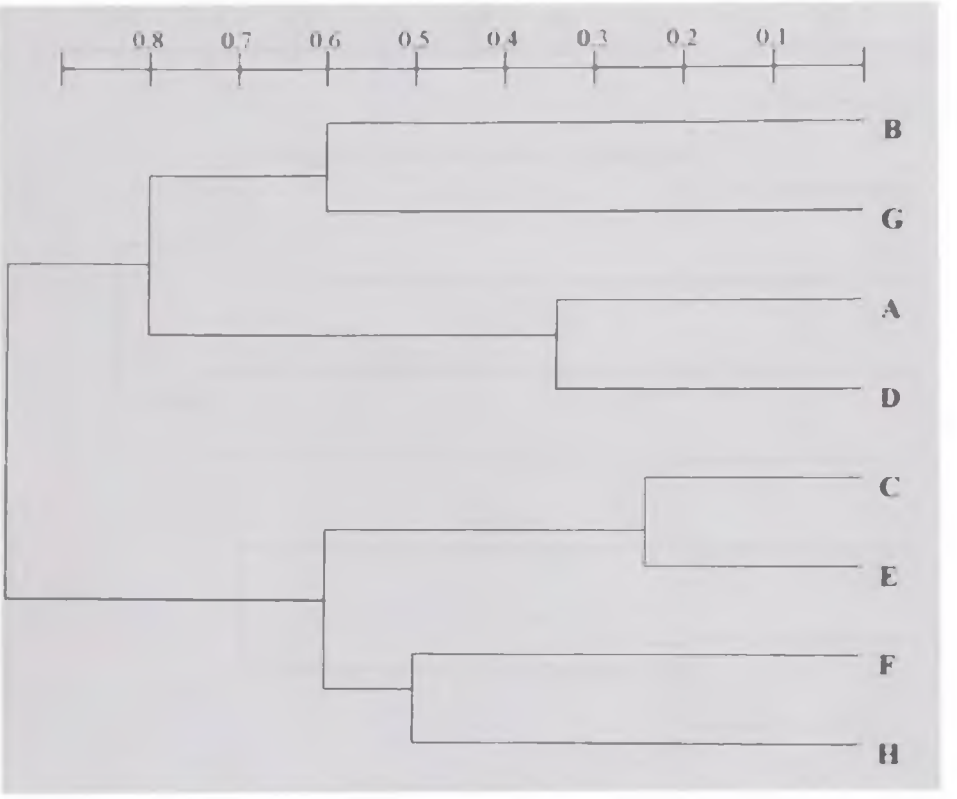


Fig. 4.12 Dendrogram (UPGMA) showing relationship among genomic species of fast-growing cowpea bradyrhizobia isolates as determined by RFLP analysis of the 16s rDNA. *The matrix of pairwise genetic distances was used to construct the dendrogram.*

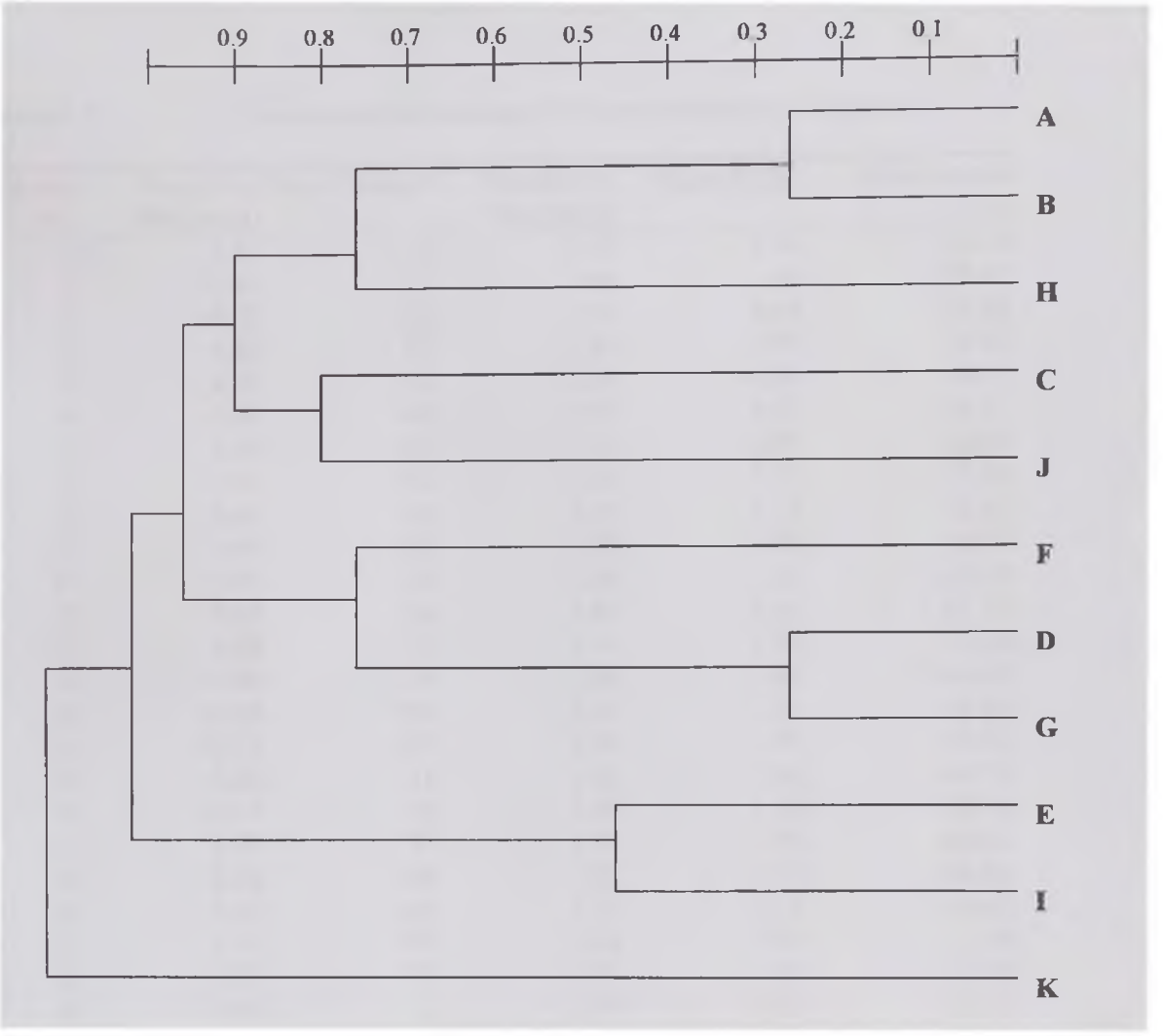


Fig. 4.13 Dendrogram (UPGMA) showing relationship among genomic species of slow-growing cowpea bradyrhizobia isolates as determined by RFLP analysis of the 16s rDNA. *The matrix of pairwise genetic distances was used to construct the dendrogram.*

Table 4.7 Symbiotic effectiveness of cowpea bradyrhizobia isolates

| Isolate No. | Shoot Dry Weight (g) | No. of Nodule | Nodule Dry Weight (g) | Shoot N (%) | Effectiveness (%) |
|---|---|---|---|---|---|
| 1 | 8.61 | 2 | 2.17 | 1.26 | 54.78 |
| 2 | 10.44 | 121 | 2.46 | 1.40 | 95.45 |
| 3 | 8.57 | 25 | 2.30 | 0.95 | 53.89 |
| 4 | 9.42 | 69 | 2.33 | 2.07 | 72.82 |
| 5 | 8.76 | 38 | 2.28 | 0.90 | 58.12 |
| 6 | 9.06 | 49 | 2.15 | 0.87 | 64.81 |
| 7 | 8.88 | 87 | 2.41 | 0.97 | 60.80 |
| 8 | 9.11 | 31 | 2.24 | 1.71 | 65.92 |
| 9 | 9.41 | 96 | 2.27 | 1.54 | 72.60 |
| 10 | 10.48 | 126 | 2.50 | 1.68 | 96.43 |
| 11 | 9.07 | 21 | 2.29 | 1.20 | 65.03 |
| 12 | 9.82 | 36 | 2.41 | 1.46 | 81.73 |
| 13 | 9.62 | 55 | 2.35 | 1.65 | 77.28 |
| 14 | 10.86 | 175 | 2.60 | 1.82 | 104.89 |
| 15 | 10.58 | 150 | 2.53 | 1.76 | 98.66 |
| 16 | 10.14 | 107 | 2.36 | 1.96 | 88.86 |
| 17 | 9.28 | 31 | 2.42 | 2.41 | 69.71 |
| 18 | 10.14 | 78 | 2.50 | 1.88 | 88.86 |
| 19 | 9.95 | 135 | 2.48 | 1.20 | 84.63 |
| 20 | 9.24 | 108 | 2.29 | 1.74 | 68.81 |
| 21 | 9.07 | 165 | 2.42 | 1.18 | 65.03 |
| 22 | 9.63 | 102 | 2.32 | 2.69 | 77.50 |
| 23 | 8.99 | 88 | 2.43 | 1.08 | 63.25 |
| 24 | 8.63 | 2 | 2.14 | 0.92 | 55.20 |
| 25 | 9.26 | 49 | 2.39 | 1.09 | 69.26 |
| 26 | 9.30 | 41 | 2.31 | 1.18 | 62.36 |
| 27 | 7.58 | 11 | 2.14 | 0.84 | 31.84 |
| 28 | 6.51 | 4 | 2.18 | 0.78 | 23.57 |
| 29 | 9.04 | 111 | 2.48 | 1.57 | 46.36 |
| 30 | 7.89 | 93 | 2.36 | 0.83 | 38.25 |
| 31 | 8.01 | 48 | 2.25 | 0.92 | 41.42 |
| 32 | 9.15 | 87 | 2.37 | 1.46 | 66.81 |
| 33 | 9.19 | 79 | 2.46 | 1.06 | 67.70 |
| 34 | 8.91 | 59 | 2.20 | 0.70 | 61.46 |
| 35 | 9.31 | 73 | 2.36 | 1.34 | 70.37 |
| 36 | 9.14 | 86 | 2.53 | 1.71 | 66.59 |
| 37 | 9.51 | 101 | 2.31 | 1.48 | 66.81 |
| 38 | 10.44 | 149 | 2.54 | 1.54 | 95.54 |
| 39 | 7.79 | 159 | 2.35 | 1.37 | 36.52 |
| 40 | 9.19 | 77 | 2.39 | 1.57 | 67.70 |
| 41 | 10.26 | 212 | 2.52 | 2.13 | 99.55 |

Table 4.7 *cont'd*

| Isolate No. | Shoot Dry Weight (g) | No. of Nodule | Nodule Dry Weight (g) | Shoot N (%) | Effectiveness (%) |
|---|---|---|---|---|---|
| 42 | 9.10 | 168 | 2.54 | 1.74 | 65.70 |
| 43 | 9.11 | 71 | 2.32 | 0.64 | 65.92 |
| 44 | 11.45 | 136 | 2.43 | 2.88 | 118.04 |
| 45 | 8.82 | 81 | 2.33 | 0.50 | 59.46 |
| 46 | 9.26 | 97 | 2.38 | 2.02 | 69.26 |
| 47 | 9.45 | 166 | 2.35 | 2.24 | 73.49 |
| 48 | 9.08 | 24 | 2.17 | 0.98 | 65.25 |
| 49 | 8.55 | 32 | 2.16 | 0.70 | 53.45 |
| 50 | 9.37 | 138 | 2.50 | 2.04 | 71.71 |
| 51 | 9.79 | 142 | 2.39 | 1.96 | 81.06 |
| 52 | 9.16 | 107 | 2.36 | 2.12 | 77.06 |
| 53 | 9.37 | 74 | 2.25 | 1.71 | 71.71 |
| 54 | 9.41 | 114 | 2.22 | 2.04 | 72.60 |
| 55 | 9.85 | 159 | 2.50 | 2.18 | 82.40 |
| 56 | 9.65 | 171 | 2.30 | 1.80 | 77.95 |
| 57 | 9.64 | 105 | 2.38 | 1.89 | 77.72 |
| 58 | 9.60 | 92 | 2.28 | 1.68 | 76.83 |
| 59 | 9.75 | 126 | 2.43 | 1.54 | 80.17 |
| 60 | 9.46 | 63 | 2.26 | 1.65 | 73.71 |
| 61 | 8.70 | 126 | 2.25 | 0.92 | 56.79 |
| 62 | 9.32 | 134 | 2.26 | 1.42 | 70.60 |
| 63 | 9.24 | 137 | 2.33 | 1.04 | 68.81 |
| 64 | 9.20 | 38 | 2.19 | 0.90 | 67.92 |
| 65 | 9.41 | 48 | 2.26 | 1.01 | 72.60 |
| 66 | 9.30 | 66 | 2.22 | 1.06 | 70.15 |
| 67 | 9.32 | 57 | 2.24 | 1.06 | 70.60 |
| 68 | 9.22 | 156 | 2.16 | 1.02 | 68.37 |
| 69 | 9.23 | 130 | 2.37 | 1.18 | 68.59 |
| 70 | 9.38 | 29 | 2.26 | 1.16 | 71.93 |
| 71 | 8.96 | 68 | 2.29 | 0.98 | 62.58 |
| 72 | 7.47 | 1 | 2.12 | 0.78 | 29.39 |
| 73 | 9.70 | 84 | 2.31 | 1.44 | 79.06 |
| 74 | 9.34 | 36 | 2.22 | 1.01 | 71.04 |
| 75 | 9.25 | 57 | 2.20 | 1.34 | 69.04 |
| 76 | 9.29 | 40 | 2.18 | 0.98 | 69.93 |
| 77 | 9.23 | 14 | 2.22 | 1.15 | 68.59 |
| 78 | 9.17 | 18 | 2.21 | 1.12 | 67.26 |
| 79 | 8.63 | 20 | 2.24 | 0.94 | 55.23 |
| 80 | 9.04 | 21 | 2.22 | 1.24 | 64.36 |
| 81 | 9.36 | 15 | 2.19 | 1.04 | 71.49 |

Table 4.7 *cont'd*

| Isolate No. | Shoot Dry Weight (g) | No. of Nodule | Nodule Dry Weight (g) | Shoot N (%) | Effectiveness (%) |
|---|---|---|---|---|---|
| 82 | 9.64 | 79 | 2.28 | 2.38 | 77.72 |
| 83 | 10.19 | 116 | 2.43 | 2.80 | 89.97 |
| 84 | 9.47 | 34 | 2.29 | 1.77 | 73.94 |
| 85 | 9.46 | 29 | 2.19 | 1.54 | 73.71 |
| 86 | 9.58 | 97 | 2.26 | 2.35 | 76.39 |
| 87 | 9.73 | 104 | 2.40 | 2.55 | 79.73 |
| 88 | 10.2 | 128 | 2.49 | 2.60 | 90.20 |
| 89 | 8.79 | 58 | 2.21 | 0.96 | 58.79 |
| 90 | 9.26 | 34 | 2.23 | 1.57 | 69.26 |
| 91 | 9.29 | 52 | 2.20 | 0.92 | 69.93 |
| 92 | 9.26 | 38 | 2.17 | 1.09 | 69.26 |
| 93 | 9.33 | 23 | 2.16 | 0.90 | 70.82 |
| 94 | 8.57 | 28 | 2.18 | 0.94 | 53.89 |
| 95 | 8.65 | 52 | 2.31 | 1.02 | 55.67 |
| 96 | 8.63 | 1 | 2.16 | 0.98 | 55.23 |
| 97 | 8.64 | 6 | 2.25 | 0.78 | 55.45 |
| 98 | 9.26 | 90 | 2.25 | 0.95 | 69.26 |
| 99 | 9.30 | 16 | 2.22 | 1.01 | 70.15 |
| 100 | 8.97 | 69 | 2.13 | 1.15 | 62.80 |
| Control | 6.82 | 0 | 0 | 0.78 | 23.60 |
| +N | 10.68 | 0 | 0 | 2.68 | 100.00 |

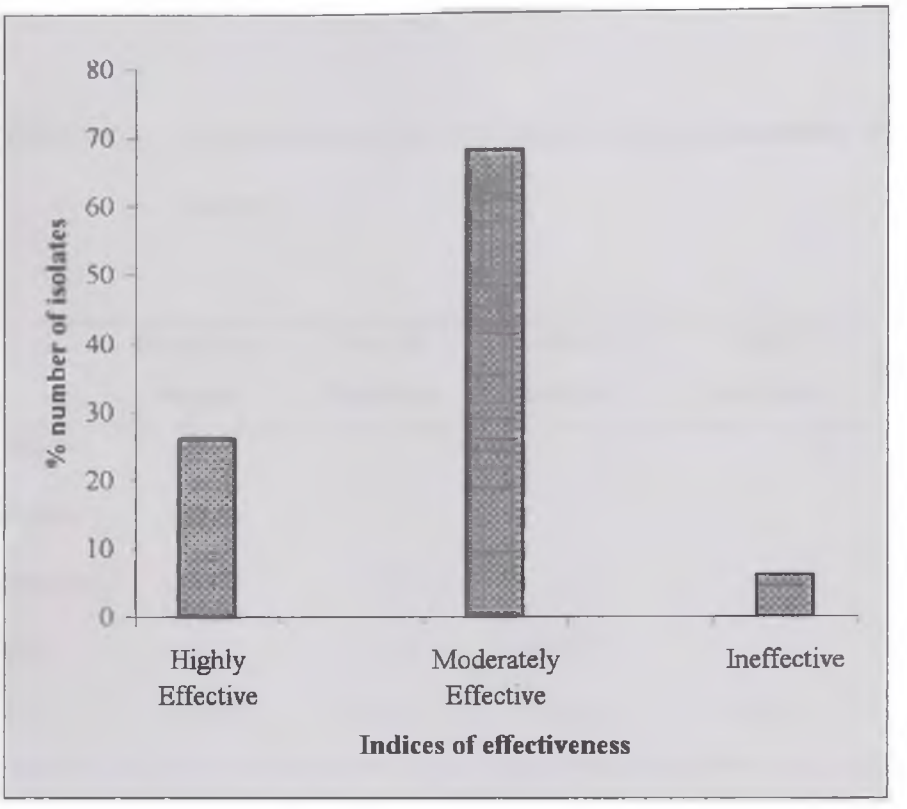


Fig. 4.14 Percent number of cowpea rhizobia isolates for groups of effectiveness

Table 4.8 Correlation between effectiveness and some parameters of nitrogen fixation

| | Shoot dry weight | Nodule Numbers | Nodule dry weight | Shoot nitrogen | Effectiveness |
|---|---|---|---|---|---|
| **Shoot dry weight** | 1 | | | | |
| **Nodule Numbers** | 0.509 | 1 | | | |
| **Nodule dry weight** | 0.553 | 0.691 | 1 | | |
| **Shoot nitrogen** | 0.617 | 0.513 | 0.527 | 1 | |
| **Effectiveness** | 0.908 | 0.494 | 0.555 | 0.615 | 1 |


Plate 4.2 **Differences in plant size, leaf colour and plant vigour of cowpea inoculated with the most effective and least effective bradyrhizobia isolates.**

zones is shown in Table 4.9. Generally, distribution of the isolates in the ecozones followed a normal distribution trend with the majority being moderately effective. A similar trend was observed when the isolates were grouped according to slow and fast growing types (Table 4.10).

### 4.3.1 Relative effectiveness of isolates in fixing nitrogen

The results of the relative effectiveness of the 10 most effective isolates against the standard strain TAL 169 is presented in Fig. 4.15. The results indicated that six of the isolates possessed symbiotic effectiveness superior to the reference strain. Four of these differed significantly from the standard strain (Fig. 4.15).

## 4.4 Competition for nodule occupancy

Few data exist on the impact of foreign genes on the fitness of an organism (Doyle *et al.*, 1995). It is required that before using any marker system for ecological studies, its effects on the most important attributes of the organism have to be studied (Sessitsch *et al.*, 1995). The potential effects of the Gus transposon on the fitness of the marked effective bradyrhizobia isolates was therefore evaluated before the actual competition studies. Each gus-marked isolate and its parent isolate were co-inoculated at equal population densities onto the same cowpea plant grown in a growth pouch. The plants were harvested after 35 days and nodules that were formed typed using the gus staining assay method described previously. The results showed that mutants and the parent isolates formed nearly equal proportions of nodules (Table 4.11), indicating that the mutants were equally competitive as the parent isolates.

Table 4.9 Effectiveness of cowpea bradyrhizobial isolates from soils of the different ecological zones.

| Ecological Zone | Number of isolates | | |
|---|---|---|---|
| | Indices of effectiveness (%) | | |
| | Highly effective | Moderately effective | Ineffective |
| Coastal Savanna | 20.8 | 75.0 | 4.1 |
| High Rainforest | 75.0 | 25.0 | 0 |
| Semi-deciduous Forest | 33.3 | 58.3 | 8.3 |
| Forest Savanna Transition | 25.0 | 66.7 | 8.2 |
| Guinea Savanna | 16.7 | 79.2 | 4.1 |

Table 4.10 Symbiotic effectiveness profiles of cowpea bradyrhizobia isolates

| Isolates | Number of isolates tested | Effectiveness (%) | | |
|---|---|---|---|---|
| | | Highly effective | Moderately effective | Ineffective |
| Fast-growing | 18 | 33.3 | 61.1 | 5.6 |
| Slow-growing | 82 | 24.4 | 70.7 | 4.9 |

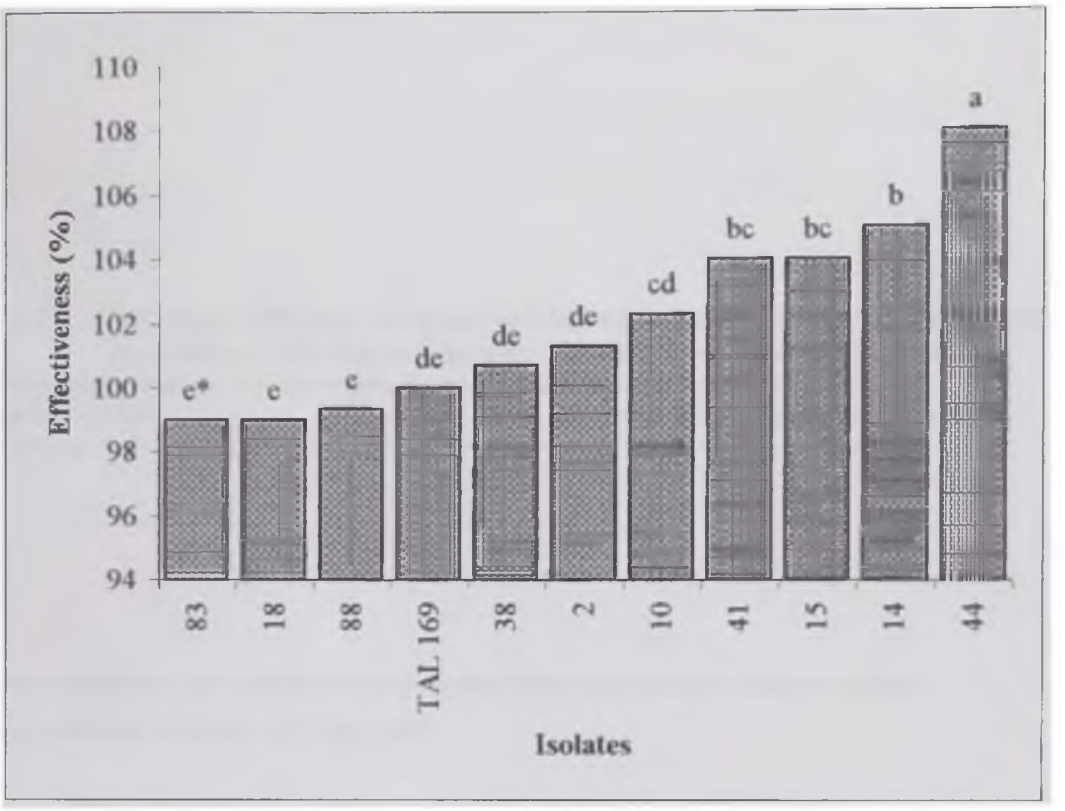


Fig. 4.15 Symbiotic effectiveness of cowpea bradyrhizobia isolates relative to TAL 169

* *Bars with same letters are not significantly different at 1% level of significance*

Table 4.11 Percentage of nodule occupancy of GUS marked cowpea Bradyrhizobia isolates co-inoculated with the corresponding parent isolates onto the same plants.

| Gus marked isolates | Blue stained nodules* (%) |
|---|---|
| G2 | 51 ± 5 |
| G10 | 53 ± 3 |
| G14 | 49 ± 2 |

* Means ± standard deviation of 3 replicates.

Results of the competition experiment between the gus-marked effective isolates and ineffective isolates are show in Table 4.12. The number of nodules formed by the different isolates and different isolate ratio combinations varied between 46 to 179 (Table 4.12). Although at the 1:1 ratio, each of the effective isolates occupied a higher proportion of nodules than by three of the five ineffective competitors, (29, 30, and 94), the reverse was true for the two remaining ineffective isolates (28 and 72), which outcompeted the three effective isolates tested. Even when the effective isolates outnumbered the more competitive ineffective isolates (28 and 72) by the ratio 2:1, the effective isolates still made no significant gains in nodule occupancy (Table 4 12).

Calculated competitive indices (Beattie *et al.*, 1989) are shown in Fig. 4.16. In each case the GUS-marked effective isolate is strain X and the unmarked ineffective isolate is designated as Y. The intercept values confirmed that the ineffective isolates 28 and 72 were more competitive than any of the effective isolates, whilst the remaining three ineffective isolates (29, 30 and 94) were less competitive.

A depression of plant yield (plant height and plant biomass) was also observed when the highly competitive ineffective isolates 28 and 72 competed with the effective isolates. Interestingly, this depression in plant yield occurred even when the effective isolates occupied 50% or slightly more of the nodules. In contrast, a higher plant yield was observed with the combination of effective isolates plus ineffective isolate, 94, irrespective of the ratio of combination and proportion of nodules occupied by the competing isolates (Plate 4.3a and b).

Table 4.12 Competitive characteristics of GUS-marked effective cowpea rhizobia isolates against ineffective isolates*

| GUS-marked effective isolates | Ineffective isolate designation | Ratio (GUS-marked isolate: ineffective isolate) | Plant height (cm) | Shoot dry weight (g) | Number of nodules | % GUS-marked nodules | % ineffective nodules |
|---|---|---|---|---|---|---|---|
| G2 | | - | **28.0** | **9.6** | **52** | **100** | - |
| | 28 | 0.5 | 24.6 | 9.2 | 46 | 4.3 | 95.6 |
| | | 1 | 22.8 | 9.2 | 50 | 28.0 | 72.0 |
| | | 2 | 23.8 | 9.3 | 54 | 15.4 | 84.5 |
| | 29 | 0.5 | 34.4 | 9.7 | 96 | 55.2 | 44.7 |
| | | 1 | 30.9 | 9.6 | 95 | 61.0 | 39.0 |
| | | 2 | 30.9 | 9.6 | 80 | 86.2 | 13.7 |
| | 30 | 0.5 | 28.8 | 9.3 | 75 | 62.6 | 37.3 |
| | | 1 | 28.9 | 9.3 | 142 | 62.6 | 37.3 |
| | | 2 | 31.1 | 9.7 | 147 | 62.5 | 37.4 |
| | 72 | 0.5 | 22.8 | 8.4 | 110 | 12.7 | 87.2 |
| | | 1 | 24.9 | 8.0 | 67 | 34.0 | 66.0 |
| | | 2 | 24.4 | 8.9 | 99 | 67.6 | 32.3 |
| | 94 | 0.5 | 32.8 | 10.4 | 127 | 53.5 | 46.4 |
| | | 1 | 33.8 | 9.8 | 134 | 60.6 | 39.3 |
| | | 2 | 36.0 | 10.0 | 161 | 86.3 | 13.6 |

* Table continued on next page

Table 4.12* *cont'd*

| GUS-marked effective isolates | Ineffective isolate designation | Ratio (GUS-marked isolate: ineffective isolate) | Plant height (cm) | Shoot dry weight (g) | Number of nodules | % GUS-marked nodules | % ineffective nodules |
|---|---|---|---|---|---|---|---|
| G10 | | - | **30.2** | **9.2** | **106** | **100** | - |
| | 28 | 0.5 | 21.7 | 8.5 | 114 | 33.3 | 66.6 |
| | | 1 | 26.0 | 8.4 | 98 | 45.9 | 54.0 |
| | | 2 | 27.8 | 8.9 | 102 | 66.6 | 33.3 |
| | 29 | 0.5 | 28.8 | 8.9 | 107 | 35.5 | 64.4 |
| | | 1 | 30.1 | 9.1 | 67 | 59.7 | 40.2 |
| | | 2 | 35.4 | 9.4 | 80 | 76.2 | 23.7 |
| | 30 | 0.5 | 34.4 | 8.4 | 141 | 53.1 | 46.8 |
| | | 1 | 34.6 | 9.2 | 123 | 78.8 | 21.1 |
| | | 2 | 32.9 | 9.6 | 142 | 83.0 | 17.0 |
| | 72 | 0.5 | 28.9 | 9.1 | 122 | 39.3 | 60.6 |
| | | 1 | 29.7 | 9.2 | 124 | 45.1 | 54.8 |
| | | 2 | 24.2 | 8.6 | 114 | 60.5 | 39.4 |
| | 94 | 0.5 | 35.5 | 10.8 | 118 | 55.9 | 44.1 |
| | | 1 | 37.1 | 9.8 | 148 | 54.0 | 46.0 |
| | | 2 | 35.0 | 10.1 | 146 | 70.5 | 29.4 |

* Table continued on next page

Table 4.12 *cont'd*

| GUS-marked effective isolates | Ineffective isolate designation | Ratio (GUS-marked isolate: ineffective isolate) | Plant height (cm) | Shoot dry weight (g) | Number of nodules | % GUS-marked nodules | % ineffective nodules |
|---|---|---|---|---|---|---|---|
| G14 | | - | **28.8** | **9.4** | **96** | **100** | - |
| | 28 | 0.5 | 21.0 | 8.9 | 88 | 33.0 | 67.0 |
| | | 1 | 23.6 | 8.9 | 73 | 16.4 | 83.5 |
| | | 2 | 24.6 | 8.7 | 86 | 62.7 | 37.2 |
| | 29 | 0.5 | 33.5 | 10.0 | 119 | 63.0 | 36.9 |
| | | 1 | 32.0 | 9.7 | 89 | 60.6 | 39.3 |
| | | 2 | 33.6 | 10.0 | 125 | 84.8 | 15.2 |
| | 30 | 0.5 | 29.9 | 9.7 | 48 | 72.9 | 27.0 |
| | | 1 | 26.8 | 9.1 | 96 | 69.4 | 30.5 |
| | | 2 | 27.5 | 9.3 | 109 | 78.8 | 21.1 |
| | 72 | 0.5 | 22.2 | 8.5 | 98 | 12.2 | 87.7 |
| | | 1 | 20.2 | 8.7 | 84 | 32.1 | 67.8 |
| | | 2 | 26.1 | 9.1 | 126 | 57.1 | 42.8 |
| | 94 | 0.5 | 38.4 | 10.1 | 115 | 66.9 | 33.1 |
| | | 1 | 32.3 | 9.8 | 136 | 63.7 | 36.2 |
| | | 2 | 40.2 | 11.1 | 179 | 75.4 | 24.5 |

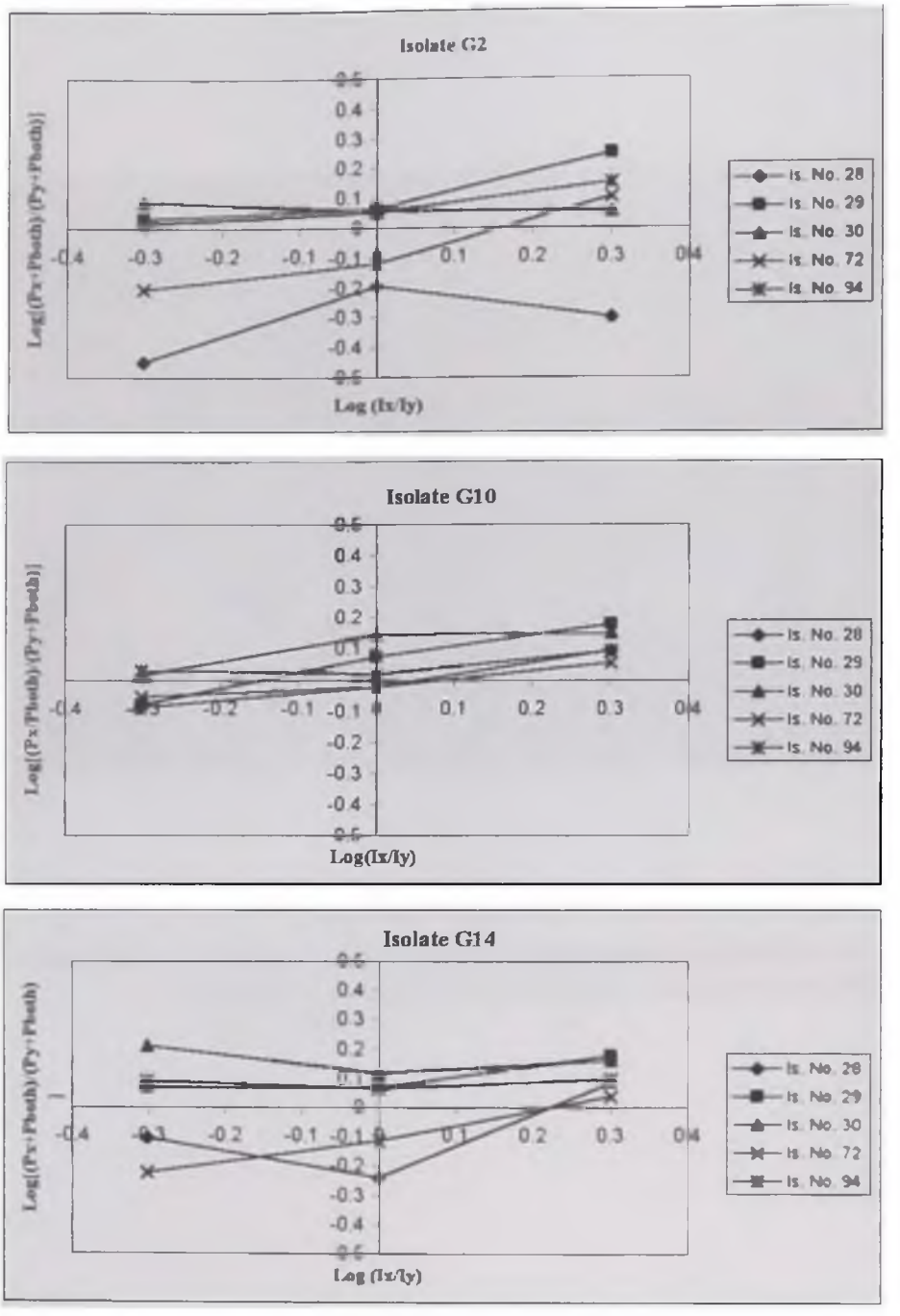


Fig. 4.16 Linear regression of Log [(Px+Pboth)/(Py+Pboth)] against Log (Ix/Iy) for GUS-marked Isolates 2,10,14 when competed against ineffective Isolates 28, 29, 30, 72 and 94.


Plate 4.3a Depression of plant height and biomass associated with ineffective isolate 28 in competition with gus-marked effective isolate 2 (G2).


Plate 4.3b Higher plant yield associated with ineffective isolate 94 in competition with gus-marked effective isolate 2 (G2).

### 4.4.1. Effect of placement and time of placement

The yield enhancement or suppression effect observed with the inoculation of isolates 94 and 28, respectively, was further examined by evaluating the effect of time of placement of the competing isolates relative to the other on the seed. It was observed that the suppressive effect of isolate 28 was removed as its placement was delayed, whilst the enhancement effect of 94 was also reduced as its relative time of placement was delayed (Figs. 4.17 to 4.22). The suppressive effect of 28 was more pronounced when it was placed first followed by the effective isolate. This was, however, not the case with the beneficial effect exerted by isolate 94.

### 4.4.2. Time of formation of nodules by competing isolates

There were differences in the time of appearance of nodules formed by the different strains. The earliest nodule was observed 9 days after inoculation, by the ineffective isolate 29 (Fig. 4.23). The longest period for the first nodule formed to be observed was, 14 days after inoculation and this was by isolate 72. The total number of nodules formed during the 14 days post inoculation period by all the isolates (Fig. 4.23) showed no significant inter-strain differences (mean nodule number plant$^{-1}$ = 8).

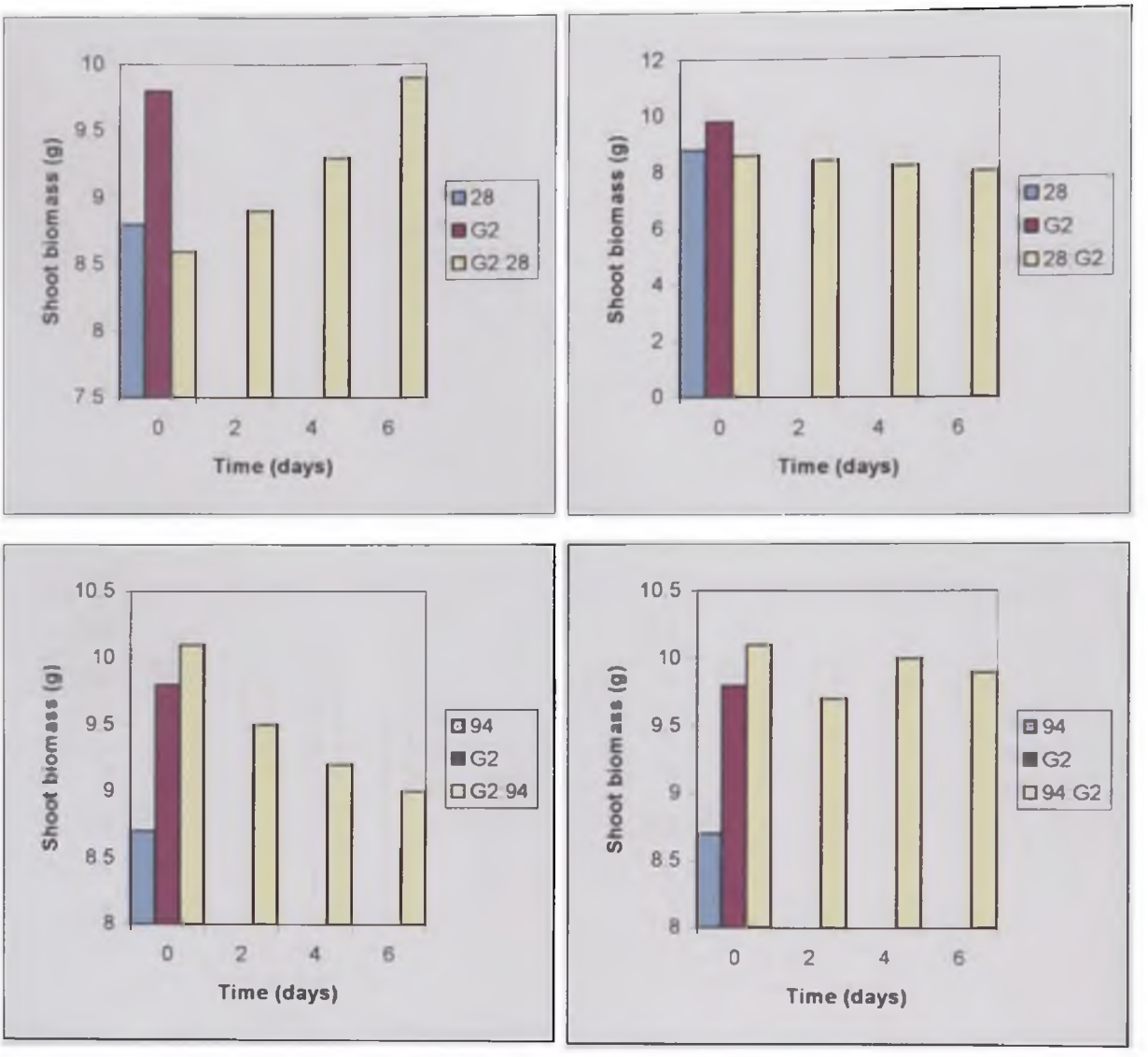


Fig. 4.17 Effect of placement and time of placement of effective isolate (G2) and ineffective isolates (28 and 94) on shoot biomass.

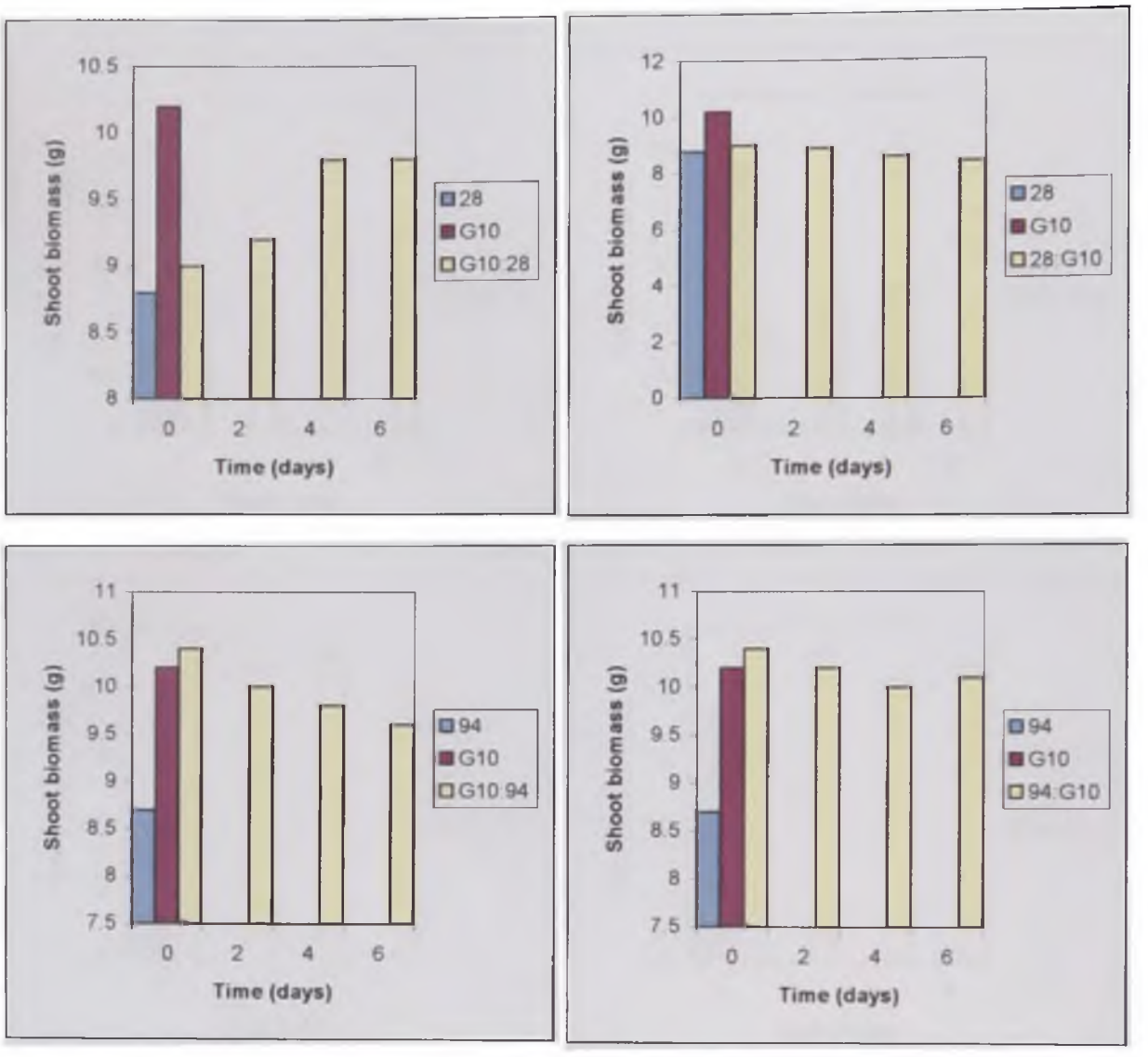


Fig. 4.18 Effect of placement and time of placement of effective isolate (G10) and ineffective isolates (28 and 94) on shoot biomass.

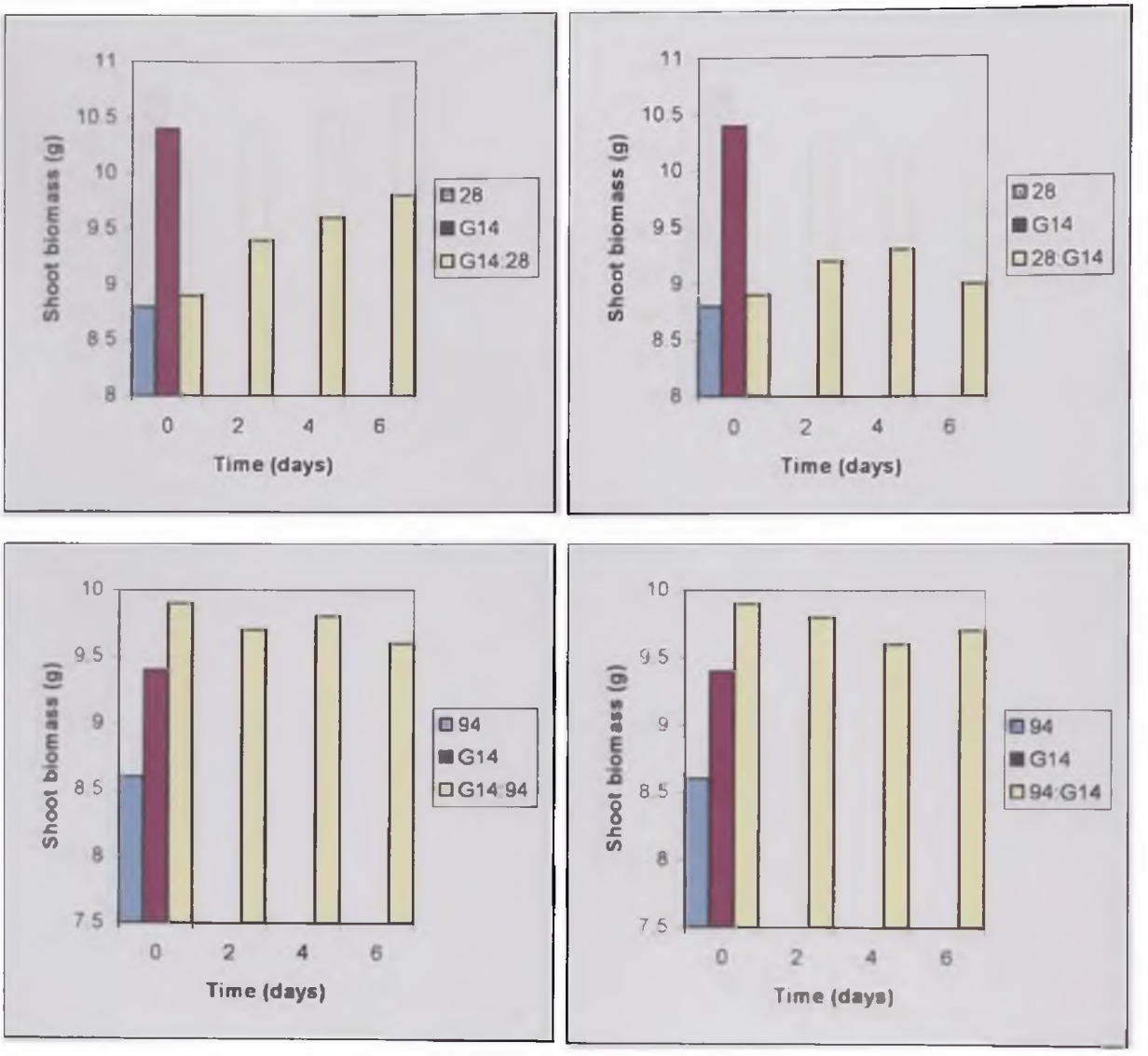


Fig. 4.19 Effect of placement and time of placement of effective isolate (G14) and ineffective isolates (28 and 94) on shoot biomass.

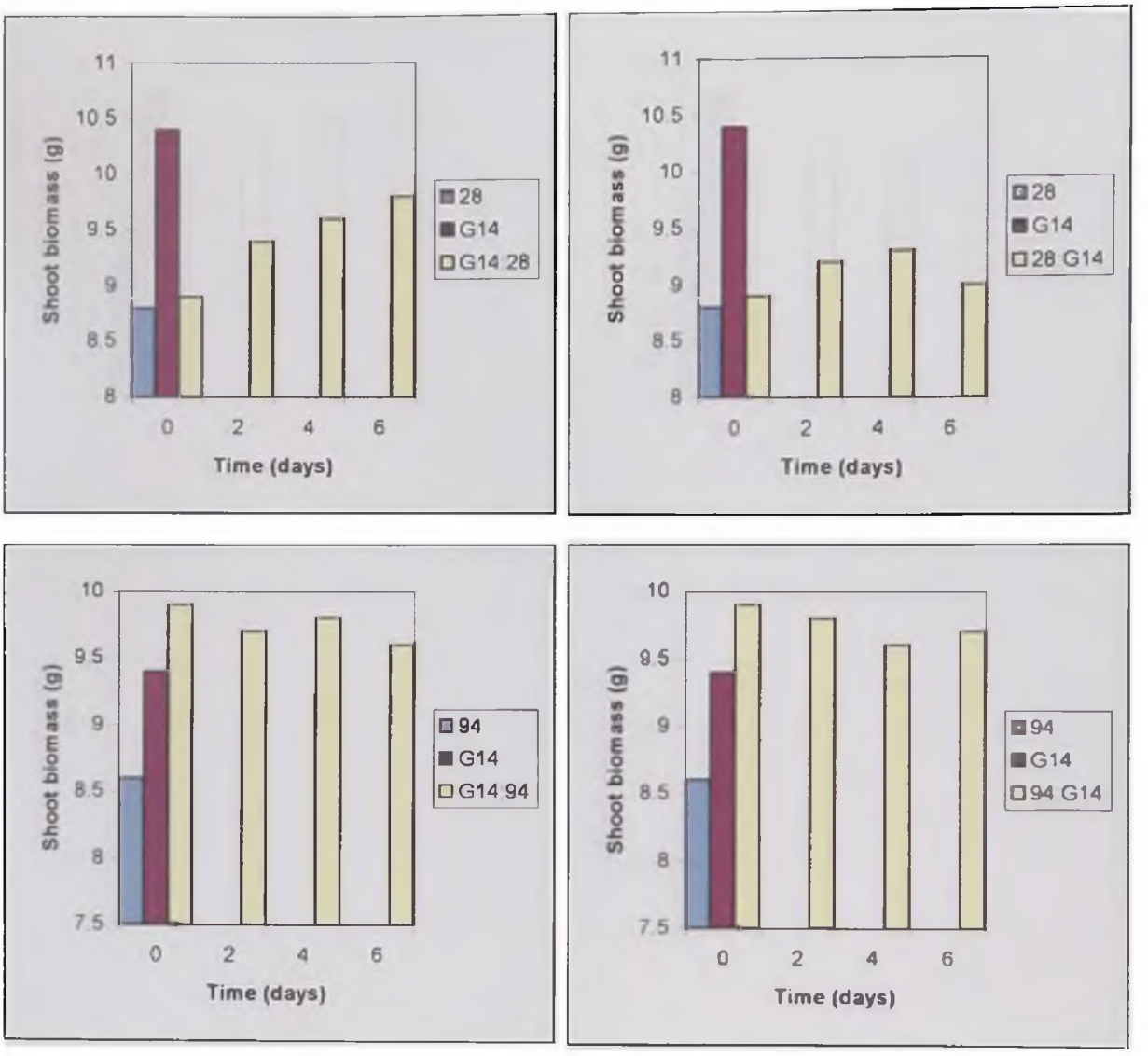


Fig. 4.19 Effect of placement and time of placement of effective isolate (G14) and ineffective isolates (28 and 94) on shoot biomass

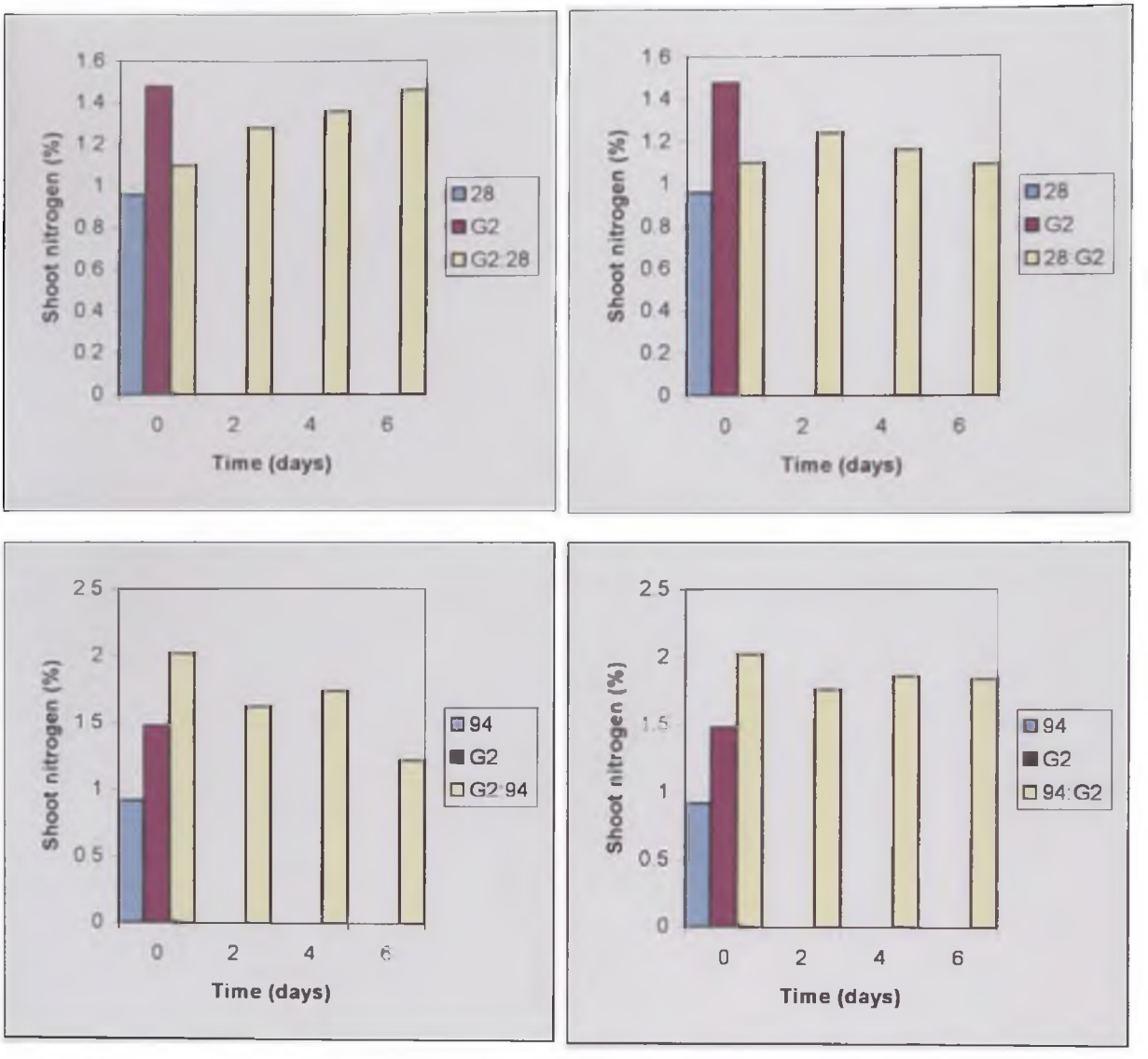


Fig. 4.20 Effect of placement and time of placement of effective isolate (G2) and ineffective isolates (28 and 94) on shoot nitrogen

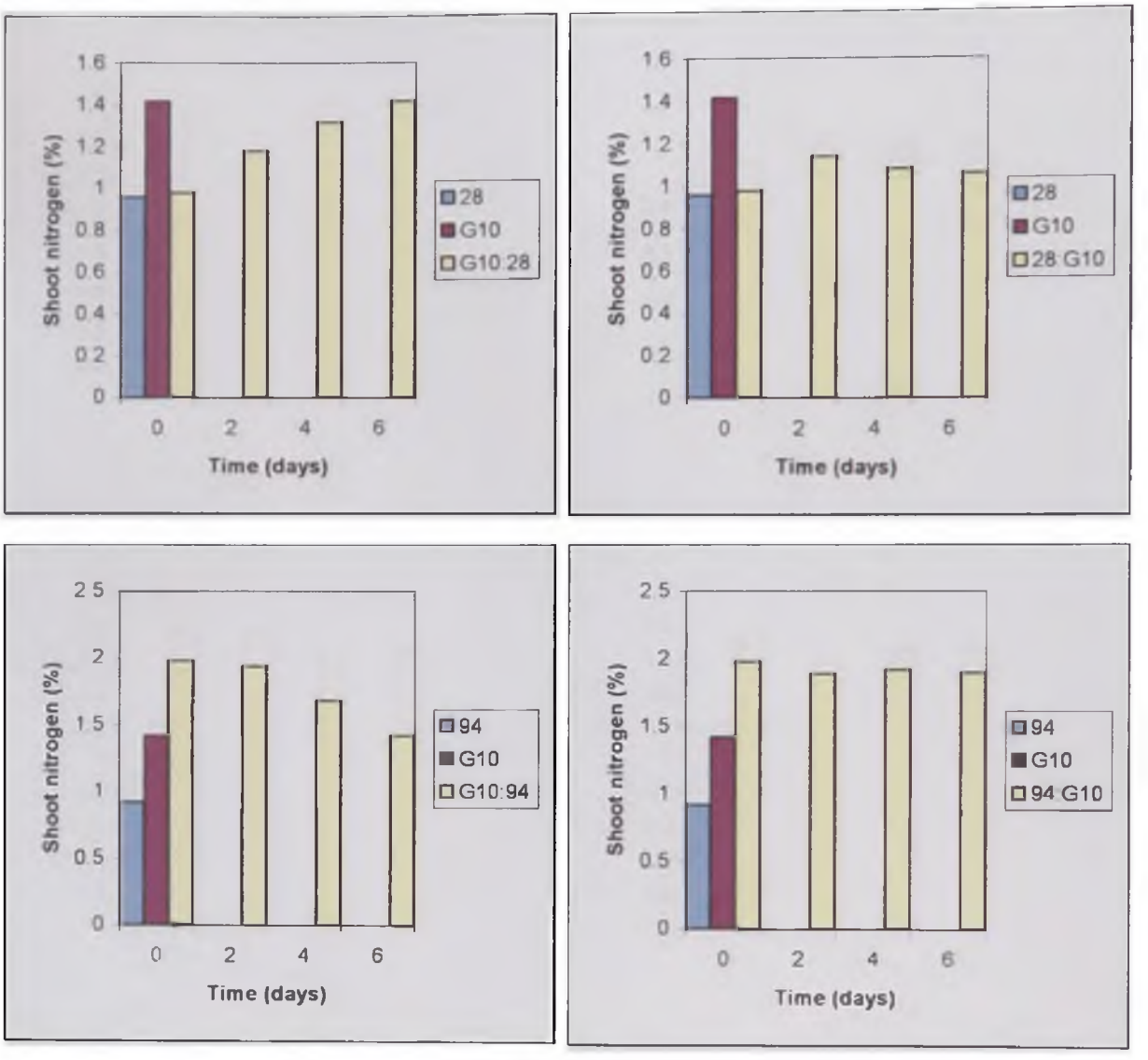


Fig. 4.21 Effect of placement and time of placement of effective isolate (G10) and ineffective isolates (28 and 94) on shoot nitrogen

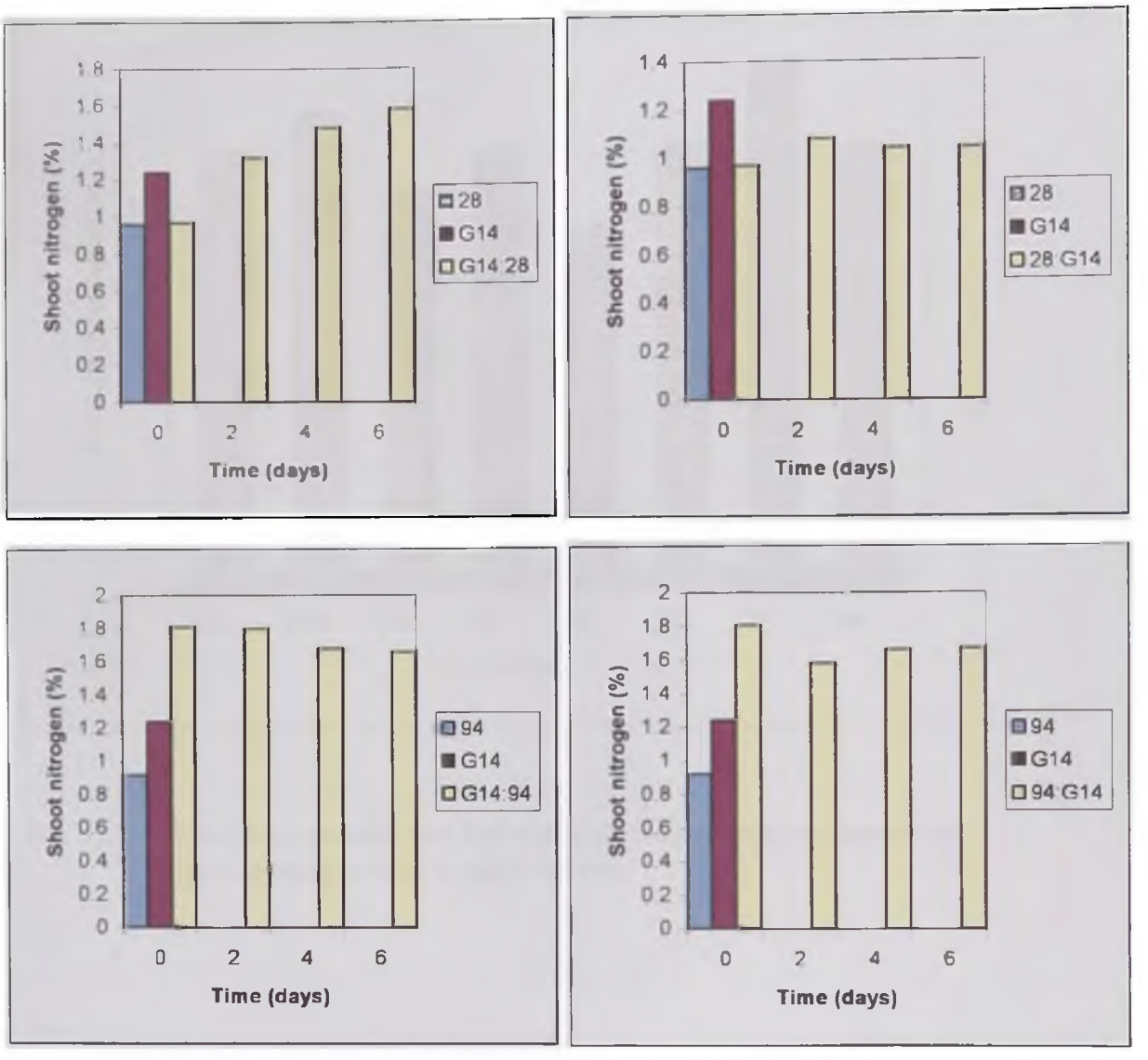


Fig. 4.22 Effect of placement and time of placement of effective isolate (G14) and ineffective isolates (28 and 94) on shoot nitrogen

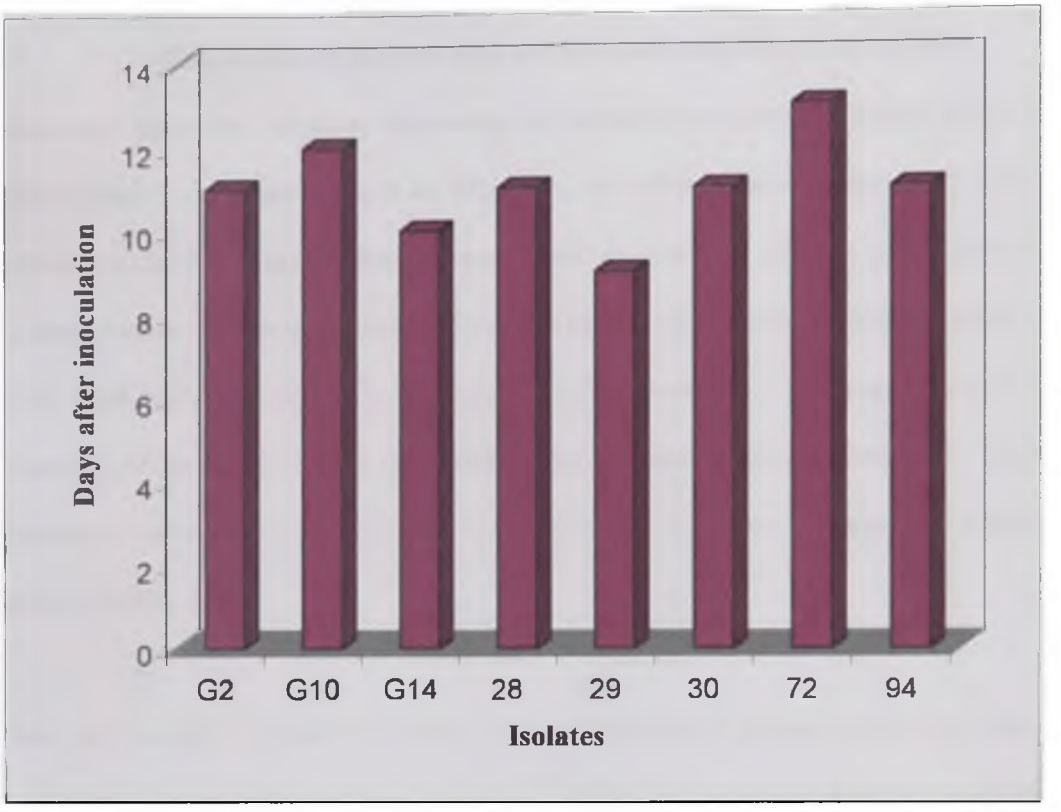


Fig. 4.23 Time when nodules were first visible on cowpea roots inoculated with the competing isolates in single cultures.

## 4.5 Inoculation of cowpea with indigenous *Bradyrhizobium* isolates

Inoculation generally increased the number of nodules produced by cowpea plants in all the soils (averaged) as shown in Fig. 4.24. However, the soil-inoculated treatments recorded higher nodule numbers than the seed-treated ones. In certain cases, for instance in the Lima soil series, the mean number of nodules produced by plants in the soil-inoculated treatment was double that of the seed-inoculated ones. In both seed and soil inoculation, the mixed strain inoculation consisting of the three isolates produced higher number of nodules recorded. Response to inoculation in terms of nodulation was best realised in Lima and Ankasa soils and poorest in Akuse soil (Fig. 4.24).

Shoot dry weight of inoculated cowpea plants showed a greater yield over those of the uninoculated control (Fig. 4.25). The trend of shoot dry matter yield of the inoculated plants was similar to that obtained for nodulation (Fig. 4.24). Table 4.13 summarises the data for percent and total nitrogen of cowpea plants and shows that percent nitrogen values associated with the various inoculation treatments were significantly different from each other in the different soils. Plants inoculated with the mixed inoculants were superior in percent nitrogen content (Table 4.15). Again significant differences between the inoculated and the uninoculated control were best seen in Lima soil (Table 4.14).

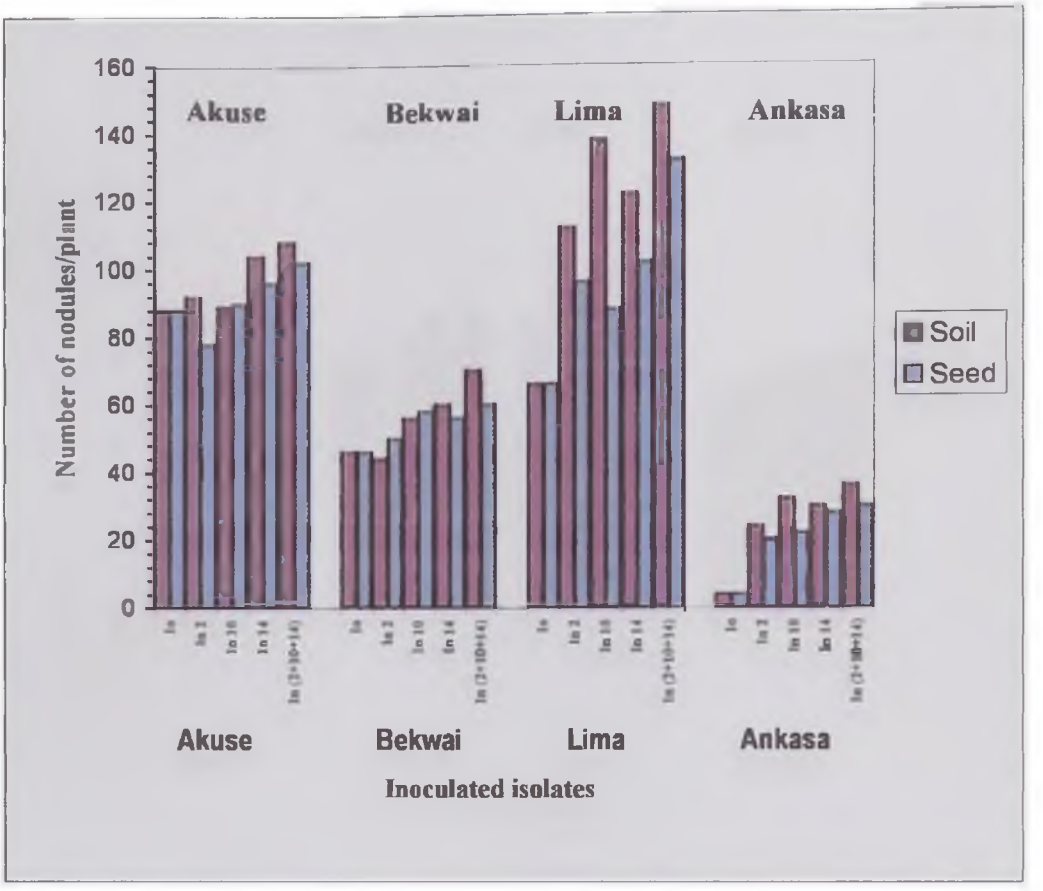


Fig 4.24 Nodulation of cowpea (Asontem) plants inoculated with isolated native cowpea bradyrhizobia isolates.
*Io = Uninoculated; In 2, In 10 and In 14 = isolates 2, 10 and 14, respectively*

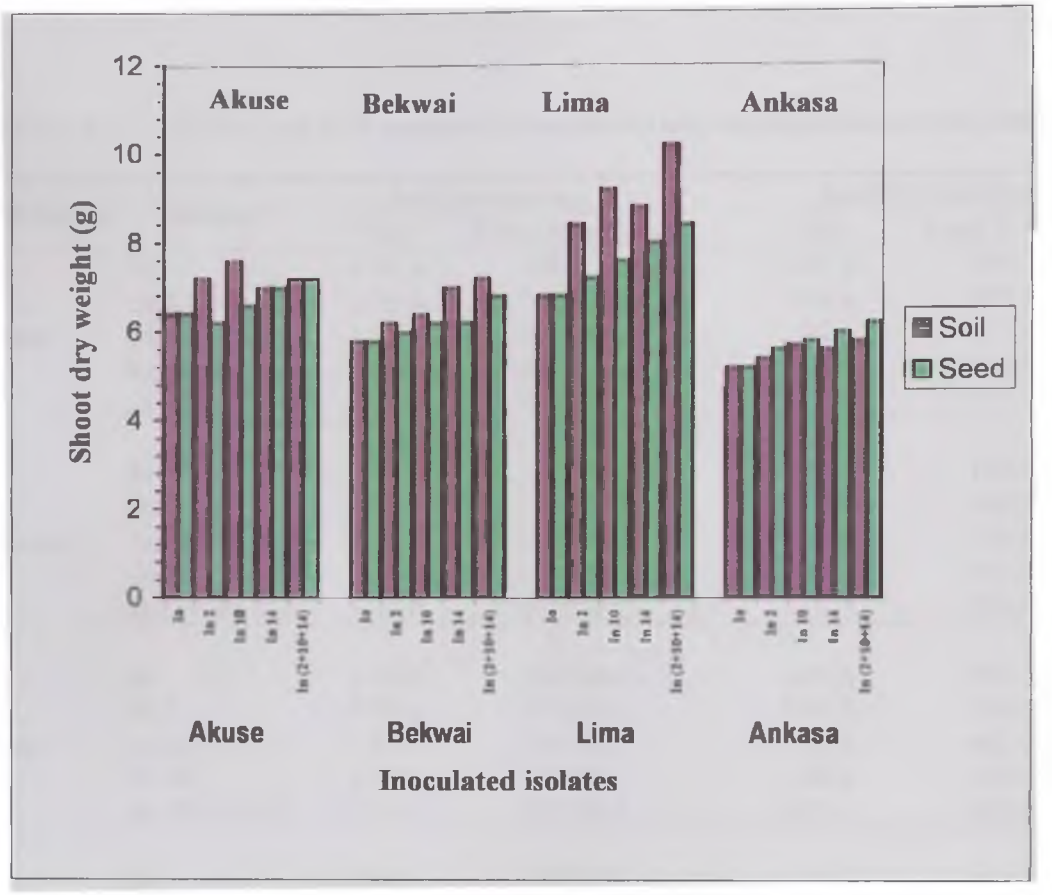


Fig.4.25 **Shoot yield of cowpea (Asontem) plants inoculated with isolated native cowpea bradyrhizobia isolates.**

***Io = Uninoculated; In 2, In 10 and In 14 = isolates 2, 10 and 14, respectively***

Table 4.13 Total N and % N in shoots of inoculated and uninoculated cowpea plants

| Soil Series | Inocula* | Soil inoculation | | Seed inoculation | |
|---|---|---|---|---|---|
| | | %N | Total N (mg/kg) | %N | Total N (mg/kg) |
| Akuse | Io | 4.48 a | 286.72 a | 4.48 a | 286.72 a |
| | In 2 | 4.78 a | 344.16 a | 4.61 a | 285.82 a |
| | In 10 | 4.90 a | 372.40 ab | 5.12 b | 337.92 b |
| | In 14 | 5.58 b | 390.60 b | 4.85 b | 339.50 b |
| | In (2+10+14) | 5.61 b | 403.92 c | 5.24 b | 377.28 c |
| Bekwai | Io | 2.68 a | 155.44 a | 2.68 a | 155.44 a |
| | In 2 | 2.90 ab | 179.80 ab | 2.72 a | 163.20 ab |
| | In 10 | 3.20 bc | 204.80 b | 3.01 b | 186.62 ab |
| | In 14 | 2.97 ab | 207.90 b | 2.99 b | 185.38 ab |
| | In (2+10+14) | 3.42 c | 246.24 c | 3.01 b | 204.68 b |
| Lima | Io | 4.42 a | 300.56 a | 4.42 a | 300.56 a |
| | In 2 | 4.50 a | 378.00 b | 5.48 b | 394.56 b |
| | In 10 | 5.86 b | 539.12 c | 5.29 b | 402.04 b |
| | In 14 | 5.77 b | 507.76 c | 5.86 c | 468.80 c |
| | In (2+10+14) | 6.14 b | 626.28 d | 6.02 c | 505.68 d |
| Ankasa | Io | 2.99 a | 155.48 a | 2.99 a | 155.48 a |
| | In 2 | 4.00 b | 216.00 b | 4.20 b | 235.20 b |
| | In 10 | 4.42 b | 251.94 c | 4.40 b | 255.20 b |
| | In 14 | 4.74 b | 265.44 c | 4.48 b | 268.80 b |
| | In (2+10+14) | 5.80 c | 336.40 d | 5.68 c | 352.16 c |

Means followed by same letters in a column are not significantly different at 0.01 level for same soil series.
* Io = uninoculated; In 2, In 10 and In 14 = Inocula 2, 10 and 14, respectively.

Table 4.14 Comparison of Means of Total N and %N by Soil Series

| Series | Total N (mg/kg) | | %N | |
|---|---|---|---|---|
| | Soil inoculation | Seed inoculation | Soil inoculation | Seed inoculation |
| Lima | 469.88 a | 414.37 a | 5.33a | 5.38a |
| Akuse | 359.56 b | 325.45 b | 5.10b | 4.89b |
| Ankasa | 245.05 c | 253.37 c | 4.39c | 4.35c |
| Bekwai | 198.84 d | 179.06 d | 3.03d | 2.88d |

Means followed by same letters in a column are not significantly different at 0.01 level for same soil series.

Table 4.15 Comparison of Means of Total N and %N by Inocula

| Inocula* | TOTAL N (mg/kg) | | %N | |
|---|---|---|---|---|
| | Soil inoculation | Seed inoculation | Soil inoculation | Seed inoculation |
| **Io** | 224.55 a | 224.55 a | 3.68a | 3.68a |
| **In 2** | 279.49 b | 269.69 b | 4.05b | 4.21b |
| **In 10** | 342.06 c | 295.44 c | 4.59c | 4.45c |
| **In 14** | 342.35 c | 315.62 d | 4.77c | 4.55c |
| **In (2+10+14)** | 403.21 d | 360.00 e | 5.24d | 4.99d |

Means followed by same letters in a column are not significantly different at 0.01 level for same soil series.
* Io = uninoculated; In 2, In 10 and In 14 = Inocula 2, 10 and 14, respectively.

# CHAPTER FIVE

## 5.0 Discussion

### 5.1 Potential to improve nitrogen fixation of cowpea

The potential to improve nitrogen fixation of cowpea in Ghanaian soils was evaluated in this study by conducting experiments to assess the natural nodulation potential of cowpea, estimate bradyrhizobia numbers capable of nodulating cowpea in the soils and to determine the response of cowpea to inoculation and nitrogen fertilization.

The formation of adequate number of nodules on legumes in any soil is dependent on the presence of high numbers of the homologous rhizobia in the soil. Estimating rhizobial population density is an indirect means of predicting whether or not a legume will respond to inoculation (Thies *et al.*, 1991). Although there is no direct way of measuring the density of indigenous rhizobial populations in soils, the most probable number (MPN) plant infection assay provides an indirect estimate (Vincent, 1970). The most probable number infection test is based on the assumption that organisms are randomly distributed and that the presence of one *Rhizobium* cell is capable of inducing nodulation on an appropriate host (Woomer *et al.*, 1988). Counts of indigenous bradyrhizobial population capable of nodulating cowpea showed that most of the soils in Ghana harbour large populations that are able to nodulate cowpea. This is not unusual since Vincent (1980) reported the presence of rhizobia even in virgin soils, although in general, in such soils, they may occur in low numbers. In this study, cowpea bradyrhizobia were found in soils never planted to cowpea (Bekwai and Nzema). This indicates that either bradyrhizobia are naturally part of the indigenous soil microflora or that other native legumes

(including grain and cover legumes, shrubs and trees) are serving as hosts and thus inoculant sources of bradyrhizobia for cowpea. The higher numbers that have previously supported the growth of a host legume has been attributed to the high rate of rhizobial multiplication in the host rhizosphere compared to non-host rhizosphere (Cregan and Keyser, 1989). However, the use of legume inoculants is not common in Ghana, and non-existent at the locations where the soils used in this study were sampled. It is therefore reasonable to assume that the high bradyrhizobia cells encountered in many of these soils truly represent long established indigenous populations. However, as much as forty percent of the soils examined contained 100 or less rhizobia per gram of soil, which gives an indication that a yield response to inoculation is most likely to be obtained in these soils. This does not preclude the possibility that response to inoculation in the other soils is possible. Response to inoculation can be obtained if competitive and highly effective strains are introduced in high quality inoculants.

The studies of Woomer *et al.* (1988) have confirmed, with five different legume species (*Trifolium repens*, *Medicago sativa*, *Vicia sativa*, *Leucaena leucocephala*, and *Macroptilum atropurpureum*), the importance of the appropriate host legume on the occurrence and preponderance of a particular *Rhizobium* species. It is therefore not surprising that the soils that contained the highest numbers of bradyrhizobia happened to be in those from areas where cowpea is commonly cultivated.

Other factors such as soil pH, soil organic matter, soil nitrogen, soil phosphorus and soil texture may also influence the population of indigenous rhizobia (Danso and Alexander, 1974). Depending upon their severity, these factors may bring about wide differences in the rhizobial

numbers in different soils. Low soil pH for instance results in poor persistence of rhizobia and can affect in addition to population, the spatial distribution of indigenous rhizobia (Munns and Keyser, 1981). This was evident in this study as very low (e.g. 6 rhizobia/g of soil) and in some instances complete absence of bradyrhizobia was recorded in the soils with pHs of less than 4.5.

There was a direct relationship between nodulation and bradyrhizobial numbers; nodulation was observed to be low in soils, such as Ankasa, Boi and Tafali, with low incidence of native cowpea bradyrhizobia. This is in contrast to the abundant nodulation observed in soils like Akuse, Agawtaw, and Bediesi where high bradyrhizobia counts were obtained. These result indicate that nodulation and nitrogen fixation of cowpea can be improved significantly through studies to assess the population densities of bradyrhizobia in different soils as an indication of the potential need for inoculation to achieve higher nodulation. In similar studies by Thies *et al.* (1991), the results suggested that cowpea inoculation response is likely to be obtained in soils containing less than 100 cells of bradyrhizobia per gram of soil, with reduced chances of improving nodulation by inoculation above this threshold value. Further improvements in the benefits of inoculation can be expected through the exploitation of the enormous genetic variability for nodulation and nitrogen fixation that would be found to exist between the indigenous bradyrhizobia and also in host genotypes.

The response of cowpea to nitrogen fertilizer as measured was the net effect of nitrogen uptake and nitrogen fixation over the growing period. Of all the mineral nutrients, nitrogen has the most pronounced influence on nitrogen fixation in legumes (Horst, 1986). Although in general mineral nitrogen depresses nodulation and nitrogen fixation (Eardly *et al*., 1984; Streeter, 1985;

Carrol and Mathews, 1990), low levels have been found in some cases to exert beneficial effects on the symbiotic process and on yield (Hardarson *et al.*, 1984). When the nitrogen levels of the soils were increased in this study from 0 to 40 kg N/ha, shoot dry weight of the cowpea cultivars increased except Benga, which did not increase in Akuse and in Tafali soils. The observed increment was not realised in Adenta soil. The shoot dry weight significantly increased in some of the cultivars and in some of the soils as the nitrogen levels were increased from 40 kg N/ha till a peak was reached. However, the N level at which a peak was attained differed in the different soils, reflecting the influence of the different levels of soil nitrogen already present in the different soils. Similarly, the cultivars showed different responses to different levels of nitrogen application. The interesting observation may be an indication that some of the cultivars were capable of high $N_2$ fixation at both low and high soil inorganic levels, as observed in soybean by Hardarson *et al.* (1984).

The response of the cultivars to increasing levels of nitrogen is an indication that nitrogen fixation was not supplying the plants with all the nitrogen they required for maximum yield. The diminishing of the positive response beyond a certain nitrogen level is not an unexpected result. As the levels of nitrogen increase, nitrogenase activity, as an expression on nitrogen fixation rate has been found to decline drastically (Horst, 1986).

High external concentrations of nitrogen inhibit root infection by *Rhizobium* (Carrol and Gresshoff, 1983). It is therefore not surprising that nodulation of cowpea in the present study declined as the nitrogen levels were increased (Figs. 4.3 and 4.8). Some species and varieties of legumes support greater nitrogen fixation than others when soil nitrogen is high (Hardarson *et*

*al.*, 1984; Senaratne *et al.*, 1987). Proper use when made of known genetic variability among existing species and cultivars can result in high $N_2$ fixation and high crop yields even when soil nitrogen is high or when fertilizer nitrogen needs to be applied to associated non-fixing crop. In this study, cowpea displayed a remarkable ability to support nodulation at as high as 160 kg N/ha. Lower levels up to 100 kg N/ha have been reported to severely decrease nodulation in other legumes such as soybean (Hardarson *et al.*, 1989) and common bean (Ruschel *et al.*, 1979). The results obtained in this study and that of Awoniake *et al.* (1990) may indicate that cowpea is a suitable candidate for intercropping.

### 5.2 Diversity of indigenous cowpea bradyrhizobia isolates

The results of the physiological and metabolic analysis obtained have demonstrated and confirmed the results of other workers such as Mpepereki *et al.* (1997), that cowpea is nodulated by both fast and slow growing rhizobia. As much as 18% of the isolates were the fast growing type. A significant proportion (73%) of the isolates (both fast and slow growing) tolerated acidic conditions which seems to confirm the reports of Keyser *et al.* (1979); Lowendorf, (1980) and Zablotowicz and Focht, (1981), that cowpea rhizobia are acid-tolerant. This acid tolerance may be an indication of adaptation to acid environments, although in the present study, only two of the isolates were isolated from soils with pH of less than 4.5. The mechanism for rhizobial tolerance to low pH is not well understood, even though plasmids (Chen *et al.*, 1993) and extracellular polysaccharide slime (Cunningham and Munns, 1984) have been implicated. Graham *et al.*, (1994) reported that acid tolerance was not an adaptive response nor was it plasmid mediated nor was it correlated with extracellular polysaccharide slime production, or related to synthesis of polyamines. Graham *et al*, (1994) suggested that pH tolerance may be

associated with outer membrane composition and structure after observing that strain UMR 1899 cells accumulated glutamate under acid stress and became more hydrophobic. This adaptive response according to Tiwari *et al.* (1996), is induced by an acid protection system controlled by different pH regulated genes, which cause increase resistance to acid stress. The growth exhibited by some of the isolates in laboratory media at low and high pH suggest the versatility of these indigenous isolates to survive under different soil conditions.

High salt tolerance was also found among the isolates. This contrasts reports of low and narrow salt tolerance ranges observed among rhizobial populations from other regions such as Nigeria, where cowpea isolates failed to grow at 2% NaCl (Eaglesham *et al.*, 1987). The results, however, are in agreement with that of Mpepereki *et al.* (1997), who found that the majority of both fast and slow growing indigenous cowpea rhizobia from Zimbabwe tolerated 5.5% NaCl. None of the soils used in this study was sampled from a saline environment. The observed NaCl tolerance of the isolates could therefore be an indication of intrinsic resistance to high osmotic stress.

Fast growing rhizobia are reported to be able to utilize as sole carbon sources certain carbon compounds (Elkan, 1992). However, no nutritional diversity differences were found between the fast and slow growing isolates in this study.

The legume *Rhizobium* symbiosis exhibits widely differing degrees of specificity. In some instances, the symbiosis is highly specific in that a particular *Rhizobium* species can form a symbiotic association with only one particular legume species. This was evident in the results

obtained. Fourteen percent of the isolates failed to nodulate any of the other eight host legumes except their homologous host, cowpea. Isolate 8 was also the only isolate among the 100 isolates that nodulated *Mimosa* spp. The fact that *Mimosa* was nodulated by only one out of the 100 isolates is an indication that *Mimosa* spp probably associates with a highly specific sub-group of the indigenous rhizobial population. There are also intermediate cases where varying degrees of cross inoculation capabilities are exhibited as demonstrated in the results obtained. At the opposite extreme are those with broad host range, in which a diversity of legumes may be infected by one or more of several rhizobia. In this study however, none of the isolates could nodulate all the tested legumes.

The high cross inoculation affinity of soybean with cowpea isolates, suggested by Norris (1965) and Christian *et al.* (1997), was confirmed with the present results. This is in contrast with the results obtained by Bromfield and Roughley (1980), and Eaglesham (1985). Also 75% of the cowpea isolates tested nodulated groundnut. This observation is at variance with the results of Habish and Khari (1968), and Doku (1969), who reported that groundnut was not nodulated by isolates from cowpea nodules. Five out of the eight legume hosts *Mimosa*, *Leucaena*, *Crotalaria, Pueraria, Calopogonium* tested were on the other hand nodulated by less than 40% of the cowpea bradyrhizobia isolates tested. This results disagrees with the assumption made by Dobereiner (1978) and Halliday (1985), that tropical legumes are non-selective in the rhizobial types they require for effective symbiosis and emphasised that more work is needed on diversity of rhizobia isolates from tropical soils.

The indirect ELISA method was used to determine the serogrouping of the isolates. The initial step in the determination involved testing for ability of the isolates to induce antibody production in mice. Results obtained showed that immunization of mice with rhizobial antigens induced species specific antibodies. The results of the serological work indicated that only a small fraction (8.5%) of isolates tested reacted strongly with antisera of each other and therefore closely related. These isolates could be classified as belonging to the same serogroup. The remaining 91.5% showed differential relatedness or did not share detectable antigenic determinants. The fact that only 8.5% of the isolates were closely related suggests that the isolates are highly diverse.

Early reports by Jordan (1982), indicated that the micro symbionts of cowpea were typically slow growing bacteria with the characteristics of *Bradyrhizobium* species. Later studies however, have suggested fast growers as typical symbionts Nevertheless a high predominance of *Bradyrhizobium* species has generally been observed. The previous descriptions were based solely on growth features and the cross inoculation concept and thus did not provide precise information about the real nature and structure on the rhizobial population. Considering the broad range of specificities either of rhizobial species towards their host or of the legume species towards their symbionts, molecular identification has become a prerequisite to any study of rhizobial population structure (Lafay and Burdon, 1998). In this study the 16S rRNA gene of the isolates were analysed in order to characterise the isolates. Four tetrameric restriction enzymes (Dde*I*, Hae*III*, Msp*I* and Rsa*I*) were used to determine similarities among the isolates. The choice of the enzymes was based on the results of Laguerre *et al.* (1994) and a recent computer based study (Moyer *et al.*, 1996), which evaluated the efficacy of selected tetrameric

restriction enzymes for rDNA-RFLP analysis of rhizobial isolates. Results obtained using dendrogrames constructed from the similarity matrix of the isolates by the method of Nei and Li (1979) indicated that diversity of 100 isolates was high, suggesting the presence of several yet unidentified rhizobial strains in our soils.

The high genotypic diversity revealed by the PCR-RFLP analysis in this study was in good agreement with the great diversity based on serotyping and host range analyses. However, isolates that had identical rDNA genotypes did not display similar phenotypic characteristics. This illustrates lack of correlation between phenotypic and genotypic based methods for grouping *Bradyrhizobium* strains. Similar observations were made by So *et al.* (1994) and van Rossaum *et al.* (1995). Several reasons may account for this observation. For instance, it was observed in this study that the patterns of serological response to isolates used as immunogens was not always reciprocal. Also the indirect ELISA techniques used in this study is dependent on interaction between an antibody and an antigen, which is attached to a solid phase (microtitre plate) by passive adsorption. Results may therefore be biased depending on the type of microtitre used. During a trial in this study, it was found that ELISA plates type Dynatech and sumilon C, gave different optical density results. Furthermore, grouping of isolates is based on a range of absorbance values, which may fail to detect widespread differences among the isolates. The cross inoculation grouping method may also be influenced by the number of test legumes used and also, different rhizobial cell number may be required to initiate nodules on different legumes. Age of cell cultures and different batches of PCR reagents were also observed in this study to affect the reproducibility of PCR results. Further studies may therefore

be necessary to assess the use or otherwise of these methods for grouping bradyrhizobial isolates.

### 5.3 Symbiotic effectiveness

Symbiotic effectiveness of indigenous rhizobial population is an important parameter for the selection of strains for inoculant production. It is also a primary factor for the determination of incidence and magnitude of legume response to inoculation (Singleton and Tavares, 1986, Thies et al., 1991). Twenty six percent (26%) of the isolates tested showed high nitrogen fixing capabilities, which were comparable to plants fertilized with nitrogen. This implies the presence of potential indigenous rhizobia that can be used for inoculant production. The pattern of effectiveness of the isolates followed a normal poisson distribution, with most (68%) of the isolates being moderately effective, few of the high (26%) or low (6%) effectiveness. This is in agreement with the results of Thies *et al.*, (1991), who showed that the effectiveness patterns of indigenous rhizobia on cowpea followed a normal distribution curve. The results indicate however, that cowpea in the various soils used in the study are nodulated primarily by rhizobia that exhibited suboptimal symbiotic effectiveness.

The wide range of effectiveness obtained and the fact that some of the isolates showed superiority in symbiotic effectiveness relative to the standard strain TAL 169 give the indication that a potential exits for developing legume inoculants from indigenous cowpea *Bradyrhizobium* strains. Obviously much more information about the indigenous rhizobial population is needed. Nevertheless, these native isolates, highly adapted to the environmental conditions of these soils may be a useful source of strains to resolve practical problems in field inoculation of cowpea.

The results are in agreement with those obtained in other countries (Chatel and Parker, 1973; Chatel *et al.*, 1973). Fredericks *et al.* (1990), working with isolates from native Ethiopia clover species found significant differences among the rhizobial strains, which as a group, showed higher rates of nitrogen fixation than commercial Nitragin inoculant. The results also showed that the soil type did not influence the distribution of isolates in terms of effectiveness in nodule formation. Rather the results pointed to the heterogeneity of the native clover rhizobia isolates in symbiotic effectiveness. The fact that the fast growing isolates were as effective in fixing nitrogen as the slow growers on cowpea indicates that the fast growing isolates are not a relic association.

### 5.4 Competition for nodule occupancy

The abilities of some rhizobial strains to occupy greater percentages of nodules on the legume than other competing strains has been well established (Dowling and Broughton, 1986; Triplett and Sadowsky, 1992). What is most uncertain is sometimes, the reliability and ease of the methods used. The results of the present competition studies suggest that the β-Glucuronidase (GUS) marking technique is a potential tool for ecological studies of rhizobia, as it was able to distinguish between differences in the competitive abilities of different cowpea bradyrhizobia isolates.

Interstrain variation in nodulating ability was demonstrated by high differences in the percentage of nodule attributable to the different competing isolates that were used in this study. Although the genetic make up of a *Rhizobium* strain has been found to influence its competitive success (Danso and Owiredu, 1988), nodulation success by many strains on the other hand is

influenced by the relative number of the strains in the mixture of strains (Amarger and Lobreau, 1982; Owiredu and Danso, 1989; Beattie *et al.*, 1989; George and Robert, 1992). In this case, relative nodule occupancy can change if the ratio of strain is changed (Furhmann and Wollum, 1989; George and Robert, 1992). When the ratios of the competing isolates were changed in the inoculum mixture in this study, we observed that the percentages of nodules formed by the individual isolates also changed by about 50% in most cases, especially the ineffective isolates.

The depression of plant yield associated with isolate 28 is interesting but not strange. Similar results have been reported in a study in Uruguay by Labandera and Vincent (1975). Such traits are by themselves sometimes useful indicators of success of competing strains of contrasting effectiveness and could provide insight into how and when some ineffective strains might exert a negative effect on legume growth in the field, including the influence of an effective inoculant strain. Other studies on clover plants by Demezas and Broughton (1986), showed that subclover plants grew poorly when less than half of the nodules were occupied by the suboptimally effective strain, WS2-01, and with more than 50% of the nodules on the same plants occupied by strain WS1-01 which exhibited superior effectiveness. There have also been reports of strains on *B. japonicum* (Teaney and Furhrmann, 1992) and *R. tropici* (O'Connel *et al.*, 1990) that exert negative effects on legume growth. What accounted for the plant growth suppressive effect of isolate 28 and perhaps of similar strains in other studies need further studies. In the case of a strain of *R. leguminosarum* bv *trifolii*, Triplett and Bartha (1987) reported that this strain inhibited the growth and nodulating ability of other strains of the same biovar it competed with. If this should similarly apply in this study, then this would possibly explain why the detrimental influence of isolate 28 diminished when its inoculation was delayed relative to the effective

isolates. The stimulation of plant growth by isolate, 94 is not only a contrast to that of isolate 28, but makes studies on microbial (rhizobial) interaction more interesting and more difficult. Similar symbiotic and plant growth enhancement by ineffective rhizobial strain has been reported (Wardisirisuk *et al.*, 1989), but no detailed and convincing evidence as to the underlying reason were given. Whether these beneficial changes have genetic basis, for example, the transfer of plasmids between such rhizobia in the rhizosphere (Broughton *et al.*, 1987, Sullivan *et al.*, 1995) would need to be investigated. On a practical although on a somewhat cautious note, the results obtained would suggest that it should be possible to formulate more effective inoculants containing selected ineffective and effective strains. Cautious, because unless well documented in several test and the potential consequence have been well established, the presence and persistence of an otherwise symbiotically incapable bradyrhizobial strain could in the long run, prove disastrous. Nevertheless, the science behind this is worth pursuing.

Many reasons could account for a strain being highly competitive. One that has been shown in this study and discussed above is, the relative abundance of the different strains in soil or at the nodulation site. Another one that has been examined is the speed of infection. However, the result of the time course studies even though revealed differences in nodulation with regards to speed of formation of the first nodule, these were not related to competitiveness in nodule fomation. For instance, although isolate 72 was the slowest in forming the first nodule, it was more competitive than all the isolates except 28.

### 5.5 Inoculation response of cowpea

Maximum nitrogen fixation in a legume requires that the legume be adequately nodulated. Where nodulation is poor and scanty, rhizobial inoculation is necessary to ensure optimum nitrogen fixation. Results obtained in the inoculation experiment in soils in pots showed that a good percentage of the nodules formed on cowpea plants were attributed to the inoculated isolates. None of the inoculated isolates showed a consistent trend in its influence on nodulation, shoot dry matter yield or percent nitrogen of the plants. This suggests that soil characteristics may have influenced the performance of the isolates. The dominance of the mixed inoculant in attaining the highest number of nodules, the highest shoot dry matter yield and the highest total nitrogen content of the inoculated plants, may be explained by the synergistic effect of the three isolates that make up the mixed inocula, which together were probably more competitive and effective than when they were inoculated as single isolates. The advantage of using mixed inocula over single inocula had earlier been indicated by Owiredu and Danso (1988), and its outcome may be due to the interaction among them.

Values obtained for nodule numbers, shoot dry weight and percent nitrogen in Ankasa soil were consistently lower than those obtained in the other three soils. The persistence of the difference suggests that some property of Ankasa soil had a depressing effect on nodulation and nitrogen fixation of the isolates. The observation has been made that the number of nodules formed on plants and the effectiveness of *Rhizobium* strain is affected by soil pH (Keyser *et al.*, 1979). It has also been proved that not only infection of the host plant is adversely affected by acidity (Lie, 1971), but also the functioning of the established nodule can be inhibited (Munns, 1970). These facts may explain why lower values of nodule numbers, shoot dry weight and percent

nitrogen were obtained in Ankasa soil, which has a pH of 4.0. The results outlined in Figs. 4.24 -4.26 and Tables 4.13 and 4.14 showed that there was no significant difference between the uninoculated control and the inoculate treatments in Akuse and Bekwai soils. This may suggest that the native bradyrhizobia isolates in these soils may be efficient in fixing nitrogen or that the native strains outcompeted the inoculated strains in nodule formation. This as well as any underlying factors need to be studied.

The method of inoculation has been shown to influence nodulation parameters of legumes (Kamicker and Brill, 1987; Danso and Owiredu, 1988; Hardarson *et al.*, 1989). Results obtained in this study indicate that soil was superior to seed inoculation. This might probably be due to the uniform distribution of rhizobial cells in the whole soil in the case of the soil inoculation method. Consequently, the chance of a growing root and a rhizobial cell getting into contact was increased as opposed to when the inoculation was localised on the seed. Similar observations were made on soybean by Danso and Bowen (1989), Hardason *et al.* (1989) and Danso *et al.* (1990). Practical ways of inoculating rhizobia directly into the bulk of the soil may therefore enhance nodulation response and nitrogen fixation, especially where native strains abound.

Several reports have shown that because cowpea *Bradyrhizobium* strains abound in most soils of the major cowpea growing areas (Doku, 1969; Sellschop, 1962), cowpea rarely responds to bradyrhizobial inoculation (Doku, 1969; Rhodes and Nangju, 1979). Even though substantial quantities of nodules were observed on the uninoculated plants, which gives a good reflection of the number of indigenous cowpea bradyrhizobia present in the different soils, the results

obtained in Lima and Ankasa soils contrast the postulate of no response to inoculation in tropical soils. Rather the results indicated that response to inoculation in the presence of native rhizobia can occur in some soils if not all. The type of inoculant strain used, the variety of cowpea used and the type of soil may all influence the results that may be obtained.

# CHAPTER SIX

## 6.0 CONCLUSION

The primary aim of this work was to study the characteristics of the indigenous bradyrhizobia population in relation to cowpea, in order to enhance its nitrogen fixation and yield. A preliminary study was done to assess the potential to improve nitrogen fixation by cowpea. Following this, 100 indigenous bradyrhizobia isolates were obtained from nodules of cowpea grown in 20 different soils sampled from five major ecological zones in Ghana. The diversity of the isolates was examined using phenotypic, serological and molecular characters. To understand the potential to improve nitrogen fixation of a legume, the ranges of effectiveness of the indigenous rhizobia is also required. Consequently the symbiotic effectiveness of the 100 isolates was determined. Low symbiotic nitrogen fixation is in many cases a result of the superior competitive abilities of ineffective over effective rhizobia for nodule occupancy (May and Bohlool, 1983). Understanding the interaction between the effective and ineffective rhizobial isolates so as to bias competition in favour of the more effective ones is therefore essential for improving symbiotic efficiency. Competitive abilities of selected isolates of known effectiveness were therefore assessed. Exotic rhizobia inoculants have been considered as aliens and less successful (Brockwell, 1981). The strains most competitive in nodule formation and persistent in a particular environment are often those isolated from similar environments (Chatel and Parker, 1973). This hypothesis was also tested using selected isolates in selected soils.

Results of the natural nodulation of cowpea indicated that large variations in nodulation exist in cowpea. Such variations are not uncommon in legume species, but have practical implications. In this study, for instance, cowpea cultivar Asontem was found to be the most prolific nodulator which makes it a favourable candidate for breeding purposes. The fact that none of the 20 soils used could support nodulation of all the 45 cowpea cultivars suggests the paucity of the soil resident rhizobia that were compatible with all cowpea cultivars. The nodulation in each soil therefore alludes to the nature of the rhizobial composition in affinities of the cultivars that the soil supports. The pattern of nodulation reflected the varying sizes of indigenous bradyrhizobial populations in the different soils. Inadequate infectivity or efficacy of the indigenous bradyrhizobia isolates was demonstrated by the positive response of cultivars to nitrogen fertilization which together with the variability in nodulation and population sizes of indigenous bradyrhizobia indicate a potential for improvement by use of bradyrhizobial inoculants.

Although cowpea is generally thought to symbiose with slow-growing rhizobia, it was clear from the investigations of the phenotypic characteristics of the isolates that both fast and slow growing rhizobia were represented in nodules of cowpea. This provides evidence to dismiss the myth that all cowpea rhizobia are slow growing. The results also provide a contribution towards dismissing the view about the exaggerated nodulation promiscuity of tropical rhizobia. It is possible that subgroups of rhizobia exist based on symbiotic specificity. The diversity of the isolates revealed by the phenotypic characteristics was further supported by results of PCR analysis that target specific chromosomal loci of the 16S rRNA gene. Twenty distinct genotypic groups were found according to the RFLP analysis of the 16S rRNA gene. Thus one intriguing observation made in this thesis is that irrespective of the method employed to analyse for

diversity the results showed great diversity among the indigenous bradyrhizobia isolates, even though there was lack of correlation among the methods. This points to the possible existence of several unique and as yet unidentified species of *Bradyrhizobium* strains in our soils. The presence of large diversity of native bradyrhizobial strains may suggest the need for diverse bradyrhizobia strains as inoculants for successful inoculation of cowpea. Again, the presence of large diversity of bradyrhizobial strains may contribute to the negative response of cowpea to inoculation observed in some soils in the tropics

Even though the soils studied with the exception of those from the high rainforest region contain sufficient numbers of indigenous rhizobia that could nodulate and efficiently fix nitrogen in cowpea, determination of the symbiotic effectiveness of the isolates showed that the majority (68%) were moderately effective. Interestingly, the proportion contained both fast- and slow-growing isolates which were somewhat evenly distributed in all the soils.

The utilization of the GUS A marker technique to assess competitiveness between effective and ineffective isolates for nodule occupancy resulted in a high differentiation of isolates as all the nodules on each plant were analysed for nodule occupancy. The results showed that the insertion of the GUS A marker gene did not affect the nodulation behaviour of the marked isolates making the technique highly suitable for the study of rhizobial competition. Competitive ability of rhizobia strains as observed in this study was not linked to effectiveness of isolates. An interesting feature about interaction between effective and ineffective isolates as revealed in this study was that such an interaction could lead to either depressed or enhanced

plant yield which suggests the possibility of formulating mixed inoculants containing such selective strain combinations.

Response of cowpea to inoculation has been a controversial issue for some time now. Lack of a positive response is attributed to the presence of large population of indigenous rhizobia. However, other causes such as (1) the unnecessary application of inoculants on cultivars capable of nodulating effectively with indigenous strains, (2) the use of wrong strains in the inoculants, (3) diversity of the indigenous rhizobia and (4) an unknown factor limiting full expression of symbiosis may be responsible. The present study has shown that cowpea responds positively to inoculation in some soils. Further work will allow identification of cultivars requiring inoculation or not in certain areas and to evaluate the importance of the environment on the performance of particular cultivar/rhizobia strain combinations. Meanwhile, as revealed in this study, the use of selected indigenous rhizobia isolates for the preparation of inoculants may be considered a better option.

It is clear, from the foregoing discussion, that to ensure that $N_2$ fixation by cowpea is enhanced, several factors have to be considered. These may include the selection of efficient $N_2$-fixing cultivars and a comprehensive knowledge of the diversity of the indigenous rhizobia particularly their effectiveness and competitiveness. Inoculant strains have often been recommended based on good symbiotic performance in a particular environment, while the soil status or the agro-ecological zone of the final application has not been considered. The soils of the tropics contain a vast genetic pool of indigenous rhizobia which have not yet been identified. Such strains and species can provide a variety of inoculant strains that may show better performance. Correlation

between the size of the indigenous rhizobia population and performance of inoculant strain has been established (Thies *et al.*, 1991). However, the effect of the diversity of indigenous rhizobia on the performance of an inoculant strain has not been determined. Probably different strategies are required in order to out-compete the well-adapted indigenous isolates for nodule occupancy.

Cowpea though has an essential role to play in the improvement of tropical agriculture; it seems that various aspects essential for efficient nitrogen fixation of cowpea are presently not linked sufficiently. Enhanced nitrogen fixation and high yield of cowpea could be realised if a database containing data on soil properties, environmental conditions, rhizobial diversity and population size, as well as the competitive ability and effectiveness of rhizobial strains in combination with particular cultivars existed

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## APPENDIX

Composition and preparation of media used

1. Yeast Extract mannitol agar (for the isolation of rhizobia)

| | |
|---|---|
| Mannitol | 10.0 g |
| Dipotassium phosphate | 0.5 g |
| Magnesium sulphate | 0.2 g |
| Sodium chloride | 0.1 g |
| Yeast extract | 0.5 g |
| Agar | 15 g |
| Distilled water | 1000 ml. |

2. PBS. (Phosphate Buffered Saline)

| | |
|---|---|
| Nacl | 8.0 g |
| $Na_2HPO_412H_2O$ | 2.7g |
| $Na.H_2PO_4$ | 0.4g |
| Distilled water | 1000ml |
| pH | 7.2-7.4 |

3. Running buffer (10 x TBE)

| | |
|---|---|
| Tris base | 108g |
| Boric acid | 55g |
| 0.5M EDTA | 40ml (pH 8.0) |

4. Loading Buffer

| | |
|---|---|
| Bromophenol blue | 0.25% |
| Sucrose (w/v) | 40% |

5. LB Medium

| | |
|---|---|
| Bacto-tryptone | 10g |
| Yeast extract | 5g |
| Sodium chloride | 5g |

6 N-free Nutrient solution

| **Stock solution** | **Form** | |
|---|---|---|
| 1 | $CaCl_2.2H_2O$ | 294.1g/l |
| 2 | $KH_2PO_4$ | 135.1g/l |
| 3 | Fe-citrate | 6.7g/l |
| | $MgSO_4.7H_2O$ | 123.3g/l |
| | $K_2SO_4$ | 87.0g/l |
| | $MnSO_4.H_2O$ | 0.338g/l |
| 4 | $H_3BO_3$ | 0.247g/l |
| | $ZnSO_4.7H_2O$ | 0.288g/l |
| | $CuSO_4.5H_2O$ | 0.100g/l |
| | $CoSO_4.7H_2O$ | 0.056g/l |
| | $Na_2MoO_2.2H_2O$ | 0.048g/l |

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