ISOLATION AND CHARACTERISATION OF INDIGENOUS RHIZOBIA AND RESPONSE TO INOCULATION BY PROMISCUOUS SOYBEAN IN THE NIGERIAN SAVANNA

BY

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BY

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APRIL, 2015

DECLARATION

I declare that the work in this thesis entitled 'Isolation and characterization of indigenous rhizobia and response to inoculation by promiscuous soybean in the Nigerian savanna' has been carried out by me in the Department of Soil Science. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

Abubakar, Jibrin Fatima

_____	_____	_____
Name of student	Signature	Date

CERTIFICATION

This thesis entitled 'ISOLATION AND CHARACTERIZATION OF INDIGENOUS RHIZOBIA AND RESPONSE TO INOCULATION BY PROMISCUOUS SOYBEAN IN THE NIGERIAN SAVANNA' by Fatima Jibrin ABUBAKAR meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello university, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To ALLAH, the Almighty, who gave me the grace to pursue this research.

ACKNOWLEDGEMENT

My sincere and profound gratitude goes to Dr. A.A. Yusuf, Chairman of my supervisory team who despite his tight schedule, graciously supervised this work out of his wealth of experience. To members of my supervisory team, Prof. I.Y. Amapu and Dr. W.B. Malgwi, your contributions towards making this work a success is highly appreciated.

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while working, I pray that Allah grants you your hearts' desires and bless you with His infinite Mercy.

ABSTRACT

Laboratory and screenhouse experiments were conducted to isolate and characterize rhizobia from soils collected from the northern Guinea, southern Guinea and Sudan savannas of Nigeria and to assess response of promiscuous soybean, TGx1448-2E to inoculation in the same soils. Bulk top soils were collected from 45 sites spread in 15 local government areas of Kaduna and Kano states. The treatments used in the screenhouse pot experiment were uninoculated control, mineral nitrogen and Legumefix, a commercial rhizobia inoculant. The treatments were arranged in Randomized Complete Block Design (RCBD) and replicated three times. Nodulation and biomass were assessed at eight weeks after planting. Response to inoculation and relative efficiency were also calculated. Nodule number and fresh weight were higher in inoculated plants than those treated with mineral nitrogen (N) across all the agro-ecological zones (AEZs). Generally, mineral N gave the highest dry matter yield (DMY) than the uninoculated control. Only about 40% of inoculated plants had DMY similar to mineral N while the remaining fall within the same range as the control. The disparity between mineral N and the other treatments provides opportunity to evaluate more rhizobia strains or inoculants that can improve the performance of soybean in the study sites. The results showed that response to inoculation was higher in the Sudan savanna than in the northern Guinea savanna. These were observed in 12 out of 21 and 9 out of 19 sites in the Sudan savanna and northern Guinea savanna respectively. Of these, inoculation response reached 62 and 90% in two sites only in the Sudan savanna while it barely reached 20% in the remaining sites. The results also showed a relative efficiency of greater than 100% in some locations which implies that the indigenous rhizobia populations were superior to mineral N and Legumefix inoculant. Notable among the locations were Albasu 1, Garko 1, Soba 4, and Kolosok which spread across all the agro-ecological zones. Strains from

such locations have high potential for use as inoculants. A total of 350 strains of rhizobia were isolated following standard procedures. The strains were assigned culture collection numbers SAMFIX 1-350 and characterized based on their colony morphology which included: colour, shape, size, elevation, margin, texture and opacity. The growth rate was also assessed and 94% were found to be fast growers while 6% were intermediate growers. A total of 20 strains were characterized based on their physiological properties which included, pH, temperature and salt tolerance.. Four strains were found to grow well at pH 4.0, fourteen of the strains grew weakly while two strains did not grow at all. At pH 8.5, eighteen of the strains grew weakly while two did not grow at all. For the temperature, seven of the strains were able to grow well at 8^{o}C, eleven strains grew weakly while two strains did not grow at all. At 35^{o}C only two strains grew well, twelve strains grew weakly while six strains did not grow at all. Also as the concentration of NaCl increased from 1% to 3%, some strains were still able to grow while others did not. However, three strains did not grow even at 1% NaCl. The results show the diversity of rhizobia in the three agro-ecological zones and also their potential for use as inoculant based on their tolerance to extreme pH, temperature and salinity.

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CHAPTER ONE

1.0. INTRODUCTION

Rhizobia are generally referred to as bacteria belonging to the genera *Rhizobium* or *Bradyrhizobium*. Other genera include *Sinorhizobium, Mesorhizobium, Allorhizobium* and *Azorhizobium*. They are classically defined as soil bacteria, which are gram-negative, aerobic, rod-shaped, chemo-organotrophic, non-spore forming and fix atmospheric nitrogen symbiotically. Rhizobia are usually motile, and have bipolar, subpolar or peritrichous flagella.

The soybean plant is capable of symbiotic relationship with different bacterial symbionts, including *Bradyrhizobium japonicum* (Jordan, 1982), *Bradyrhizobium elkanii* (Kuykendall *et. al.,* 1992), *Bradyrhizobium liaoningense* (Xu *et al.,* 1995) and *Sinorhizobium fredii* (Chen *et al.,* 1988). As a result, it can contribute to the N economy of nutrient-poor soils.

Soybean has been variously described as a "miracle bean" or a "golden bean" because it is a cheap, protein-rich grain. It contains 40% high quality protein, 20% edible vegetable oil, and a good balance of amino acids (Singh, *et. al.,* 1987; Weingartner, 1987) and has, therefore, tremendous potential to improve the nutritional status and welfare of the families of resource-poor farmers. It is used as livestock feed, and has numerous industrial purposes (Myaka *et al*, 2005). The 40 percent protein content of soybeans is approximately twice that of other legumes (Greenberg and Hartung, 1998). Since the 1960s, the plant has been the dominant oilseed (Smith and Huyser, 1987).

It is estimated that rhizobia can fix about 50 to 300 kg N ha^{-1} (Bokhtiar and Sakurai, 2005). Thus, their contribution to the N economy of the soil can be quite substantial. Soybean has the capability for soil fertility improvement in cereal based cropping systems in the Guinea savanna

(Yusuf *et. al.,* 2006), as such its cultivation can fix as much as 100 kilograms of nitrogen per hectare (Sanginga *et. al.,* 2003).

In the early 1980s, it was assumed that most tropical countries did not have the facilities and personnel required for inoculum production, storage, and distribution and were dependent upon importation of the final product (Pulver *et al.,* 1982). In order to overcome the difficulties associated with inoculation as experienced by African farmers, the International Institute of Tropical Agriculture (IITA) in Nigeria developed promiscuous soybean varieties (the so-called TGx or Tropical *Glycine* cross) that nodulate effectively with indigenous *Bradyrhizobium* spp. (Abaidoo *et al.,* 2007). Although 'traditionally-bred' soybean varieties do not contribute to the soil N status because most of the N fixed is removed by harvesting the grains, the 'dual-purpose', promiscuous soybean varieties developed by the International Institute of Tropical Agriculture (IITA) between the mid- 70s and early 90s produce a substantial amount of grain and leafy biomass, resulting in a relatively low N harvest index (Sanginga *et al.,* 2003). The promiscuous nature allows these varieties to nodulate freely with the indigenous *Bradyrhizobium* spp. population, avoiding inoculation, a technique that has often failed in sub-Saharan Africa (Mpepereki *et al.,* 2000). TGx soybean genotypes in some locations, however, are reported to develop nitrogen deficiency symptoms (Okereke and Eaglesham, 1993), suggesting that the TGx soybean may require inoculation with bradyrhizobia at some locations if high soybean yields are to be obtained.

Sanginga *et al.* (1999) found that need for inoculation for some elite promiscuous soybean breeding lines depended on effectiveness of indigenous Bradyrhizobia in a given locality. Okereke and Eaglesham (1993) reported an increase in nodulation and N_2 fixation of promiscuous soybean cultivars using a commercial *Bradyrhizobium* inoculant. Sanginga *et al.*

(1997) showed that inoculation increased total N, N_2 fixation and grain yield of early maturing promiscuous cultivars of soybean but did not affect late maturing varieties. Recently, Wasike *et al.* (2009) reported nodulation of promiscuous soybean varieties by indigenous rhizobia in Western and Eastern Kenya. Therefore to better understand the degree to which bradyrhizobia populations in Africa nodulate TGx soybean, it is essential to characterize these *Bradyrhizobium* spp. populations.

1.1 Statement of the Problem

Often, the *Rhizobium* population in the soil is too low or the strain of the *Rhizobium* species present is not effective even in areas where soybean has been cultivated for several years (Brady and Weil, 2008). Therefore, effective and competitive strains of rhizobia populations need to be evaluated for use as inoculants. Recent studies with these promiscuous soybean varieties have shown considerable variability in the effectiveness and population of communities of indigenous Bradyrhizobia in a given location (Sanginga *et al.,* 1999; Fening and Danso, 2002). Sanginga *et al.* (1995) also found a direct relationship between Bradyrhizobia cell counts and promiscuous soybean response. Thus, promiscuous soybean may also need inoculation with exotic bradyrhizobia depending on effectiveness and population of indigenous bradyrhizobia in the locality (Okereke *et al.,* 2000), as well as the degree of promiscuity of variety (Sanginga *et al.,* 1999).

Nitrogenous fertilizers are subject to losses due to denitrification leading to release of N_2 and NO_2 gases, volatilization of ammonia, run-off and leaching which can contaminate surface and underground water. Under tropical conditions, the efficiency of applied nutrient has been estimated to be less than 50% N, 10-30% for P and about 40% for K (Baligar and Bennett,

1986). For instance, Subbiah *et al*. (1985) reported that when 120 kg N ha^{-1} (contained in N fertilizer compounds) was applied to maize in a single dressing, only 21% could be recovered from soils. High rates of N fertilization especially when using ammonium sulphate have also been shown to acidify soils (Stumpe and Vlek, 1991). In addition to environmental problems, farmers in the Nigerian moist savanna (NMS) have to contend with the rising costs of N fertilizer, the availability of which is often erratic. Therefore, technologies that will reduce N fertilizer input by resource-poor farmers in the NMS are urgently needed. Nitrogen input through biological N_2 fixation (BNF) by grain legumes such as soybean can help to maintain soil N reserves as well as substitute for N fertilizer requirement for large crop yields (Peoples and Craswell, 1992). However, even where yield responses are not evident, inoculation may still have benefits by increasing seed N levels and N levels in plant residues (McKenzie *et al.*, 2001).

1.2 Justification

The effect of biological nitrogen fixation (BNF) vary depending on survival of the rhizobial strains under different soil conditions like salinity, drought, acidity, soil temperature (Zaharan, 1999). It was reported by O'Hara *et al.* (2002) that the abundance of diversity in the soil populations of rhizobia provides a large resource of natural germplasm to screen for desired characteristics present in the natural pool. This requires rigorous screening for efficient rhizobial strains with adaptation to different soil conditions (Zaharan, 1999). To achieve this, indigenous rhizobial strains need to be characterized under different conditions in the laboratory and then later tested in the field for their effectiveness. In addition, there is a need to improve crop productivity in an eco-friendly manner and this will lead to the promotion of commercial biological products intended to restore or enhance the fertility and organic matter content of soils

(Laditi *et al*., 2012). Increasing and extending the role of biofertilizers such as *Rhizobium* inoculants would reduce the need for chemical fertilizers and decrease adverse environmental effects. Therefore, in the development and implementation of sustainable agriculture techniques, biofertilization is of great importance in alleviating environmental pollution and the deterioration of nature (Elkoca *et al.*, 2008).

1.3 Null Hypotheses

- Response of promiscuous soybean to inoculant may vary among the different agro-ecological zones.
- Rhizobium isolates are variable in colony morphology and tolerance to environmental stresses which include salinity, soil pH and temperature variations.

1.4 Aim and Objectives

The goal of this work is to create and develop a collection of indigenous rhizobia strains that would improve biological nitrogen fixation and soybean yield in the Nigerian savanna. The specific objectives are:

- To determine response to inoculation by promiscuous soybean in the Nigerian savanna
- To characterize the isolates based on morphological and physiological properties.
- To create and develop a culture collection of rhizobia nodulating promiscuous soybean in soils of the Nigerian savanna.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Development of Improved Soybean Varieties and Utilization Technologies in Nigeria

Soybean may have been introduced to Nigeria as early as 1908, but its cultivation as a crop can be attributed to the introduction of the Malayan variety in 1937 by British colonial officers in Benue State (Singh *et al.,* 1987). Until recently, the Malayan variety was virtually the sole variety grown by farmers. This variety is low yielding, susceptible to bacterial diseases and is late maturing (Smith *et al.* 1995). The latter characteristic exposes soybean to pod shattering due to the desiccating action of the seasonal Harmattan wind. The expansion of the crop was limited by the lack of suitable varieties. It was reported by Dashiell *et al.* (1987) that most soybean varieties could not nodulate in association with the native rhizobia indigenous to African soils and the seed quickly lost viability, which made it difficult for farmers to store it until the next cropping season (Sanginga *et al.,* 1999).

2.2. Soybean Production in Nigeria

The total national output of soybean between year 1999 and 2005 was about 265,589,000 tonnes. (AMREC, 2007). Annual production is estimated by the Ministry of Agriculture at around 550,000 tons.

2.2.1. Economic Importance of Soybean

Soybean is among the major industrial and food crops grown in every continent. The crop can be successfully grown in many states in Nigeria using low agricultural input. Soybean cultivation in

Nigeria has expanded as a result of its nutritive and economic importance and diverse domestic usage. It is also a prime source of vegetable oil in the international market. Soybean has an average protein content of 40% and is more protein-rich than any of the common vegetable or animal food sources found in Nigeria. Soybean seeds also contain about 20% oil on a dry matter basis, and this is 85% unsaturated and cholesterol-free (Dugje *et al.,* 2009).

The rapid growth in the poultry sector in the past five years has also increased demand for soybean meal in Nigeria. It is believed that soybean production will increase as more farmers become aware of the potential of the crop, not only for cash/food but also for soil fertility improvement and Striga control. The market for soybean in Nigeria is growing very fast with opportunities for improving the income of farmers. Currently, SALMA Oil Mills in Kano, Grand Cereals in Jos, ECWA Feeds in Jos, AFCOT Oil Seed Processors, Ngurore, Adamawa State, and PS Mandrides in Kano all process soybean. IITA along with partners has developed improved technologies for soybean production (Dugje *et al,* 2009).

2.2.2. Agronomic Requirements for Soybean Production

Soil and climatic requirements: Soybean growth is influenced by climate and soil characteristics. Soybean performs well in the southern and northern Guinea savannas of Nigeria where rainfall is more than 700 mm. However, short-duration varieties can thrive in the much drier Sudan savanna when sown early and with an even distribution of rainfall throughout the growing period. The time for planting soybean depends upon temperature and day length. Soybean is a short-day plant and flowers in response to shortening days. It can be grown on a wide range of soils with pH ranging from 4.5 to 8.5. Soybean should not be planted in sandy, gravelly, or shallow soils to avoid drought stress. It should not be grown in waterlogged soils or soils with surfaces that can crust, as this will lead to poor seedling emergence.

Planting date: Soybean produces well over a wide range of planting dates, if moisture is available. The recommended dates for planting soybean in different ecological zones in Nigeria are early June –early July for southern Guinea savanna, mid-June – early July for northern Guinea savanna and July, weeks 1–2 for Sudan savanna.

Plant density: Seed rate recommended is about 50–70 kg in order to obtain a population of 444,444 plants/ha for soybean varieties. Plant spacing for soybean is 3 to 4 seeds/hole at a spacing of 75 cm between rows and 10 cm between stands. For the early maturing varieties, a spacing of 50 cm between rows and 5 – 10 cm within rows is recommended because they respond better to narrow spacing than the late-maturing varieties.

Fertilizer requirement: A good fertilizer recommendation for soybean production depends on a good soil test. Under normal conditions, soybean as a legume should provide itself with nitrogen through biological nitrogen fixation. Until nodulation occurs, the soybean plant depends on soil nitrogen for growth. Phosphorus is often the most deficient nutrient; therefore, optimum phosphorous fertilizer is applied for good yield. Phosphorus is applied at the rate of 30 kg P/ha in the form of single super phosphate fertilizer (SUPA) (3 × 50 kg bags) in addition to 2½ × 50 kg bags of compound fertilizer NPK 15:15:15. Nitrogen and potassium fertilizers are needed only when there are obvious deficiencies. Incorporate the fertilizer into the soil at land preparation during harrowing and leveling the field (Dugje *et al.,* 2009).

Choice of variety: Soybean variety selection is based on maturity, yield potential, lodging, drought tolerance, and resistance to pests and diseases. The maturity period should be the first consideration when choosing a variety suited to your geographical zone. Table 2.1 shows the recommended soybean varieties for the Guinea savanna ecological zones in Nigeria. (Dugje *et al.,* 2009).

Table 2. 1 Recommended soybean varieties for Guinea savanna ecological zones in Nigeria.

Variety	Ecology	Characteristics	*Striga* control
TGx 1448-2E	Southern and Northern Guinea savannas	Medium maturing, high yield, low shattering, high oil content, excellent grain color	Good
TGx 1835-10E	Guinea savanna	Early maturing, rust Resistant, pustule resistant	Not known
TGx 1485-1D	Guinea savanna	Early maturing, pustule resistant,rust susceptible	Not known

N.B. Early and extra-early maturing varieties are strongly recommended in the Sudan savanna because of the low amount and duration of rainfall in the zone.

Source: Dugje *et al.*, 2009

2.2.3. Pests and Diseases of Soybean

Weeds and their control

Manual weed control: the first weeding is carried out at 2 weeks after planting and the second at 5 – 6 weeks after planting. Avoid weeding immediately after a rainfall as this would lead to transplanting the weeds. Poor hoe weeding or delay in weeding could cause significant reductions in soybean yields (Dugje *et al.*, 2009).

Chemical weed control: Herbicides, if used properly, are safe and effective in controlling weeds in soybean. The choice of herbicide, however, depends on the predominant weed species and the availability of the herbicide. Herbicides are available for pre-emergence or post-emergence weed control in soybean. If herbicide is applied at planting, one weeding may be required at 5–6 weeks after planting (Dugje *et al.*, 2009).

Insect pests and their control

Several different insects occur in soybean fields but few are normally of any economic importance, and the species that cause damage are usually not abundant enough to warrant control measures. In the vegetative stage, the crop is very tolerant of caterpillars but very susceptible to silverleaf whitefly attack (Dugje *et al.*, 2009).

Diseases and their control

Soybean diseases normally result in major yield losses in Nigeria. Some of the common diseases caused by fungi, bacteria, and viruses are mentioned below.

Fungal and bacterial diseases

Rust: Asian soybean rust, caused by *Phakopsora pachyrhizi,* is one of the most important foliar diseases in Nigeria. The infected leaves have small tan to dark brown or reddish brown lesions on which small raised pustules (or 'bumps') occur on the lower surface of the leaves. Pustules produce a large number of spores. Brown or rust-colored powder falls when severely infected leaves are tapped over a white paper or cloth. Severe infection leads to premature defoliation and yield losses up to 80%. The disease is of great economic importance in the derived savanna and southern Guinea savanna zones where rainfall and humidity are high (Dugje *et al.*, 2009).

Bacterial pustule: The disease is caused by *Xanthomonas axonopodis pv. glycines*. Symptoms appear as specks to large, irregular spots with raised light-colored pustules in the elevated centers of the spots on the lower surface. The elevated pustules sometimes have cracks in them. Later lesions join together and the dead areas tear away to give a ragged appearance to the leaves. Symptoms of rust and bacterial pustule sometimes appear similar (Dugje *et al.*, 2009).

Phytophthora seedling blight and root and stem rot: *Phytophthora sojae* causes seedling blight, and root and stem rot. Young seedlings that appear to be established turn off-color to yellow, wilt, and die. The stems of these plants may show a brown discoloration that begins at the soil line and extends up the stem. The brown, dead leaves remain attached to the plant, and the dead seedlings are obvious symptoms of the disease in the field. The root rot phase of the disease is rapidly becoming a very destructive disease in Nigeria. The Phytophthora fungus can kill plants at all stages of growth. Infected stands may survive but are less productive than healthy stands. Infection generally occurs in fields with poor drainage, but it can occur in

normally well-drained fields that are waterlogged for 7–14 days after irrigation or very heavy or prolonged rainfall (Dugje *et al.,* 2009).

Frogeye leaf spot: The fungus *Cercospora sojina* that survives in infected soybean residue and seeds causes this disease. Symptoms appear as brown, circular to irregular spots with narrow reddish brown margins on the leaf surfaces. The central areas of the spots turn ash gray to light brown. Sometimes lesions can develop on stems and pods from where mature seeds are infected. Infected seeds may show discoloration of the seed coat that ranges from small specks to large blotches of light to dark gray or brown.

To control these diseases, the following steps need to be considered:

• Plant resistant varieties. This is the best option to control disease.

• Plant in a good seedbed. Avoid poorly drained or compacted soil.

• Plant seeds treated with fungicides as mentioned earlier under 'seed dressing'.

• Rotate crops with maize to prevent the increase in inoculum levels in a field.

• Use of a foliar fungicide is seldom warranted, except on high-value fields (e.g., seed production fields) or in years when the weather is especially favorable for disease development (Dugje *et al.,* 2009).

Virus diseases

Soybean is susceptible to several viruses transmitted by aphids, beetles and whiteflies prevailing in Nigeria. Most of the virus infection results in foliar symptoms such as mosaic and mottling, thickening/brittling of older leaves, puckering, leaf distortion, severe reduction in leaf size, and

stunting of plants. Mixed infection with more than one virus is common under field conditions. Features of the three most common virus diseases on soybean in Nigeria are presented here.

Mosaic disease: Cowpea mild mottle virus (CPMMV; genus Carlavirus, family Flexiviridae) transmitted by whitefly (Bemisia tabaci Gennidius) is the most prevalent virus associated with soybean mosaic disease in Nigeria. In addition, Bean pod mottle virus (genus Comovirus, family Comoviridae), Alfalfa mosaic virus (genus Alfamovirus, family Bormoviridae), Cucumber mosaic virus (genus Cucumovirus, family Bormoviridae), and Southern bean mosaic virus (genus Sobemovirus) were also detected in mosaic diseasevaffected plants either singly or in mixed infections, particularly with CPMMV. Depending on genotype and age of infection symptoms range from mosaic and mottling, leaf curling, green vein banding, and stunting. Most severe symptoms are observed in plants infected at early stages of growth (preflowering) and significant reduction in pods (Dugje *et al.*, 2009).

Yellow mosaic disease: It is caused by whitefly (B. tabaci)-transmitted different viruses belonging to the genus Begomovirus, family Geminiviridae. Soybean yellow mosaic virus was found to be the most prevalent virus associated with this disease. Soybean mottle mosaic virus, which also causes similar symptoms, was found to be less frequent in the fields. Virus-infected plants produce bright yellow mosaic or specks, and develop into large blotches on the leaf lamina, but this infection does not result in leaf distortion or reduction in lamina size. Mixed infection of these two begomoviruses and CPMMV are common in the fields and such infection results in bright yellow mosaic symptoms and leaf puckering (Dugje *et al.*, 2009).

Dwarf disease: The causal virus responsible for soybean dwarfing disease is not known. This disease occurs in low frequency in the fields. Leaves and shoots of the infected plants are severely stunted with severe reduction in leaf lamina. Infected plants do not produce any pod.

Cultivate virus disease-resistant varieties. This is the most convenient, economical, and effective approach for controlling soybean virus diseases. If resistant varieties are not available, the following approaches can contribute to the management of virus diseases in the field.

• Many viruses involved in mosaic disease are seed transmitted in soybean. Use certified seed to avoid seed-borne infection or use seed that are produced away from the infection source.

• Do not plant seeds obtained from mosaic-affected plants.

• Rouge (uprooting and destruction) symptomatic plants. This can reduce the incidence of insect-transmitted viruses.

• Eradicate the weeds and voluntary plants in the vicinity of the soybean farms.

• Treat seeds with systemic insecticides and apply one or two foliar sprays of insecticides to reduce the insect vector activity during preflowering stage (most vulnerable to virus infections) of the plant (Dugje *et al.,* 2009).

2.2.4. Soil Fertility Enhancement

Soybean improves soil fertility and fixes nitrogen in the soil for the succeeding maize. When grown in rotation with maize, it serves as a catch crop in controlling *Striga hermonthica,* a parasitic weed that attacks maize, by causing suicidal germination of *Striga* (Dugje *et al.,* 2009). Yusuf *et al.* (2009) enumerated several benefits of soybean grown in rotation with maize in northern Guinea savanna of Nigeria. The authors found improvement in maize yield and soil microbial biomass. Grain legumes are an excellent component for a good quality soil in any farming system because if provides residual N and reduces the need for mineral N fertilizer by associated non-legumes (Yusuf *et al*., 2009).

2.3. Breeding for Promiscuous Nodulation

Breeding for promiscuous nodulating genotypes was one of the approaches IITA followed to enhance biological nitrogen fixation of tropical soybeans. Soybeans that nodulate effectively with diverse indigenous rhizobia are considered as promiscuous, and the characteristic promiscuity (Kueneman *et al.,* 1984). Hence, promiscuous genotypes of soybean form symbiotic association with available *Rhizobium* strains in the soil and thus fix atmospheric nitrogen whilst non-promiscuous genotypes need specific rhizobial strains to fix nitrogen from the air. In Africa cowpea-type rhizobia are indigenous and are abundant (Hailu, 2011).

In the late 1970s, breeders at IITA observed that most high yielding soybean cultivars from USA have specific requirements for *Rhizobium japonicum* (Pulver *et al.,* 1982) and inoculation of these varieties was found to be essential when growing them under tropical conditions of low soil nitrogen. In the early 1980s, it was assumed that most tropical countries did not have the facilities and personnel required for inoculum production, storage, and distribution and were dependent upon importation of the final product (Pulver *et al*., 1982). The non abundance of

commercial *R. japonicum* inoculants and nitrogenous fertilizers led to the option of breeding promiscuous cultivars in IITA since soybean genotypes that do form symbiotic association with indigenous cowpea-type rhizobia were identified. Generally, soybean varieties developed for promiscuous nodulation with the indigenous rhizobia were considered to increase production of soybean in tropical Africa with minimum cost affordable to small-scale farmers (Hailu, 2011).

2.3.1. Identification of Promiscuously Nodulating Soybean Germplasm

Observation on nodulation of some soybean cultivars in soils where soybean has not been cultivated previously and the non-nodulation of exotic varieties that were bred in the USA indicated the existence of genotypic variation in soybean for the ability to recognize and form symbiosis with diverse species of rhizobia. Pulver *et al.* (1982) reported genotypic variation in six soybean genotypes in their ability to form an effective symbiosis with local *Rhizobium spp.* These workers noted that local cultivars were more promiscuous as compared to improved cultivars from the USA. IITA screened 400 geographically diverse soybean germplasm accessions for their compatibility with indigenous rhizobia in a range of tropical environments and assessed the efficiency of symbiosis under greenhouse and field conditions (Pulver *et al.*, 1985). Of the 400 genotypes screened for promiscuity, 10 germplasm accessions were found forming effective symbiotic relationships with the soil rhizobia at five locations in Nigeria. The source of these germplasm accessions were tropical Africa and south East Asia (Hailu, 2011).

2.3.2. Promiscuous Varieties Released

A total of 21 IITA bred tropical soybean varieties have been released in Africa. Most of these varieties were released in Nigeria. Some were released by the National Agricultural Research Systems (NARS) of Ghana, Benin, Togo, Democratic Republic of Congo, Uganda and Ethiopia.

In terms of maturity groups, seven varieties each were released from the early, medium and late, respectively. Grain yields ranged from 1 - 2.1 t ha^{-1} in the early maturing varieties depending on locations. In medium maturing varieties grain yields ranged from 1 - 2.7 t ha^{-1}. In the case of late maturing varieties grain yields ranged from 1.3 - 2.3 t ha^{-1}. These yields were achieved through natural nodulation and only starter fertilizers were applied. In addition to grain yield and promiscuous nodulation, several other traits were incorporated into these varieties. Some of them are fodder yield, seed longevity, resistance to shattering and lodging, suitability for processed products such as soymilk, resistance to major foliar diseases such as bacterial blight and pustule, frogeye leaf spot and tolerance to rust in recently released varieties (Hailu, 2011).

2.3.3. Dual-purpose Soybean Lines

Most of the IITA promiscuous soybean varieties and lines have been bred for dual-purpose to fit into the mixed farming system of the savanna. For some of the varieties specific studies were carried out to identify their feed value. From all maturity groups of soybean, lines were identified with high grain and stover yields and good stover quality assessed based on crude protein, neutral detergent fiber and dry matter digestibility (IITA, 2000). Lines that possess these qualities from the early maturity group are TGx 1878-30E, TGx 1880-3E, TGx 1019-2EB, and TGx 1871-12E and from medium maturity group they are TGx 1873-6E, TGx 1869-14E, TGx 1880-15E, and SAMSOY-1. From the late maturity group TGx 1869-13E, TGx 1440-1E, TGx 1871-6E, and TGx 1872-23E are identified as the best lines (Hailu, 2011).

2.3.4. Promiscuous Soybeans in Cropping Systems of the Savanna

The role of promiscuous soybeans for soil health and effect on productivity of subsequent cereal crops after soybean has been investigated in a greater detail by IITA scientists in the past two

decades. The study carried out by Carsky *et al.* (1997) estimated the value of residual soybean nitrogen on subsequent maize grain yield under the prevailed situation of soybean residue removal at 10 sites in the Guinea savanna of Nigeria using one early (TGx 1456-2E) and one medium maturing (TGx 1660-19F) varieties of soybean. These researchers reported that the yield increase following the medium duration soybean variety was similar to that from 40 kg ha^{-1} nitrogen applied four weeks after planting to maize preceded by maize. Their study also revealed that the total nitrogen in the 0-10 cm depth of the previous TGx 1660-19F plots (0.063%) was significantly greater ($p<0.05$) than in the previous maize plots (0.058%) considering all sites of the study. Carsky *et al.* (1997) concluded that the effect of previous soybean crop on maize grain yield was due to residual nitrogen availability either from the roots, fallen plant parts of soybean or nitrate-sparing effect. Nitrogen fixation and nitrogen contribution by different promiscuous nodulating soybean lines were further studied in the southern Guinea savanna of Nigeria by Sanginga *et al.,* (1997). These workers reported that nitrogen derived from the atmosphere and nitrogen derived from the soil were the major sources of nitrogen accounting for 84 and 75 kg N ha^{-1} or 46% and 43%, respectively, of the plant total nitrogen. They observed a great variation among soybean genotypes in that a late maturing genotype, TGx 1660-19F, derived on the average 126 kg ha^{-1} (52% of plant total nitrogen) from N_2 fixation as compared to the early maturing line IAC 100 with 37 kg N ha^{-1} (38%). Sanginga *et al.* (1997) estimated a net contribution of 18 kg N ha^{-1} to soil on the average after grain removal and it ranged from -8 to 43 kg N ha^{-1} depending on the soybean line grown (Tefera, 2011). Integration of legumes into the cropping systems of the northern Guinea savanna of Nigeria has been considered a vital resource management technique for reduction of energy use, cost and pollution potential of inorganic fertilizer usage (Yusuf *et al*., 2009)

Further study by Sanginga *et al.* (2002) substantiated the beneficial effect of soybean to maize in that 1.2 – 2.3-fold increase in maize yield was obtained when grown after soybean as compared to maize after maize. On-farm study in southern Guinea savanna using TGx 1456-2E and TGx 1660-19F indicated that these lines fixed 39-54% of their total nitrogen requirement that amounted to 56-70 kg N ha^{-1} in TGx 1456-2E and 51-78 kg N ha^{-1} in TGx 1660-19F (Osunde *et al.,* 2003). A maize grain yield of 3 t ha^{-1} was reported by these workers indicating the tremendous contribution from a 2-year soybean rotation. They recommended growing of promiscuous soybeans in rotation with maize even without the residues of soybean being returned to the farm land. Overall, studies indicated that under the Guinea savanna condition of Nigeria, promiscuous lines of soybean derive 46% of their plant total N (85 kg N ha^{-1}) from the atmosphere and the balance 43% (75 kg N $ha^{-1)}$ from the soil. Thus, Sanginga (2003) suggested that breeding for higher nitrogen derived from the atmosphere lines should continue along with the development of efficient *Bradyrhizobium* strains as inoculants (Hailu, 2011).

2.4. Symbiotic Nitrogen Fixation

Various bacterial species that belong to the α-proteobacteria and the order Rhizobiales (Madigan *et al.*, 2000) can engage in a symbiosis with plants of the leguminous family (Spaink *et al.*, 1998). These bacteria, which are collectively called rhizobia, based on this symbiotic behavior have the unique capacity to induce the formation of root nodules in the host plant by the production of specific signal molecules called Nod factors (Lerouge *et al.,* 1990, Spaink *et al.,* 1991, Truchet *et al.,* 1991). The rhizobia are also able to invade the plant tissue via infection threads, which results in a differentiated root nodule-inhabiting form of the bacteria that exists inside the cells of the host plant (Spaink *et al.*, 1998). This differentiated form of the bacteria, called bacteroids, can fix gas phase nitrogen into ammonia (Spaink *et al.,* 1998). The fixed

nitrogen, in the form of ammonia or alanine (Waters *et al.*, 1998), is supplied to the host plant, which, in turn, supplies the bacteria with various nutrients (Spaink *et al.*, 1998). The rhizobial species are genetically a very diverse group (van Berkum and Eardly, 1998), as is illustrated by their recent division into four different families, the Rhizobiaceae, the Phyllobacteriaceae, the Hyphomicrobiaceae, and the Bradyrhizobiaceae (Madigan *et al.*, 2000). Within these four families, only a limited number of genera have the capacity to engage in a nitrogen-fixing symbiosis with leguminous plants. Currently these genera include *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium*, and *Allorhizobium* (De Lajudie *et al.*, 1998).

Despite this genetic diversity, it has become clear that these genera of bacteria have many common genetic and biochemical characteristics related to their capacity to establish a successful symbiosis. These common factors include the capacity to recognize specific signal molecules, such as flavonoids (Schlaman, *et al.*, 1998), from the host plants and to produce special signal molecules, such as the Nod factors, which apparently are not produced by other related genera. In addition, common factors include specialized structural adaptations and special regulation of classes of molecules also occurring in most other soil bacteria. These molecules serve specialized functions during symbiotic conditions, such as (*a*) different growth conditions, (*b*) the presence of many potentially toxic compounds (e.g. phenolics and enzymes), and (*c*) the need to avoid a defense response of the plant host. For instance, it can be expected that a wide variety of cell surface characteristics of the rhizobia are different from those of other related soil bacteria. However, the identification of common adaptations is hampered by the fact that different plant host species offer very different habitats for the guest bacteria (leading to host specificity) and that the evolution of various bacterial traits has occurred convergently from many different genetic backgrounds, as shown by the genetic diversity mentioned above. The identification of

various common rhizobial characteristics that are involved in symbiosis was facilitated by the apparent clustering of various groups of genes encoding these characteristics on transmissible genetics elements, such as large plasmids in all rhizobial genera (Broughton and Perret, 1999, Flores *et al.*, 1998), and large transposable elements, such as the so-called symbiosis islands, in *Mesorhizobium* spp. (Sullivan and Ronson, 1998). Other common rhizobial characteristics have been identified by a detailed analysis of factors that were expected to be involved in a symbiotic capacity, such as specialized nitrogen and carbon metabolism (Spaink *et al.*, 1998), cell surface characteristics (Spaink *et al.*, 1998), secreted factors (Finnie *et al.*, 1997, Finnie *et al.*, 1998, Viprey *et al.*, 1998, York and Walker, 1997), and specialized uptake systems (Spaink *et al.*, 1998, Willis and Walker, 1999). Regarding the recognition processes underlying the specialization of various subgroups of rhizobia for different host plants (i.e. host specificity), the rhizobia as a group are specialized for the structure and regulation of production of their cell surface and secreted glycans, including glycolipids. This statement applies, namely, for the characteristics of the Nod factors, the extracellular polysaccharides [EPSs], the K-antigens, the cyclic glucans , and the lipopolysaccharides [LPSs] (Spaink *et al.*, 1998). Although the extent of the specialization, compared with that of non-rhizobial species from the α-proteobacteria, is not always clear (except perhaps for the Nod factors, which seem to be uniquely produced by the rhizobia), there are various unique chemical features or unique combinations of chemical features that seem to be associated with the capacity to engage in a symbiosis with leguminous plants (Spaink, 2000).

2.4.1. Characterization of Soybean-Nodulating Rhizobial Communities and Diversity

Soybean establishes a symbiotic relationship with soybean-nodulating bacteria during the development of its nitrogen-fixing organ, the root nodule. The major soybean-nodulating rhizobia that have been identified are *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, and *Sinorhizobium/Ensifer fredii* (Jordan, 1982; Scholla & Elkan, 1984; Kuykendall *et al.,* 1992; Young, 2003). Owing to the complexity of their taxonomical classification, additional species of soybean-nodulating rhizobia have been extensively discussed in the literature (Xu *et al.,* 1995; Tan *et al.,* 1997; Vinuesa *et al.,* 2008). Soybean-nodulating bacteria are found over wide regions of the world, and their genetic diversity may reflect geographical and climatic differences as well as host diversity. Some strain of *Bradyrhizobium japonicum* effectively fixes atmospheric nitrogen and is expected to be an effective bacterial inoculant. Inoculation of soybean seeds with an effective inoculant can increase soybean yield, but field-indigenous rhizobia frequently compete with the inoculant (Yamakawa *et al*., 2003). Therefore, analysis of the genetic diversity and field distribution of indigenous soybean-nodulating rhizobia is important in improving our understanding of rhizobial ecology as well as inoculation methodology under various environmental conditions (Yuichi, 2011).

Many studies have investigated rhizobial ecology. In case of research for indigenous soybean-nodulating rhizobia in Japan, Sawada *et al*., (1989) isolated 85 indigenous soybean Bradyrhizobia from soybean root nodules sampled from 46 soybean fields in Japan and classified the isolates according to their serotype by using rabbit antisera prepared against USDA strains of *Bradyrhizobium* as serotype antigens (Yuichi, 2011).

Minamisawa *et al.* (1999) also characterized Japanese indigenous soybean Bradyrhizobia isolated from six fields by analysis of their fingerprints with the hybridization probes RSα, RSβ,

nifDK, and *hupSL*, and revealed the diversity and endemism of their population structures. Furthermore, phylogenetic comparison of common nod gene was also conducted between isolates in Thailand and Japan (Yokoyama *et al.*, 1996). Their results suggested that bradyrhizobia might diversify in individual fields depending on the associated host plants and local soil conditions. The soybean-nodulating rhizobial community might change depending on the host cultivar and cultivation temperature even in the same field as well as in different fields with geographical, soil texture, soil pH, salinity, and other differences (Howieson & Ballard, 2004). Previously, Suzuki *et al.*, 2008 demonstrated that soybean nodulating rhizobial communities differed greatly in nearby fields depending on whether the soil was acidic or basic. Therefore, knowledge of rhizobial ecology and biology in relation to numerous environmental gradients is needed (Yuichi, 2011).

Some soybean cultivars possess nodulation regulatory genes known as *Rj* genes, and the genotypes non-*Rj*, *rj*1, *Rj*2, *Rj*3, and *Rj*4 have been confirmed to exist in nature (Devine & Kuykendall, 1996). A non-nodulating soybean line was identified from a cross between the soybean cultivars Lincoln and Richard, and incompatible nodulation was found to be controlled by a single recessive gene; this genotype was named the *rj*1 genotype (Williams and Lynch, 1954). Subsequently, several dominant *Rj* genotypes (*Rj*2, *Rj*3, and *Rj*4) were discovered that restrict effective nodule formation in certain *Bradyrhizobium* strains (Caldwell, 1966; Caldwell *et al.*, 1966; Vest, 1970; Vest and Caldwell, 1972). The *Rj* genes play a role in controlling the plant's compatibility with specific rhizobial strains, and indigenous soybean-nodulating rhizobia may display a preference for a certain *Rj* genotype (Yuichi *et al.*, 2000). Therefore, characterization of soybean-nodulating rhizobial communities requires that compatibility and

preferences between soybean *Rj* genotypes and soybean-infecting rhizobia be taken into consideration (Yuichi, 2011).

2.4.2. Legume-rhizobium Symbiosis

A. Nodule formation

The relationship between *Rhizobium* and legume plants is selective. Individual species of rhizobia have a distinct host range allowing nodulation of a particular set of legume plants. For example, *Rhizobium leguminosarum* bv *viciae* nodulates pea and vetch, whereas *Bradyrhizobium japonicum* nodulates soybean (Stougaard, 2000). At the other extreme, the exceptionally broad host-range *Rhizobium* sp. NGR234 nodulates 353 legume species representing 122 genera (Pueppke and Broughton, 1999). Differences in both infection processes and organogenic programs are reflected in variations in root nodule morphology (Doyle, 1998), but overall there are pronounced developmental similarities as would be expected from a common ancestry.

B. Histology and nodule development

In the most studied legumes, infection occurs via an infection thread that takes the bacteria through the root hair into the root cortex and distributes them to cells, which become the infected cells of the nitrogen fixing nodule (Stougaard, 2000). The root zone susceptible to invasion is located behind the root tip where root hairs are still growing and competent for invasion. In response to attached bacteria, root hairs deform and curl setting up a pocket that provides a site for initiating the infection. The infection thread is a plant derived structure originating from plasma membrane invagination accompanied by external deposition of cell wall material. In advance of the intracellular “inward” progressing thread, root cortical cells dedifferentiate and reenter the cell cycle. Cortical cells prepared for infection thread passage appear to be arrested in the G2 phase, whereas cells completing the cycle resume division to establish the nodule

primordium. Later in the process, pattern formation and cell differentiation specify tissue and cell types (Stougaard, 2000).

The bacteria are endocytosed into a subset of cells where they differentiate into nitrogen-fixing bacteroids surrounded by the peribacteroid membrane of the symbiosome. In the mature functional nodule, peripheral vascular bundles are connected to the root vasculature and the main tissues/cell types can be distinguished cytologically and to some extent with molecular markers. In determinate nodules such as soybean the meristematic activity ceases early, and the nodule grows by expansion giving a spherical shape. All developmental stages from root hair curling to nodule senescence are consequently phased in time. Indeterminate nodules such as pea maintain an active meristem depositing cells that are subsequently infected. This results in a cylindrical shape with the organ formative developmental stages represented along the longitudinal axis. The type of nodule formed is specified by the plant host (Stougaard, 2000).

C. Mutual signal exchange starts the process

The early plant host signals secreted into the rhizosphere can be (iso) flavonoids, stachydrines, or aldonic acids. Best studied are the flavonoids that, in conjunction with the rhizobial *NodD* transcriptional activator, induce expression of the *nod* gene regulon. In turn, *nod* gene products synthesize and transport the Nod factor the major early signal molecule perceived by the host plant. A flexible interaction with the host is enabled by several mechanisms. Alternative *NodD* activators recognizing different plant flavonoids provide an extended host range in some bacterial strains. *B. japonicum* even has an alternative two component regulatory pathway for activating its *nod* regulon, and in *Sinorhizobium meliloti nod* gene expression is fine-tuned by positive- and negative-control circuits (Stougaard, 2000).

Nod factors are low-M_r b,1-4-linked *N*-acetyl glucosamine compounds (Lerouge *et al.*, 1990) typically carrying a fatty acid on the non-reducing sugar and sulfuryl, fucosyl, mannosyl, or arabinosyl groups at the reducing terminal sugar. Additional substitutions include carbamoyl, glycerol, and fucosyl derivatives (Dénarié *et al.*, 1996; Spaink, 1996). When purified and applied in the absence of bacteria these lipochitooligosaccharides (LCOs) function as mitogens or "morphogens" on some legume roots. For example, addition of nanomolar concentrations induces root hair deformation in most legumes. In more responsive plants, pre-infection threads (cytoplasmic bridges in G2 arrested cortical cells), cortical cell divisions, and empty nodule structures with an anatomy comparable to rhizobial-induced nodules also develop (Dénarié *et al*., 1996; Spaink, 1996). These responses show that some legumes encode all functions necessary to develop the nodule once the process has been switched on.

Spontaneous nodule development on certain alfalfa mutants grown axenically supports this idea.

Rhizobium strains differing in their repertoire of *nod* genes produce LCOs with different structural features. Biological activity is determined by the length of the chitin backbone, the structure of the lipid, and a suite of other substitutions on the oligosaccharidic backbone. Host specificity results at least partly from flavonoid activation of *nod* genes and plant perception of the resulting bacterial LCO signal molecules are required for nodulation to occur. For example, *R. leguminosarum* bv *viciae* can be genetically modified to produce an acetyl fucosylated LCO under control of a flavonoid-independent *NodD* activator .This modified LCO is now recognized by *L. japonicus* plants resulting in nodulation by the *nod* gene deregulated *R. leguminosarum* strain (Bras *et al*., 2000).

2.5. The Need to Inoculate

As a general concept, tropical soils often lack available nitrogen (Stowers and Elkan,1980). Of the two generally available forms of nitrogen, that fixed biologically is more economical than fertilizer N. The legume-rhizobium symbiosis may account for 100-200 KgN/ha/yr (Norris and Date, 1976). Exploitation of this phenomenon provides an important, highly economical source of available nitrogen.

The selection of appropriate strains of Rhizobium is the first key step in effective inoculation of legumes. The most important criterion in selecting a strain is ability to form effective nitrogen-fixing nodules on the appropriate legume host, preferably under a wide range of conditions. Effectiveness, as measured by dry matter yield or the amount of nitrogen fixed, is still the fundamental criterion for selection although various other tolerances are now recognized as important (Stowers and Elkan, 1980).

The ability of a strain to survive in soil and compete with native strains (Means *et al*., 1961) is important to successful nodulation and nitrogen fixation. Early nodulation over a range of soil temperature and pH; ability to grow well in broth and peat (or other inoculum carrier) (Brockwell *et al.,* 1968); tolerance to fungicides (Afifi *et al.*, 1969; Kecskes and Vincent, 1969) and the ability to nodulate and fix nitrogen in the presence of high levels of available nitrogen (Gibson *et al.,* 1971) are other important criteria. The criteria listed above provide a general overview of important considerations in the selection of Rhizobium, per se. However, specificity of the strain of microsymbiont and of the host genotype also deserve consideration (Stowers and Elkan, 1980).

Some tropical legumes demonstrate a high degree of symbiotic specialization, whereas others are relatively non-specific and will nodulate promiscuously with native rhizobia (Norris and Date, 1976). Inoculation is often required with highly specific host. Strain selection is essential for preparation of effective inocula.

2.5.1. Assessment of the Need for Inoculation

Effective rhizobia are essential to providing a beneficial symbiotic relationship with the host legume. In most parts of the world there is a broad range of rhizobia strains which vary in the degree of effectiveness and competitiveness. In some areas very effective and competitive strains may be the major constituents of the native rhizobial populations, but in other areas these strains may be: **(1)** lacking or **(2)** less effective and/or **(3)** less competitive.

In the latter cases where there is no native rhizobial population or satisfactory strain, introduction of a superior strain must be made to create a greater potential for maximum yield (i.e. increase in nitrogen fixation). Many studies have been done which establish that inoculation with a superior strain is a method for increasing yields in legumes (Dube, 1976; Ham *et al*., 1971; Subba Rao, 1975; Sundara-Rao *et al.*, 1975; Sundara Rao, 1976). Some commercially prepared inocula have also improved yield (Abel and Erdman, 1964; Chhonkar and Negi, 1971; Dube, 1976; Dunphy, 1978, Dunphy *et al.*, 1977).

An assessment of the need for inoculation is important before beginning any study on enhancing nitrogen fixation in legumes through Rhizobium strain selection. This can be accomplished two ways:

1. The method of Vincent (1970) which assesses the need for inoculation by observing nodules on uninoculated legumes already growing in the test area and recording:

a. The proportion of plants bearing nodules.

b. The abundance or sparseness of nodules on the nodulated plants.

c. The location of nodules on the root system (crown, distal; taproot, lateral).

d. The size and appearance of nodules: whether white in section (inactive; no N-fixation), pink with leghemoglobin (active, N-fixation) or with a green area to the base of a restricted red zone, indicative or previous leghemoglobin (inactive or partially active). (This last condition will have to be interpreted in relation to the stage of maturity of the nodules and conditions affecting growth of the plant and photosynthesis; indicating that this method of analysis is not absolute and certain physiological and environmental factors can effect coloration in the nodule).

2. The method of Date (1976) which tests the success of inoculation through three treatments:

a. Uninoculated control plots to check for presence of natural occurring rhizobia.

b. Plots inoculated with potentially useful strain known to be effective in controlled environment or glasshouse tests.

c. Plots inoculated with the same strain and supplied with combined nitrogen.

In this approach there is a three part evaluation of a particular strain.

- To assess the need for inoculation.
- To compare the competitiveness of a strain to the native Rhizobium population.
- In the final part the ability of the strain to produce efficient nodules in the presence of available combined nitrogen is evaluated as a method of evaluating the suitability of the strain for inoculant production.

2.5.2. Criteria for Selection of Effective Strains

Many factors should be considered when evaluating a particular strain. The three variables which affect the Rhizobium-legume symbiosis should be considered. They are:

(1) the host,

(2) the environment and,

(3) the organism, itself.

The selection of effective rhizobia is important in improving legume yield via improved nitrogen fixation. Persistence in the soil is a characteristic which is very vital. This characteristic involves factors such as:

(1) survival of *Rhizobium* while not associated with legumes,

(2) the ability to multiply in the soil such that rhizobial population is capable of nodulating the legume host, and

(3) the ability of the *Rhizobium* to persist in the soil and produce effective nodules on the roots of regenerating annuals or on new roots of perennials (Date, 1976).

Aside from these primary prerequisites for an effective strain many various secondary characteristics should be considered such as pH tolerance (Norris, 1973; Norris and Date, 1976; Roughley, 1976; Abel and Erdman, 1964), ability to survive in different soil types (Kumara-Rao *et al.*, 1974), ability to grow and nodulate in the presence of available combined nitrogen (Sundara-Rao *et al.,* 1975; Weber, 1966; Gibson *et al.,* 1971; Ham *et al*., 1976; Sistachs, 1976).

2.5.3. Effectivity in Nitrogen Fixation

Effectivity is of primary concern in increasing legume yield through symbiotic nitrogen fixation. Effectivity as evaluated by increase in dry weight yield or total-N-content is the fundamental

criterion for selecting superior strains. Effectivity is probably the easiest parameter of nitrogen fixation to evaluate.

In selecting rhizobia one should use simple tests such as dry weight yield or total-N-content in evaluating a strain for effectivity. Once an effective strain has been determined, it is necessary to establish whether that strain will nodulate and produce efficient nodules in the presence of a native rhizobial population (Stowers and Elkan, 1980).

2.5.4. Competition for Nodule Formation

An important characteristic in strain selection is the ability of a strain to successfully compete with the less effective native rhizobia for nodule (infection) sites. The Rhizobium-legume interaction, itself, may dictate whether a superior strain will compete with the native rhizobial population. In many cases, less effective strains are more competitive than highly effective strains.

It is possible to visualize that less effective native Rhizobium could saturate infection sites preventing nodulation by the applied strain. In some instances, a strain may be more efficient in one host genotype while not in another. Ideally, one should be able to introduce a highly effective Rhizobium strain to a field where a less effective native population is present and obtain an efficient symbiosis. In the greenhouse this concept holds true, but in the field where various environmental conditions exist the selection of Rhizobium tolerant to various environmental conditions are essential to achieving maximum benefit from symbiotic nitrogen fixation (Stowers and Elkan, 1980).

2.5.5. Environmental Conditions and Nitrogen Fixation

The major environmental conditions which should be considered are:

- Extremes in temperature

- High elevation
- Varying soil types
- High levels of available soil nitrogen
- Moisture levels
- Extremes in pH

A. Extremes in temperature

The effect of temperature on symbiotic nitrogen fixation has been well documented (Gibson, 1961; 1963; 1967a; 1967b). This effect can be examined in the laboratory or in growth chambers using a tube method. Effectivity is measured by total-N-content in these experiments. The optimum temperature for the growth of rhizobia is 28^{O}C. Depending on the environment where the rhizobia are intended, it may be important to select rhizobia which are tolerant to higher temperatures than 28^{O}C. The selection of high or low temperature tolerant Rhizobium should be supplemental to an overall selection of effective strains. One factor which should be considered when evaluating temperature tolerances of the Rhizobium strains is the temperature optimum of the particular host plant which in most cases is 28^{O}C (Stowers and Elkan,1980).

B. High elevations

While high elevation, in itself, probably does not influence nitrogen fixation, factors which accompany high elevation such as low temperature and low micronutrient level may limit the potential of an introduced strain.

C. Varying soil types

Damirgi *et al*. (1967) showed that particular strains of Rhizobium were associated with particular soil types. This is an important criterion for selection of effective Rhizobium in that one strain of Rhizobium may be effective in one soil type and not in another. This phenomenon may be

related to the pH of the soil, the organic content, or the ionic composition. It is recommended that strains be selected from the environment where they are intended to be used and in that selection the most effective may be isolated.

D. High levels of nitrogen

In some tropical soils high levels of available nitrogen may exist (18ppm to 400ppm). Such levels of nitrogen are likely to inhibit nodulation (Norris and Date, 1976), while not providing nitrogen necessary for optimal plant growth. This problem could be alleviated by selecting rhizobia tolerant to high nitrogen levels, but in this case a simple agricultural practice of mulching can also alleviate this problem by removing available nitrogen (Griffith and Manning, 1949; Griffith, 1951). Mulching in this case may provide a much less expensive alternative than strain selection for nitrogen tolerant rhizobia (Stowers and Elkan, 1980).

E. Moisture levels

High moisture levels reduce the number of rhizobia in a soil population. It has been shown that certain species of rhizobia withstand desiccation in field soils (Parker *et al.*, 1977).

F. pH extremes

The optimum pH for rhizobia is 6.8. Most rhizobia will not grow at pH 4.0 or lower, while the upper limit is 8.5 (Date and Halliday, 1979). All types of rhizobia grow best in a buffered environment where the pH does not vary more than 0.5 pH units. pH tolerance would allow introduction of a new strain of Rhizobium to many different soils (Stowers and Elkan, 1980).

An additional criterion which should be considered is the ability of a strain to survive and persist in the soil in various environmental conditions. Due to the diversity of environmental factors which may affect the symbiosis, strains should be selected which can overcome environmental obstacles and provide an efficient symbiosis.

2.6. Isolation, Purification and Maintenance of Rhizobia

2.6.1. Isolation

Soil:

Many selective media have been developed but are impractical (Parker *et al.,* 1977). The most practical method of isolating rhizobia from soil involves the indirect determination of rhizobia in the soil via the plant-infection technique (Vincent, 1970 and Brockwell, 1963), or nodulation-dilution frequency tests (Brockwell *et al.,* 1975). These methods are essentially the same. The experimental method involves diluting soil samples and mixing the diluted samples with surface sterilized seeds. The rhizobia are then isolated from the nodules (Stowers and Elkan, 1980).

Vincent (1970) provides a satisfactory method of isolation of rhizobia from nodules. The nodules are homogenized and plated on a suitable rhizobia media containing a fungal growth inhibitor such as cycloheximide (Actidione) at 0.002%.

Native legumes:

Isolation of rhizobia from native legumes is the most practical method of obtaining different strains for a culture collection. Strains isolated through this method provide one with a myriad of strains usually varying in nitrogen-fixing potential.

It is possible that more than one typical colony type may appear on the plate streaked from a single nodule and each of these should be taken to pure culture and held for characterization (Stowers and Elkan, 1980).

2.6.2. Purification Techniques

The absolute purification of rhizobia relies on the positive identification of the particular species and strain. A simple method of purification of Rhizobium would be the use of selective medium,

which would exclude all other organisms. Rhizobia have various characteristics which are important in distinguishing them from other organisms.

There are tests which utilize these characteristics and help determine whether Rhizobium or contaminants are present.

Gram stain:

This is a stain which divides all bacteria into two classes; they either react positively or negatively. Rhizobia are gram-negative (clear red) under light microscope. Contaminants conversely are gram-positive and appear dark violet.

The gram stain is definitely not an absolute test in that there are many gram-negative organisms in nature. However, it is a simple test and can establish the presence of a gram positive contaminant.

Ketolactose test: (Bernaerts and Deley, 1963).

This reaction involves the oxidation of lactose to form 3-Ketolactose. This reaction only occurs with *Agrobacterium spp*. and is diagnostic for them. Since *Agrobacterium spp*. are a common nodule contaminant, it is a good practice to test for 3-Ketolactose in nodule isolates. To test for 3-Ketolactose one can apply an isolate to an agar plate (1% lactose, 0.1 % yeast extract, and 2 % agar). Several isolates can be applied to one plate. The plate is incubated for one to two days at $25^{O}C$ and afterwards flooded with a shallow layer of Benedict's reagent and left at $25^{O}C$. If Ketolactose is present a yellow ring of Cu_2O becomes visible around the cell mass; after one hour it is maximal in diameter (about 2-3 cm). A positive reaction indicates a contaminant is present.

There are tests which utilize the cultural characteristics to distinguish rhizobia from contaminants.

Growth on yeast mannitol agar: Growth on YMA is slow for Rhizobium. Fast growers show moderate to excellent growth after three days while the slow growers show moderate growth after seven days.

Growth on YMA with Congo red: Rhizobium absorb very little of the red dye while some contaminants absorb the dye strongly.

Growth and change of pH on peptone glucose agar: Rhizobium grows poorly on this medium and causes little change in pH.

There are other tests which are extremely specific and are excellent in establishing the identity of the particular strain being studied.

Phage typing: The interaction between bacteriophages and bacteria results in either the formation of clear or turbid plaques. The formation of clear plaques is termed complete lysis i.e. the bacterial cell is destroyed while phage progeny is produced. In the formation of turbid plaques, not all the bacteria are destroyed, and the phage DNA is incorporated into the bacteria.

Both cases have been reported in Rhizobium and the interaction between bacteriophage and bacterium are highly specific (Schwinghamer and Reinhardt, 1963 and Kleczkowska, 1957). In phage-typing the formation of clear plaques determines phage specificity of the particular strain you are working with.

For example, if you isolate phage from the soil and determine which Rhizobium strain is lysed, you could then use that phage to determine the identity of an unknown strain.

To do phage typing in a laboratory would require the maintenance of a phage collection and in that collection there should be phage for all strains worked with in the laboratory. Phage typing can be accomplished at little expense in a very modestly equipped laboratory.

Serology: Serological techniques like phage typing are excellent in determining the identity of a specific strain. On the surface of bacterial cells there are antigens which elicit an immune response in animals. Antibodies are produced by the animal and can be used to determine the presence or absence of specific antigens.

The development of antibodies (antisera) can be accomplished by using experimental animals such as rabbits.

Vincent (1970), gives a detailed account of methodology for development of antisera as well as the methodology for the serological reaction, either agglutination or precipitation.

Cross inoculation groups: Rhizobium can be grouped together depending on which legume host they nodulate. Fred *et al.* (1932) proposed cross-inoculation or bacteria-plant groups which divide the genus of Rhizobium into groups based on their nodulation specificity. (Stowers and Elkan, 1980).

Nodulation tests: The only absolute identification of *Rhizobium* is through nodulation tests. If the unknown bacteria does not nodulate its host legume then it is not *Rhizobium*, however it should be noted that in certain instances there are examples of extreme host-strain specificity such as in soybean. The use of a highly promiscuous test plant could alleviate this problem. In practice small-seeded tropical legumes are used to determine nodulation capabilities because of their great promiscuity and small size. Several test plants other than the actual host have been used in determining the nodulating capacity of a particular strain. Siratro (*Macroptilium atropurpureum*) serves as a suitable test host for soybean and the cowpea group of legumes such as peanut, cowpea, velvet bean, poona pea, lentil, horse gram, and pigeon pea. White clover or subterraneum clover serve as suitable test hosts for *Rhizobium trifolii*. Many of the test hosts are

tropical in origin and are considered primitive evolutionary. These are generally smaller seeded which provides for easier laboratory study (Stowers and Elkan, 1980).

2.6.3. Maintenance of Isolates

As with any bacterial culture collection, accurate records must be maintained. In a Rhizobium culture collection accurate as well as descriptive records are essential. It is extremely important that data concerning the origin of a strain be noted as well as its symbiotic capacities. Vincent (1970) describes information that should be included in a culture collection file as follows:

1. collection number
2. origin
    a. host
    b. locality (soil pH, temperature range, rainfall, other environmental conditions)
    c. if applicable, name of supplier and original collection number
3. summary of important features such as symbiotic capacities and other relevant reports. It is important that these records be made and maintained so that the particular strain may be studied in reference to its true environmental niche, symbiotic capacity, or other aspect of that strain.

The preservation of rhizobial isolates and the maintenance of Rhizobium cultures is necessary to every testing laboratory as basis for further study and comparison to new isolates.

The maintenance of rhizobial cultures can be accomplished through two basic methods: 1) agar cultures and 2) dried cultures.

Agar slant cultures are the easiest method for maintenance of rhizobial cultures. Rhizobial cultures may be maintained in these agar slants for several years. These cultures remain stable under refrigeration and freezing conditions. Small screw-cap test tubes are adequate and also reduce desiccation.

Dried cultures provide the best method for maintaining rhizobia. Under these conditions the rhizobia are genetically stable, which is an important aspect to be considered. There are three types of dried cultures which can be used to maintain rhizobia (Vincent, 1970):

1) Standard lyophilization or freeze drying,
2) Porcelain bead (Norris, 1963),
3) Dried soil (Vincent, 1970).

The maintenance of rhizobial cultures is of extreme importance in keeping an active culture collection. One should maintain their collection in duplicate to prevent losses of cultures due to unfortunate circumstances such as power failures or contamination. Ideally the duplicate collection should be lyophilized while working collection could remain on agar slants for easy access. Cultures maintained on agar slants should be periodically (every six months) transferred, streaked for pure culture and tested for nodulation capability (Stowers and Elkan, 1980).

2.6.4. Evaluation of Superior Strains of Rhizobium

The evaluation of rhizobial strains involves the assessment of the relative effectiveness of the particular strain. There are criteria for the evaluation of rhizobial strains which can be employed depending on the sophistication of the laboratory:

(1) Competitive ability (saprophytic competence and the ability to nodulate and form an efficient symbiosis in the presence of a less effective native rhizobial population.

(2) Effectiveness in nitrogen fixation.

(3) Secondary characteristics (pH tolerance; survival in different soil types; grow in the presence of combined nitrogen, pesticide resistance; and characteristics important for inoculant production such as ability to survive in part culture) (Stowers and Elkan, 1980).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Need to Inoculate Trial

3.1.1. Experimental Site and Description

The experiment was set up in a screenhouse in the Department of Soil Science, Ahmadu Bello University, Zaria (11^0 9' N, 7^0 36' E). The experimental soils were collected from different parts of Sudan savanna, Northern Guinea and Southern Guinea savanna. According to USDA Soil Survey Staff (1975), the following soil types are found in the savanna agro-ecological zones: Alfisols, Inceptisols, Entisols, and Vertisols. Klikenberg and Higgins (1968) identified Alfisols as the most common of the 23 soil mapping units occurring in the savanna. Soils commonly found in the Guinea savanna zones are derived from pre-Cambrian crystalline Basement Complex. Table 3.1 shows the range of weather conditions and dominant crop species of these agro-ecological zones.

3.1.2. Soil Collection and Preparation

Bulk soil samples were collected at a depth of 0-20 cm from 45 sites in 14 local government areas (LGAs) spread across three agro-ecological zones of Nigeria (Table 3.2). The soil samples were collected randomly within each site and coordinates are shown in Appendix 1. Figure 3.1 shows the distribution of the sampling sites. The samples were air dried, crushed and sieved through a 4mm mesh (for planting) and 2mm mesh (for physicochemical analysis). Plastic pots of about 16.5cm in diameter and 16.5cm height with a capacity of 3.5litres and drainage holes were filled with 4kg of soil samples. Field capacity of the soils were determined by adding water

to the pots up to saturation level and allowed to drain over 24 hours. The moisture retained by the soil was assumed to be its requirement to reach field capacity.

Table 3.1. Distribution of annual rainfall, potential evapotranspiration, mean air temperature, tree, grass and crop species in the major savanna zones of Nigeria.

	Agro-ecological zones			
Parameters	Southern Guinea	Northern Guinea	Sudan	Sahel
Annual rainfall (mm)	1000-1500	900-1300	500-1000	100-500
Length of rainy season (days)	190-250	130-190	80-130	80
Potential annual ET (mm)	950-1300	1100-1500	1300-1800	1500-2000
Mean annual temperature (^{o}C)	23-25	24-26	26-28	28-30
Main tree spp.	*Dainella olivera*	*Isoberlina spp*	*Combretum,Acacia,*	*Acacia,*
Main grass	*Andropogon tectorum, Imperata cylindrica*	*Hyparrhenia,Andropogon*	*Andropogon gayanus*	*Cenchrus* spp
Main food crop	Yam, Maize, Sorghum	Sorghum, Maize	Millet, Sorghum	Millet
Main cash crop	Soybeans, Sesame	Cotton	Groundnuts	Cowpea

Adapted from Esu (1978)

Table 3.2. Locations of the sampling sites

S/N	LOCATION (LGAs)	AGROECOLOGY	SITE
1	Garko	Sudan Savanna	6
2	Bunkure	Sudan Savanna	2
3	Albasu	Sudan Savanna	3
4	Minjibir	Sudan Savanna	1
5	Bichi	Sudan Savanna	3
6	Wudil	Sudan Savanna	2
7	Dawakin Kudu	Sudan Savanna	2
8	Warawa	Sudan Savanna	2
9	Giwa	Northern Guinea Savanna	5
10	Soba	Northern Guinea Savanna	6
11	Igabi	Northern Guinea Savanna	4
12	Tudun Wada	Northern Guinea Savanna	4
13	Kachia	Southern Guinea Savanna	3
14	Kolosok	Southern Guinea Savanna	1
15	Zonkwa	Southern Guinea Savanna	1
	Total		45

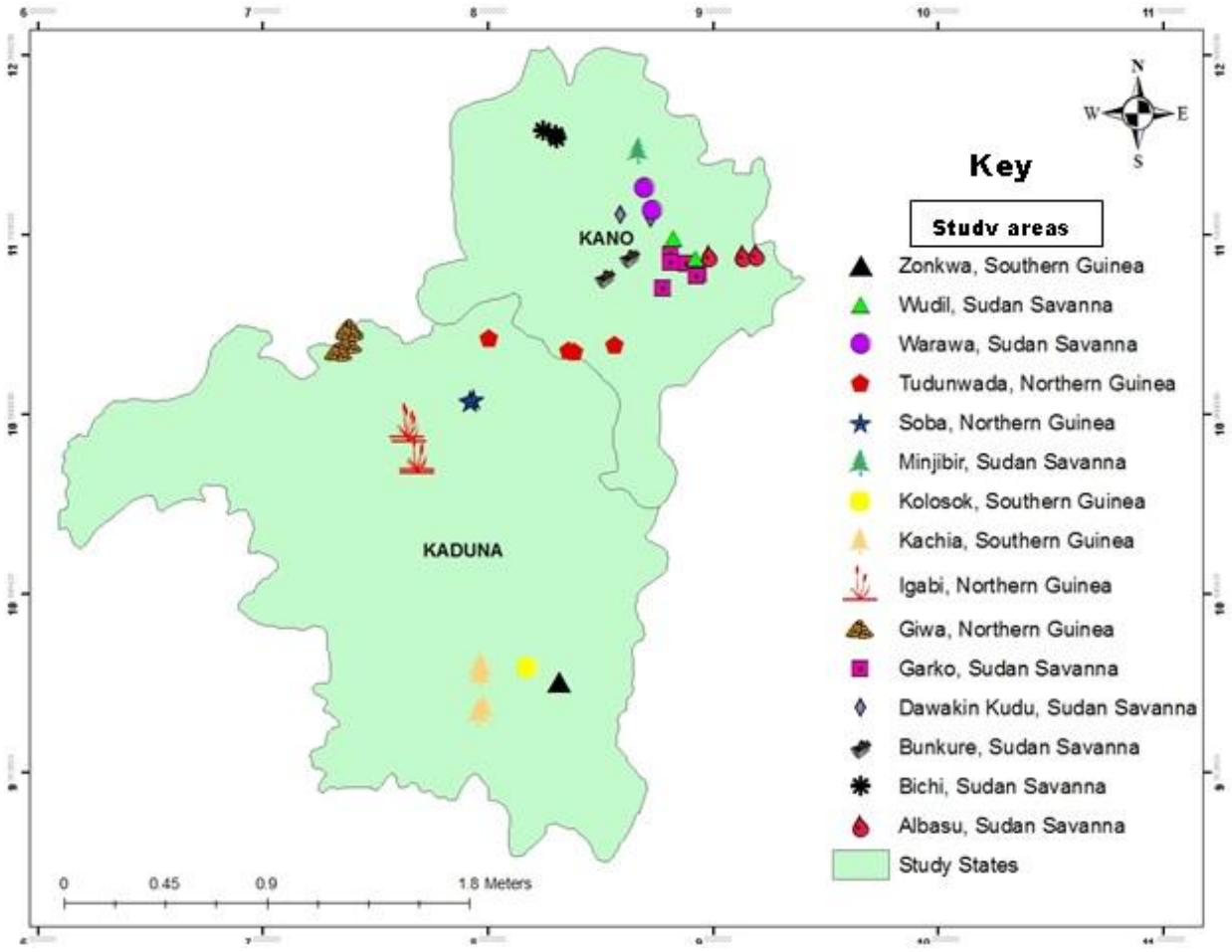


Fig. 3.1. Map showing distribution of sampling sites.

3.1.3. Physicochemical Analysis

The following analyses were conducted on the soil samples: Particle size distribution; Soil pH; Soil organic carbon; Total Nitrogen; available P; Exchangeable bases and Exchangeable acidity; Effective cation exchange capacity.

a. Particle size distribution

Particle size distribution was determined by the hydrometer method, as described by Gee and Bauder (1986), using distilled water and calgon (sodium hexametaphosphate) as dispersing agents. The soil texture was determined using the USDA soil textural triangle.

b. Soil pH

Soil pH was measured in both water and 0.01 M $CaCl_2$ solution using a 1:2.5 soil to solution ratio with a glass electrode pH meter (Hendershot *et al.,* 1993).

c. Soil organic carbon

Organic carbon was determined by the Walkey-Black (chromic acid) method as described by Nelson and Sommers (1982).

d. Total nitrogen

Total nitrogen was determined by micro-Kjeldahl digestion method (Bremner and Mulvaney, 1982).

e. Available phosphorus

Available phosphorus was determined by the Bray 1 method (Olsen and Sommers, 1982).

f. Exchangeable bases

Exchangeable bases for each soil was extracted with 1N ammonium acetate at pH 7.0 buffer (Chapman, 1965). Exchangeable K and Na was determined using flame photometer while exchangeable Ca and Mg was determined using Atomic Adsorption Spectrophotometer (AAS) (Jackson, 1958).

g. Exchangeable acidity

Exchangeable acidity was determined by titration method as described by McLean (1982).

h. Cation exchange capacity (CEC)

The soil CEC was determined by 1N ammonium acetate saturation method (Anderson and Ingram, 1993).

3.1.4. Experimental Treatments and Design

Soybean variety TGx 1448-2E was used as a trap crop. The trial was made up of 3 treatments. The first treatment had mineral nitrogen which was the reference, the second treatment had a known commercial inoculant (Legumefix) which contained *Bradyrhizobium japonicum* 532c while the third treatment was an uninoculated control with no N applied. All treatments were replicated three times and arranged in Randomized Complete Block Design (RCBD).

Fertilizer application

The following fertilizer treatments were applied to all pots to ensure adequate nutrient supply.

$ZnSO_4$	0.079mg/pot
$MgSO_4$	0.085mg/pot
TSP	0.507mg/pot
$NaMoO_4.2H_2O$	0.004mg/pot
KCl	0.117mg/pot

Urea was used as nitrogen source at the rate of 0.263mg in each of the relevant pots.

The urea was applied in split form, the first dose at planting and the second dose 14 days after planting. The fertilizers were added (excluding the second portion of N source) and mixed with the soil in each pot thoroughly to ensure uniform distribution of the nutrients before planting.

3.1.5. Seeds Sterilization, Inoculation and Planting

The seeds were surface sterilized to rid them of contamination, especially with *Rhizobium*. This was done by rinsing the seeds in a sterile beaker containing 95% ethanol for 10 seconds to rid the seeds of waxy material and trapped air. The ethanol was then drained and the seeds completely immersed in 3% sodium hypochlorite for 3-5 minutes after which they were rinsed with six changes of sterile distilled water (Somasegaran and Hoben, 1985). Five seeds were planted per pot and then later thinned to two plants per pot at two weeks after planting. The plants were irrigated once or twice daily depending on the moisture content of the soil.

3.1.6. Data Collection

At eight weeks after planting, the plants were harvested and the following parameters assessed: nodule number, nodule fresh weight, shoot dry weight and root dry weight. To retrieve the root nodules, whole plants were carefully removed from the pots to avoid detachment of the nodules from the roots. Soil from root materials was carefully and gently removed with hands. The roots were washed by carefully immersing in a bucket of water to completely remove the soil. The nodules were then detached from and placed in vials containing silica gel previously dried at 110ºC (Bala *et al.*, 2010). The roots and shoots were oven dried at 65^{o}C for 72hours. The nodules were not oven dried because they were subsequently used for isolation of rhizobia. Hence, they were preserved by placing in vials containing silica gel before the isolation.

3.2. Isolation and Characterization of Rhizobia Strains

3.2.1. Isolation and Authentication

Desiccated nodules were rehydrated by immersing in clean beaker of cool water and leaving in the refrigerator to imbibe overnight after which intact, undamaged nodules were randomly selected and immersed in sterile beakers containing 95% ethanol for 5-10 seconds to break surface tension and to remove air bubbles from nodule tissues. The nodules were then transferred to a 3% (v/v) solution of sodium hypochlorite for 3-5 min and then rinsed in 6 changes of sterile distilled water using sterile forceps for transferring. Each surface sterilized nodule was crushed with a pair of blunt-tipped forceps in a large drop of sterile water in a Petri dish. One loopful of the nodule suspension was streaked on a yeast-mannitol agar (YMA) plate (Vincent, 1970) containing bromothymol blue (BTB) and Congo Red (CR). The plates were incubated at 28 ºC for 7-10 days and daily observations were made for the appearance of typical colonies of

rhizobia. Typical colonies were isolated and re-streaked on YMA-BTB and YMA-CR and their characteristics observed. Isolates were stored on YMA slant in screw-cap tubes in the refrigerator for subsequent characterizations.

The presumptive isolates were authenticated as rhizobia by testing their capacity to form nodules on roots of legumes from which the isolates originally were obtained as described by Somasegaran and Hoben (1994) using growth pouches. Two growth units were set up for each of the isolates plus two extra units which served as uninoculated control. The soybean seeds were surface sterilized and pregerminated 0.75% (w/v) water agar in Petri dishes. About 20 seeds were evenly spread per plate and incubated at 25-30^{o}C. After germination, two seeds were aseptically transferred to each of the growth pouches. The pouches were fertilized with 50ml Broughton and Dilworth N-free medium and then inoculated with 1ml of the broth culture for each of the isolate with the exception of the controls.. The plants were removed at four weeks after planting and the presence or absence of nodules were noted.

Data Collection

Eight weeks after planting, the plants were uprooted and the nodule number and fresh weight recorded. The shoot and root dry weight were also be recorded after drying at 65^{o}C for 72hrs.

3.2.2. Characterization

a. Morphological characterization

Cultural characteristics/Colony morphology: The following colony morphological characteristics was determined on each of the isolates on the plates;

i. Colour
ii. Shape: round or irregular

iii. Size

iv. Elevation: flat or convex

v. Margin: regular, wavy or stringy

vi. Texture: mucous, greasy, creamy or dry

vii. Opacity: transparent, translucent or opaque

b. Physiological characterization

i. Salt tolerance

Tolerance to Sodium Chloride (NaCl) was tested through determining the growth on YMA medium supplemented with NaCl at a concentration of 1% and 3% (w/v).

ii. pH tolerance

Tolerance to pH was tested on YMA medium set at two pH values of 4.0 and 8.5 using NaOH and HCl.

iii. Temperature Tolerance

Their tolerance to temperature will be assessed at 8^{o}C and 35^{o}C on YMA.

3.3. Calculations

The relative effectiveness index gives an indication of the relative efficiency (RE) and inoculant response (IR) for each of the agro-ecological zones and was calculated using the following expressions:

Relative Efficiency (RE) = 100a/b

Inoculant Response (IR) = 100 (c-a)/a

Where:

a = dry matter weight in control treatment

b = dry matter weight in plus N treatment

c = dry matter weight of inoculated treatment

3.4. Statistical Analysis

The nodule number, fresh weight and plant biomass for each location was analyzed using descriptive statistics.

CHAPTER FOUR

4.0. RESULTS

4.1 Soil Physical and Chemical Properties of the Experimental Sites

The means and ranges of some of the physico-chemical properties of the experimental soils are shown in Table 4.1

4.1.1. Particle size distribution

Results of the particle size distribution of the soils show that the dominant soils in the northern guinea savanna are sandy loam in texture. In the southern Guinea savanna, the soils are sandy loam, sandy clay loam and loamy sand. In the Sudan savanna, the soils are sandy loam, loamy sand and sandy. Detailed results for each site in all the locations with their individual textural classes are shown in Appendix 2.

4.1.2. Soil reaction (pH)

The mean pH of the soils in both water and $CaCl_2$ are 6.5 and 5.3 (slightly acidic) respectively for northern Guinea savanna, with a range of 5.7-7.5 in water and 5.0-6.4 in $CaCl_2$. In the southern Guinea savanna the mean pH recorded in both water and $CaCl_2$ are 5.8 and 4.7 (moderately acidic) respectively , with a range of 5.6-6.6 in water and 4.6-4.7 in $CaCl_2$ while in Sudan savanna the mean pH is 6.2 and 5.2 (slightly acidic) and a range of 5.4-7.3 and 4.4-5.8 in both water and $CaCl_2$ respectively. These ratings are according to FMANR (1990) (Appendix 3).

4.1.3. Organic carbon

The organic carbon content of the soils are considered low (FMANR, 1990) for all the agro-ecologies with northern Guinea savanna having a mean of 3.8 g kg^{-1} and a range of 1.8-11.2 g kg^{-1}, southern Guinea savanna having a mean of 5.01 g $kg^{-1}$and a range of 4.0-11.2 g kg-^{1} and Sudan savanna having a mean of 2.3 g kg^{-1} and a range of 0.4-3.9 g kg^{-1}.

4.1.4. Total nitrogen

The mean total N of the soils for northern Guinea savanna are considered medium (1.9 g kg^{-1}), for southern Guinea savanna high (4.13 g kg^{-1}) and for Sudan savanna high (3.6 g kg^{-1}). The northern Guinea savanna recorded a range of 1.1-2.8 g kg^{-1} , the southern Guinea savanna a range of 1.4-18.9 g kg^{-1} and the Sudan savanna a range of 1.1-7.4g kg^{-1} (FMANR, 1990).

4.1.5. Available P

The available P was generally low for northern Guinea savanna (having a mean of 6.31 mg kg^{-1}). Both southern Guinea savanna and Sudan savanna respectively have a mean of 15.76 mg kg^{-1} and 16.6 mg kg^{-1} which is considered moderate. The range for northern Guinea savanna is 1.75-12.25 mg kg^{-1}, for southern Guinea savanna it is 1.75-26.3 mg kg^{-1} while for Sudan savanna, it is 1.75-31.5 mg kg^{-1} (FMANR, 1990).

4.1.6. Exchangeable Bases and Cation Exchange Capacity

Exchangeable bases: Ca^{2+}, Mg^{2+} and Na^{+} were all of high concentration while K^{+} was of medium concentration in the soils of all the agro-ecologies. The northern Guinea savanna recorded a mean of 7.26 cmol kg^{-1} and a range of 4.20-11.60 cmol kg^{-1} for Ca^{2+}, mean of 2.63 cmol kg^{-1} and range of 0.38-4.83 cmol kg^{-1} for Mg^{2+}, mean of 0.18 cmol kg^{-1} and range of 0.11-0.24 cmol kg^{-1} for K^{+} and lastly a mean of 0.39 cmol kg^{-1} and range of 0.26-0.74 cmol kg^{-1} for

Na^+. The southern Guinea savanna recorded a mean of 8.03 cmol kg^{-1} and a range of 4.60-12.20 cmol kg^{-1} for Ca^{2+}, mean of 3.70 cmol kg^{-1} and range of 2.40-5.80 cmol kg^{-1} for Mg^{2+}, mean of 0.19 cmol kg^{-1} and range of 0.09-0.26 cmol kg^{-1} for K^+ and lastly a mean of 0.31 cmol kg^{-1} and range of 0.23-0.54 cmol kg^{-1} for Na^+ . The Sudan savanna recorded a mean of 7.80 cmol kg^{-1} and a range of 4.40-10.60 for Ca^{2+}, mean of 2.88 cmol kg^{-1} and range of 0.84-4.64 cmol kg^{-1} for Mg^{2+}, mean of 0.19 cmol kg^{-1} and range of 0.06 -0.38 cmol kg^{-1} for K^+ and lastly a mean of 0.45 cmol kg^{-1} and range of 0.18-2.78 cmol kg^{-1} for Na^+ (FMANR, 1990).

4.1.7. Cation Exchange Capacity

The cation exchange capacity (CEC) for the northern Guinea savanna ranged from 6.2-17.2 cmol kg^{-1} with a mean of 11.34 cmol kg^{-1}. This falls within the range of medium fertility according to FMANR (1990). The CEC for the southern Guinea savanna ranged between 8.6-18.7 cmol kg^{-1} with a mean of 13.18 cmol kg^{-1} (high CEC). The CEC for the Sudan savanna ranged from 7.3 and 16.8 cmol $kg^{-1}$and a mean of 12.18 cmolkg^{-1} which is also considered high (see Appendix 2).

Table 4.1 Means and ranges of some of the physico-chemical properties of experimental sites

Agro-ecological zone	Clay (%)	Silt (%)	Sand (%)	pH water (1:2.5)	pH $CaCl_2$ (1:2.5)	OC (g kg^{-1})	TN (g kg^{-1})	Avail. P (mg kg^{-1})	Exchangeable bases (cmol kg^{-1}) Ca	Mg	K	Na	CEC (cmol kg^{-1})
Northern Guinea savanna													
Minimum	8	8	56	5.7	5	1.8	1.1	1.75	4.2	0.38	0.11	0.26	6.2
Maximum	18	28	84	7.5	6.4	11.2	2.8	12.25	11.6	4.83	0.24	0.74	17.2
*Mean	13.16	21.05	65.79	6.46	5.3	3.77	1.9	6.31	7.26	2.63	0.18	0.39	11.34
Southern Guinea savanna													
Minimum	14	4	66	5.6	4.6	4	1.4	1.75	4.6	2.4	0.09	0.23	8.6
Maximum	22	16	82	6.6	4.7	11.2	18.9	26.3	12.2	5.8	0.26	0.54	18.7
**Mean	18	10.29	71.71	5.8	4.7	5.09	4.13	15.76	8.03	3.7	0.19	0.31	13.18
Sudan savanna													
Minimum	8	2	70	5.4	4.4	0.4	1.1	1.75	4.4	0.84	0.06	0.18	7.3
Maximum	18	16	90	7.3	5.8	3.9	7.4	31.5	10.6	4.64	0.38	2.78	16.8
***Mean	9.9	8.09	82	6.24	5.18	2.35	3.6	19.13	7.8	2.88	0.19	0.45	12.18

*Mean of 19 sites

**Mean of 5 sites

***Mean of 21 sites

4.2. Inoculant Response (IR) and Relative Efficiency (RE) in the three agro-ecological zones

4.2.1. Sudan savanna

The total dry matter yield (TDMY) was used to determine both the inoculant response (IR) and relative efficiency (RE) for Sudan savanna as shown in Figure 4.1. The IR ranged from -26.15% to 90.5% while the RE ranged from 55.06% to 112.5%. The figure shows that WDL 1 has the highest response to inoculation (90.5%) followed by ALB 3 (65.11%) and WRA1 has the lowest negative response to inoculation of -26.15%. ALB 1 has the highest relative efficiency of 112.5% followed by GRK 1 which has RE of 111.11% while BKR 1 has the lowest RE of 55.06% (Appendix 4).

4.2.2. Northern Guinea savanna

In the Northern Guinea savanna (Figure 4.2), total dry matter yield (TDMY) was also used to determine the both the relative efficiency (RE) and inoculant response (IR). The IR ranged from -16.67% to 22.2% while the RE ranged from 67.74% to 114.7%. The highest relative efficiency was observed for SOB 4 with RE of 114.7% followed by IGB 2 with RE of 101.4% while the lowest was IGB 3 (67.74%). The highest response to inoculation was in GWA 4 (22.2%) followed by GWA 5 (21.8%) while the lowest was SOB 4 (-16.67) (Appendix 4)..

4.2.3. Southern Guinea savanna

In the Southern Guinea savanna (Figure 4.3), the IR ranged from -25% to 30.3% while the RE ranged from 82.5% to 128.6%. The highest IR was in KCH 4 (30.3%) followed by KOL (22.22%) while the lowest was in KCH 3 (-25%). The highest RE was observed for KOL (128.6%) followed by KCH 1 (100%) while the lowest RE was in KCH 4 (82.5%) (Appendix 4).

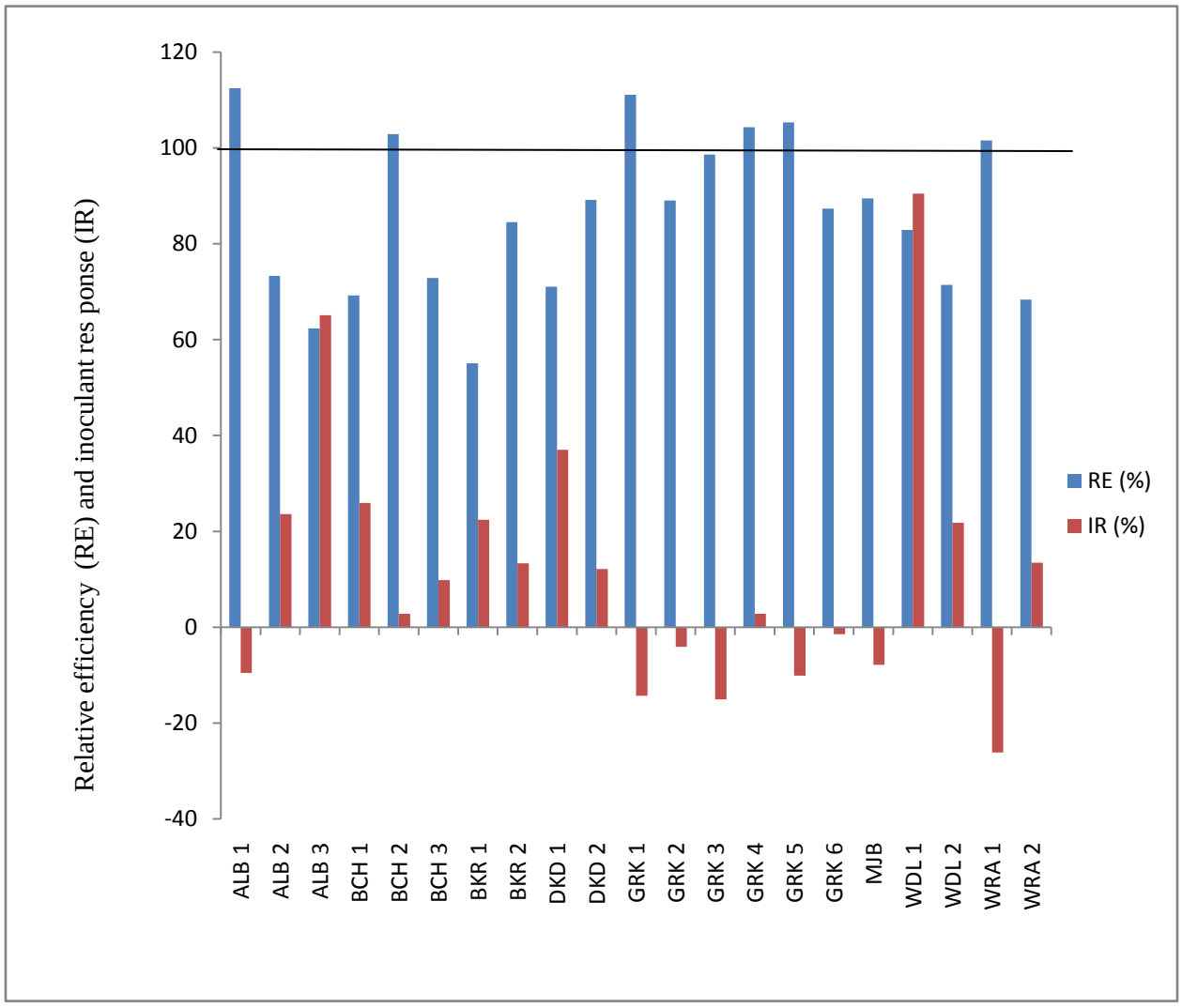


Figure 4.1. Relative Efficiency (RE) and Inoculant Response (IR) in the Sudan savanna

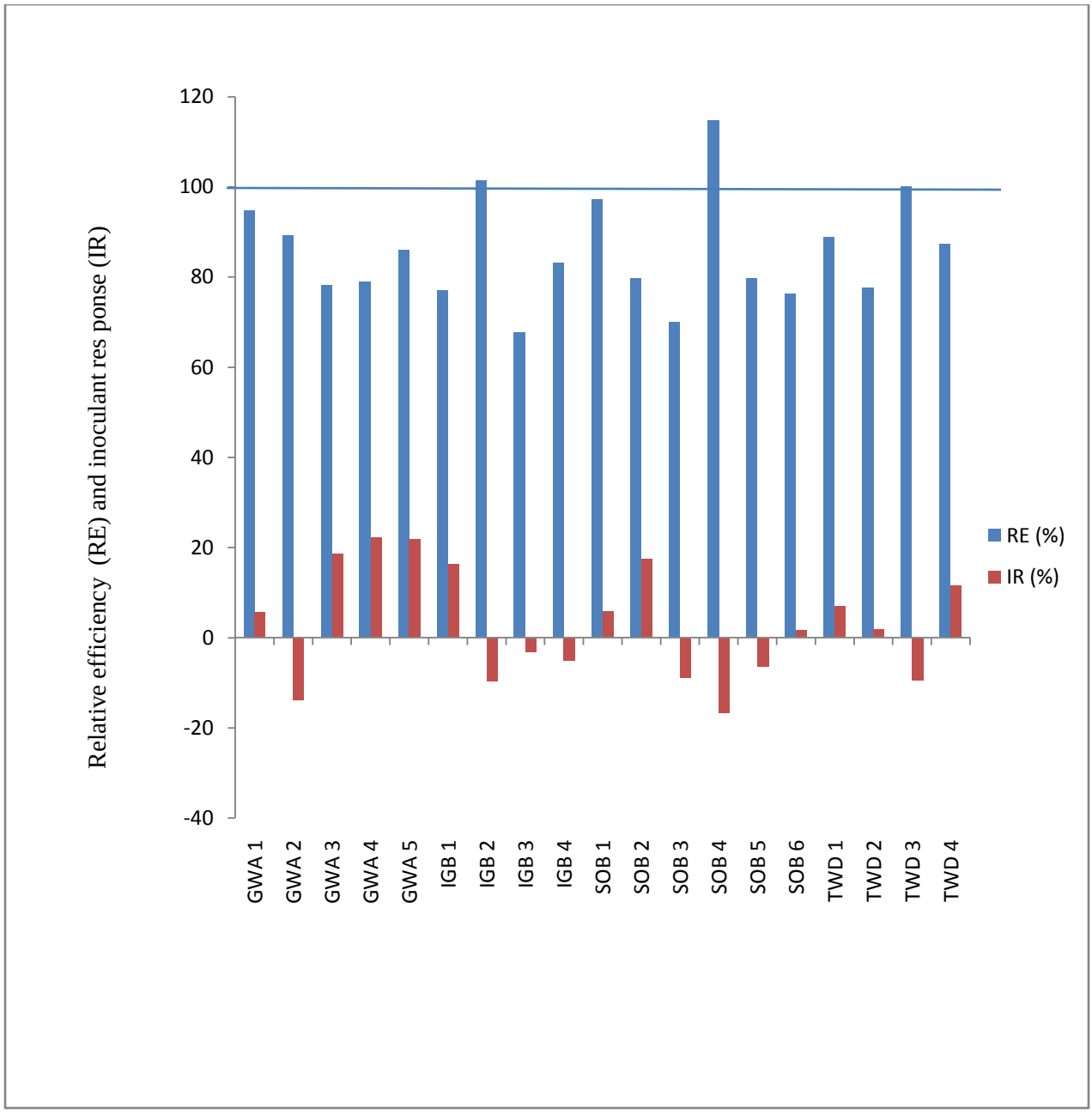


Figure 4.2. Relative Efficiency (RE) and Inoculant Response (IR) in the Northern Guinea savanna

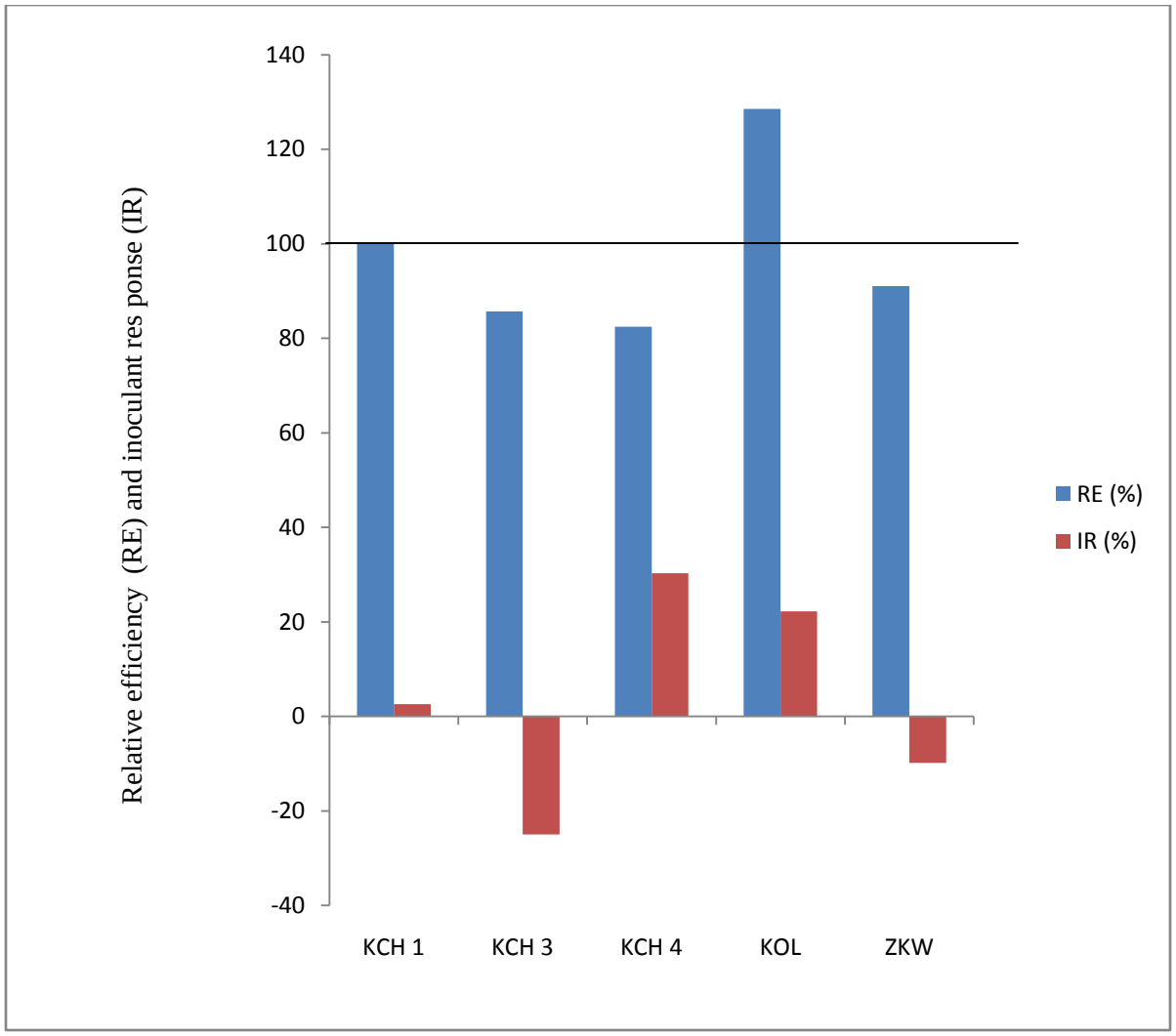


Figure 4.3. Relative Efficiency (RE) and Inoculant Response (IR) in the Southern Guinea savanna

4.3. Effect of Rhizobium inoculation and mineral Nitrogen on soybean Nodulation and Biomass

4.3.1. Nodule number

Nodule number was higher in the inoculated treatments compared to mineral N across all the AEZs. However there are some locations which showed similar response regardless of whether inoculated or mineral N was applied. The 1:1 line in Figure 4.4 indicates that nodule number was higher in inoculated treatments compared to the uninoculated control in over 95% of the experimental sites.

In the Sudan savanna, response to inoculation was higher compared to response to N application. Almost all locations had nodule number above the benchmark (Figure 4.5) suggesting that nodule number was much higher in the inoculated pots compared to uninoculated (control) pots.

In the northern Guinea savanna, there was also high response to inoculation as compared to the control (Figure 4.6). However, the mineral N treatment gave the highest nodule number.

In the southern Guinea savanna, the inoculated treatment gave the highest nodule number (Fig 4.7). Response to inoculation was higher compared to control and mineral N application. However, in some locations, similar response was observed for both the inoculated and mineral N treatments.

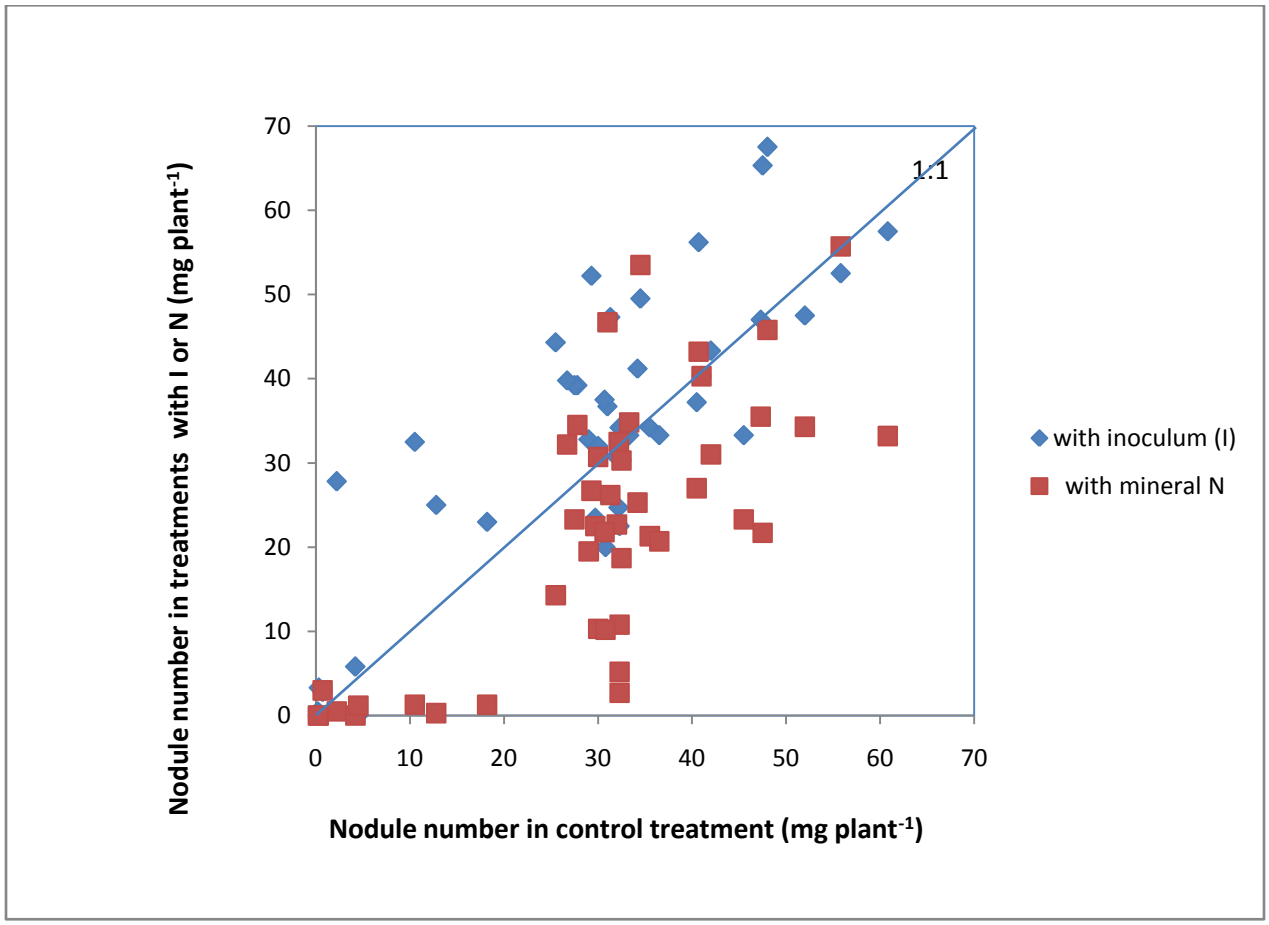


Figure 4.4. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on nodule number in all the AEZs.

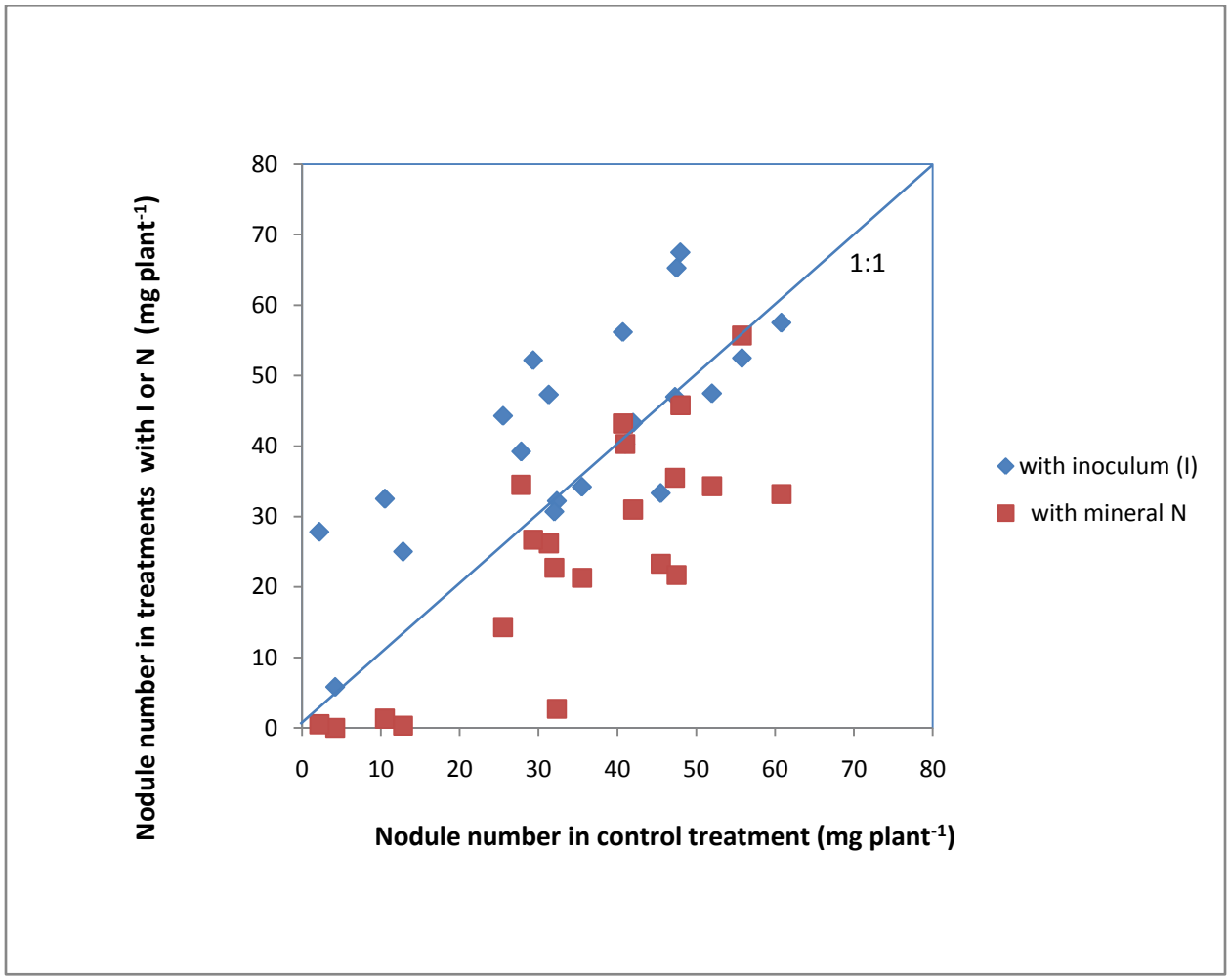


Figure 4.5. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on nodule number in the Sudan savanna.

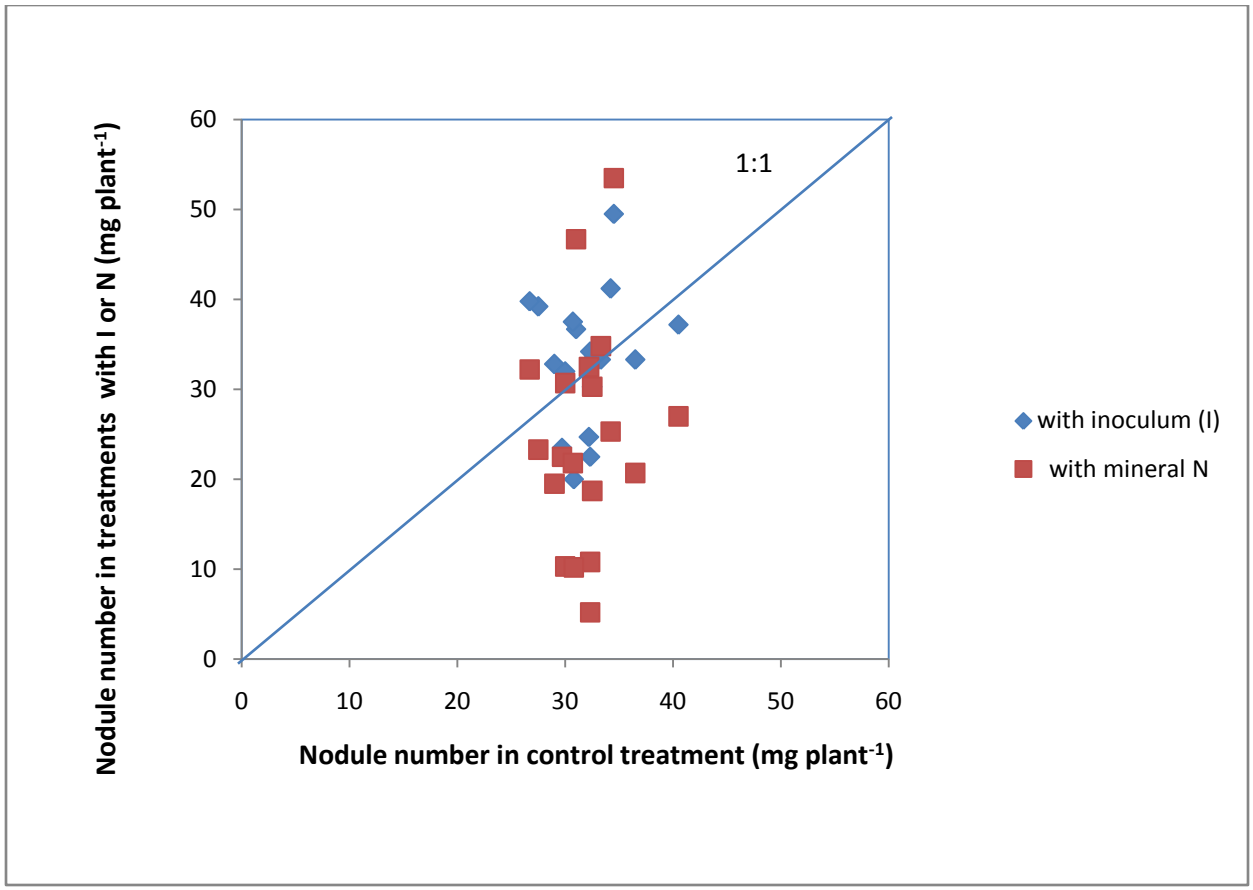


Figure 4.6. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on nodule number in the northern Guinea savanna.

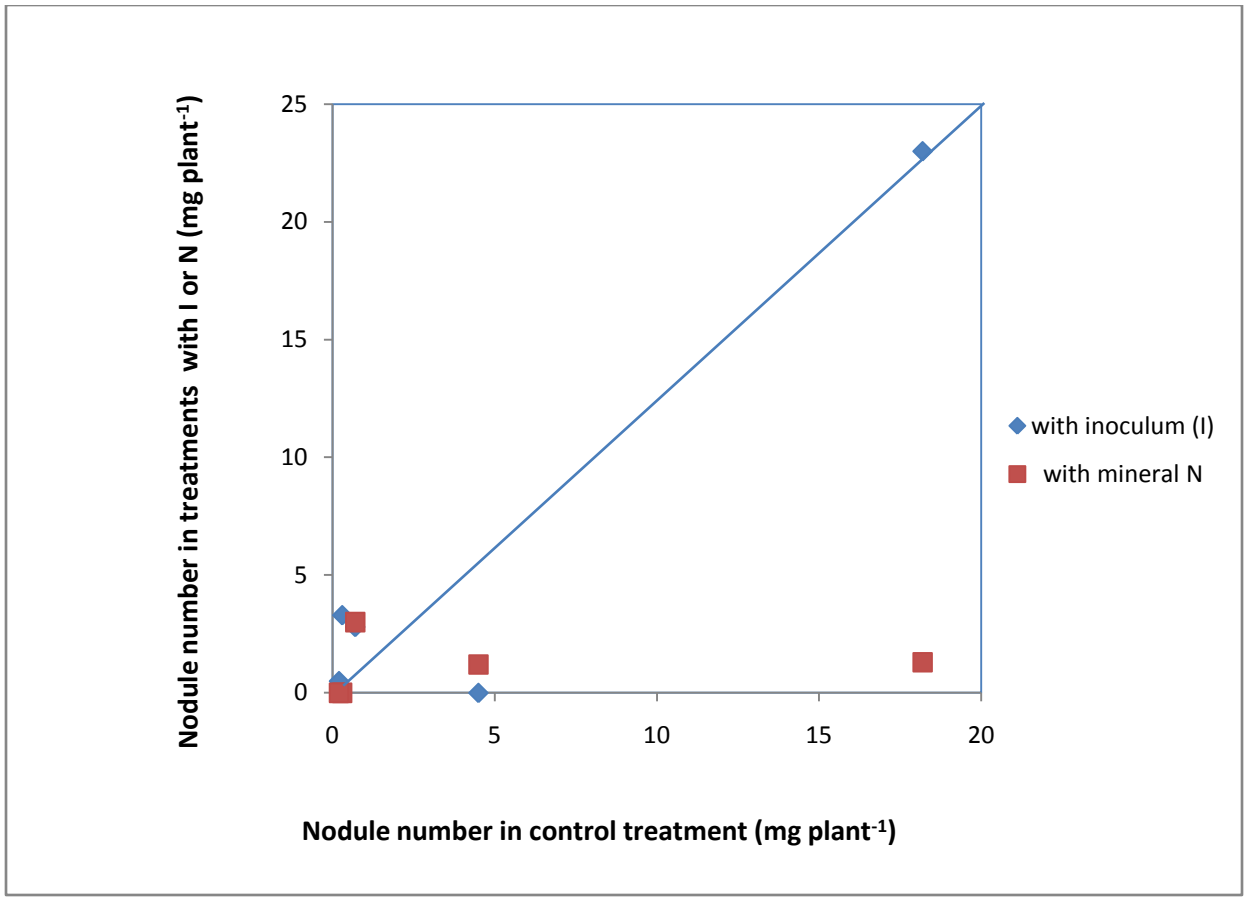


Figure 4.7. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on nodule number in the southern Guinea savanna.

4.3.2. Nodule fresh weight

Nodule fresh weight was generally highest in the inoculated treatment compared to control and mineral N application across all the AEZs. However, there are some locations which showed similar response regardless of whether inoculated or mineral N was applied i.e. they were at par with each other (Fig 4.8).

The highest nodule fresh weight was observed in the inoculated treatment in the Sudan savanna (Fig 4.9). Response to inoculation was higher in inoculated treatment as compared to control and mineral N application. However, the mineral N treatment in some locations showed similar response as that of inoculated treatments.

In the northern Guinea savanna, there was also a high response to inoculation in terms of nodule fresh weight. However mineral N treatment in most locations had nodule fresh weight which were more comparable to the control (Fig 4.10).

In the southern Guinea savanna, the inoculated treatment gave the highest nodule fresh weight as in the case of the nodule number. Response to inoculation was higher when compared with the control. However, in some locations, similar response was observed for both the inoculated and mineral N treatments (Fig 4.11).

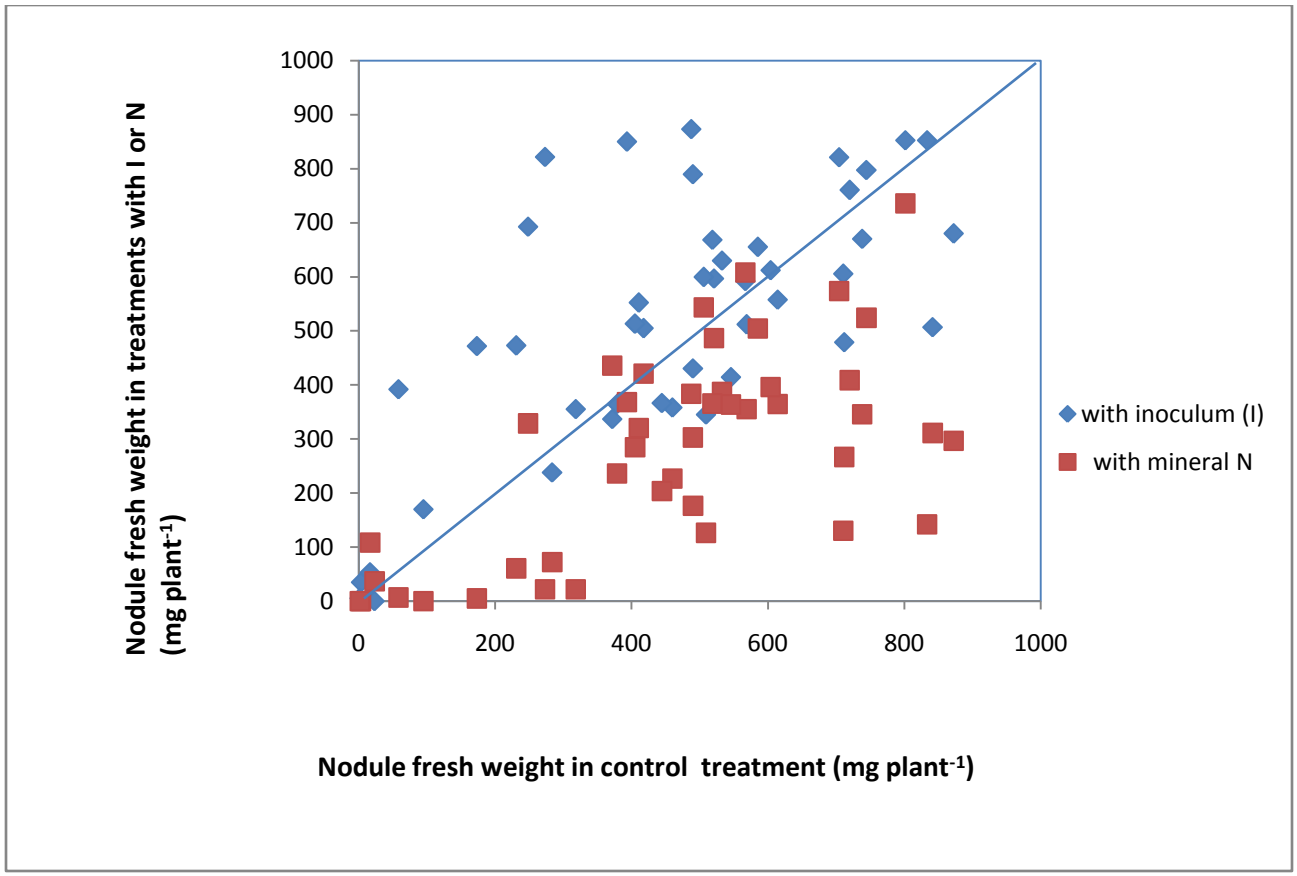


Figure 4.8. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on nodule fresh weight in all the AEZs.

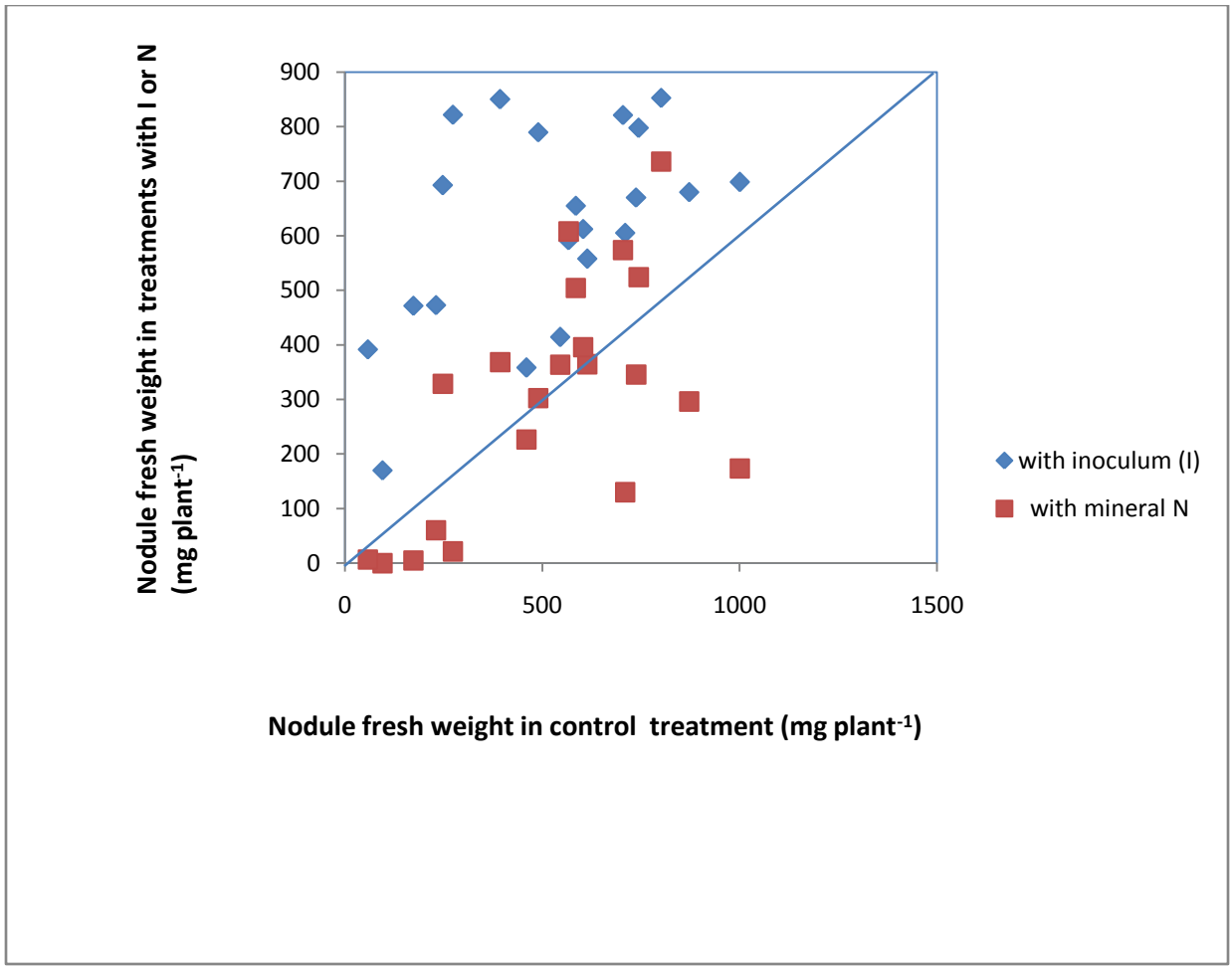


Figure 4.9. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on nodule fresh weight in the Sudan savanna.

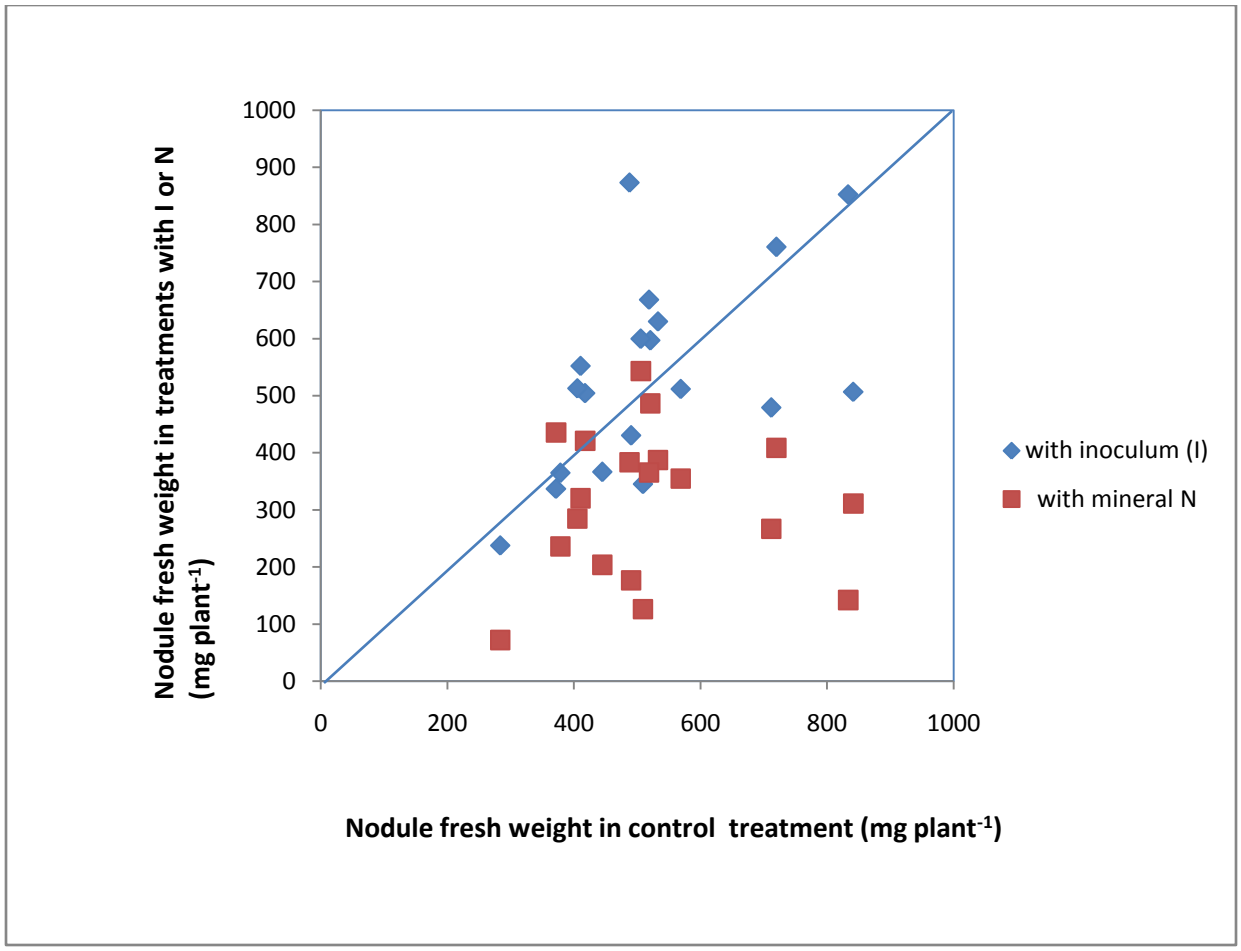


Figure 4.10. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on nodule fresh weight in the northern guinea savanna

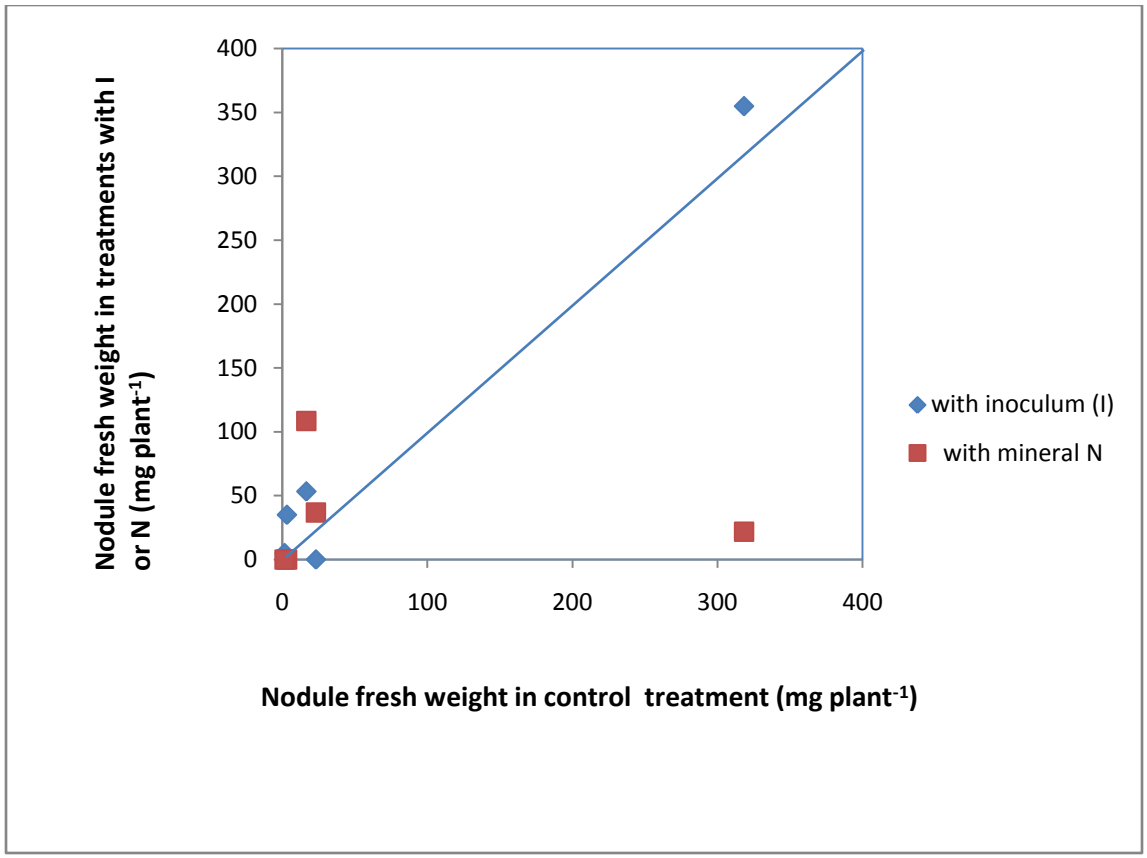


Figure 4.11. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on nodule fresh weight in the southern Guinea savanna.

4.3.3. Shoot dry matter yield

The response of soybean to rhizobia inoculation in terms of shoot dry weight also varies with location. In most of the locations, similar trend was observed irrespective of whether inoculated or mineral N was applied. This can be observed in Figure 4.12 where the points overlap each other. But generally response observed for mineral N was slightly higher than inoculated and control treatments.

In the Sudan savanna, response to mineral N was still slightly higher than inoculant. With respect to control, it was observed that even though mineral N treatment gave a slightly higher biomass, in some locations, the response was the same irrespective of whether inoculated or mineral N was applied (Figure 4.13).

In the northern Guinea savanna, there was better response when mineral N was added than when inoculated. However, there are still some locations where inoculation gave higher shoot dry matter yield than mineral N application (Figure 4.14).

The shoot dry weight for the southern Guinea savanna was higher for mineral N as compared to inoculated. However, for a certain location, a response to inoculation similar to N application, which was also high was observed. (Figure 4.15).

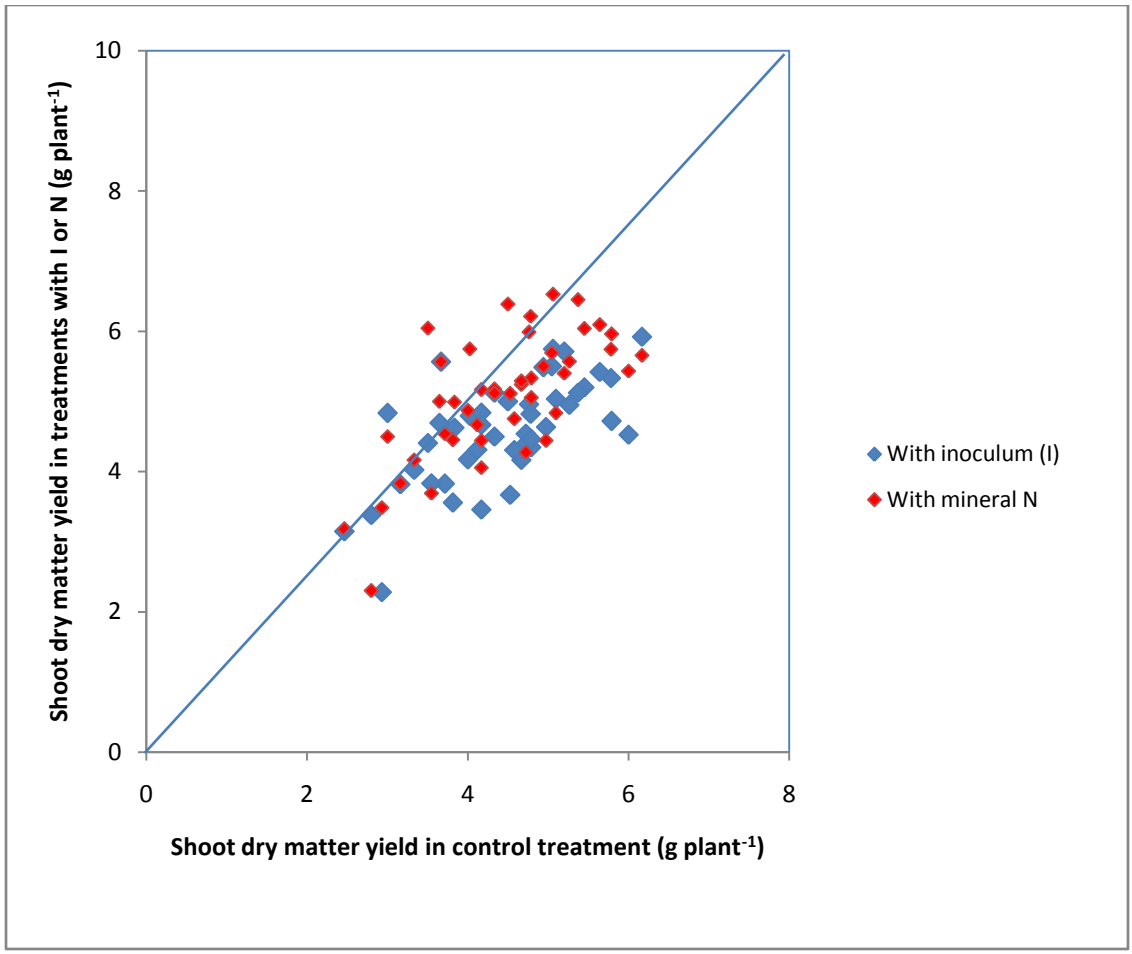


Figure 4.12. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on shoot dry matter yield in all AEZs.

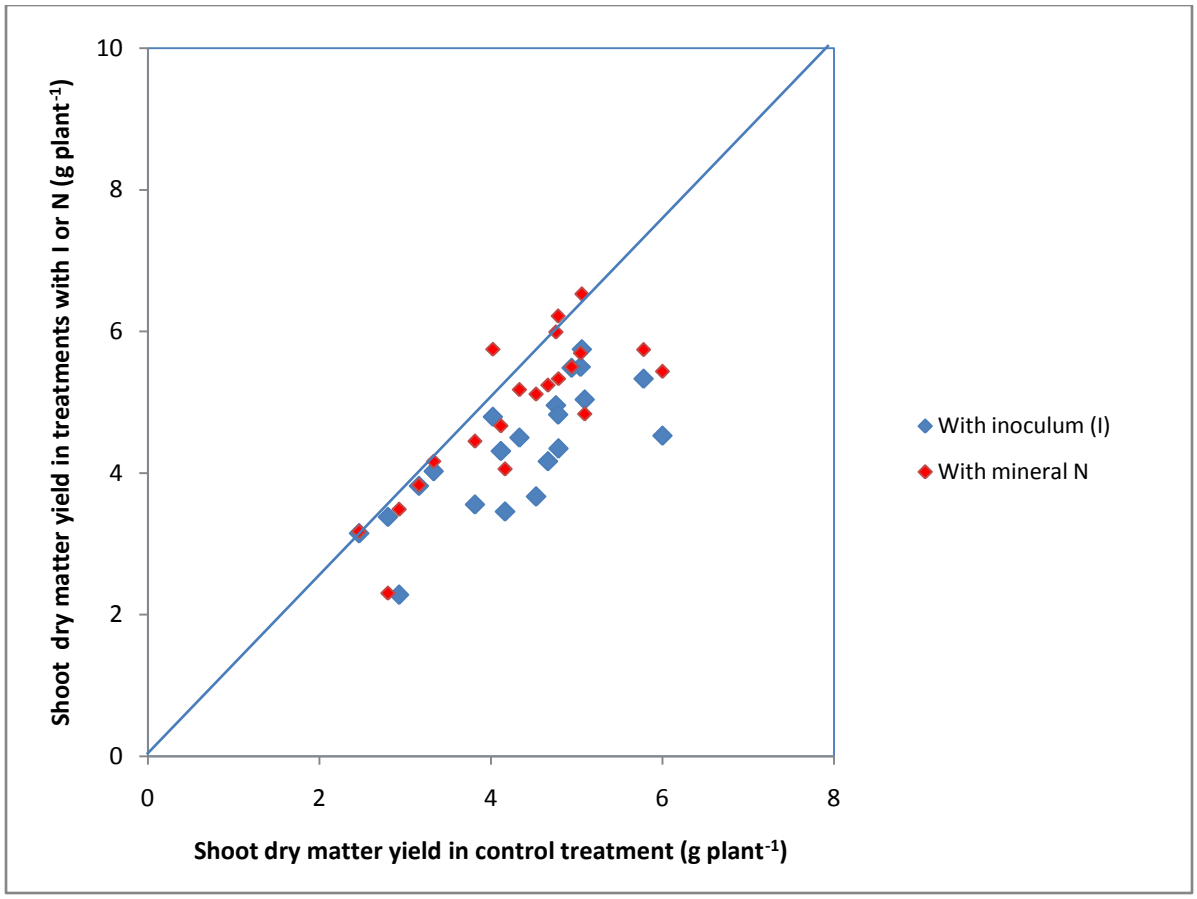


Figure 4.13. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on shoot dry matter yield in the Sudan savanna.

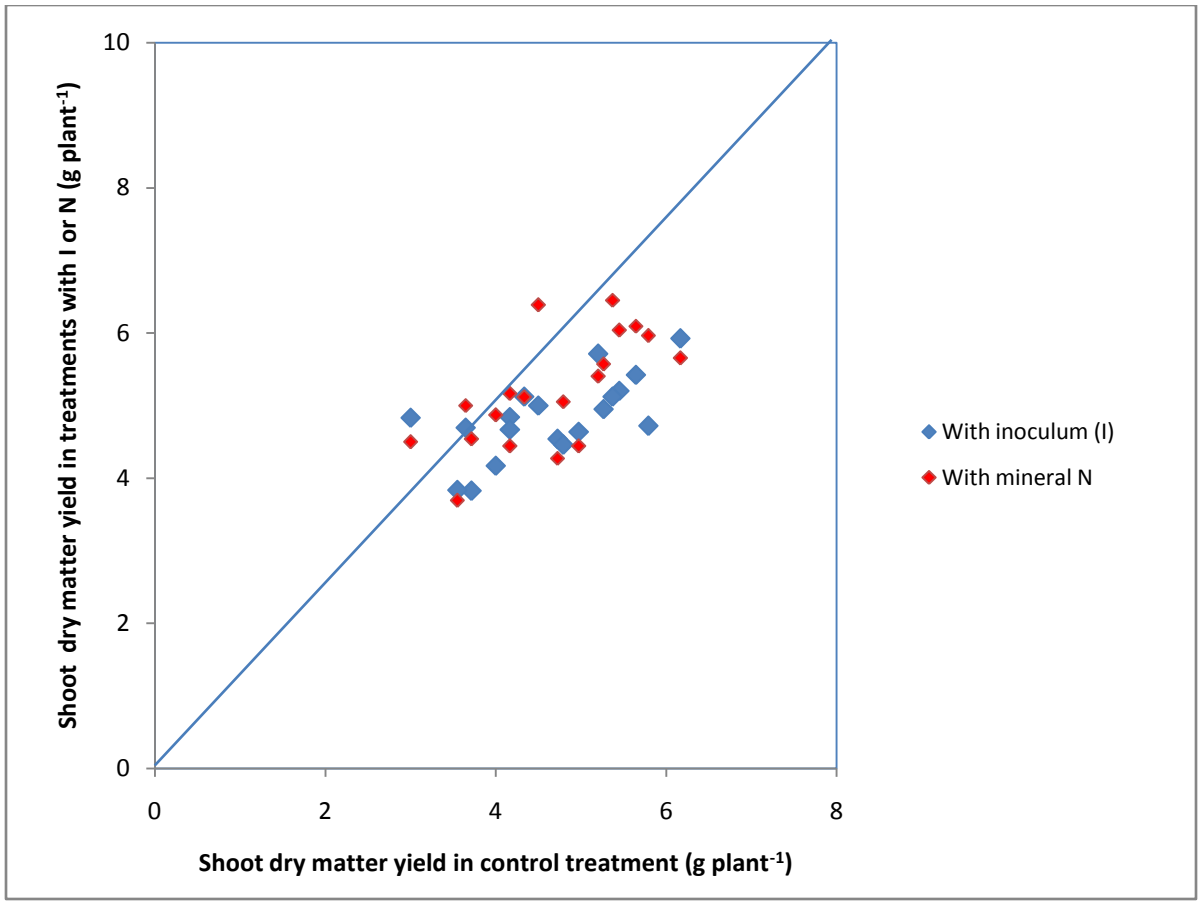


Figure 4.14. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on shoot dry matter yield in the northern Guinea savanna.

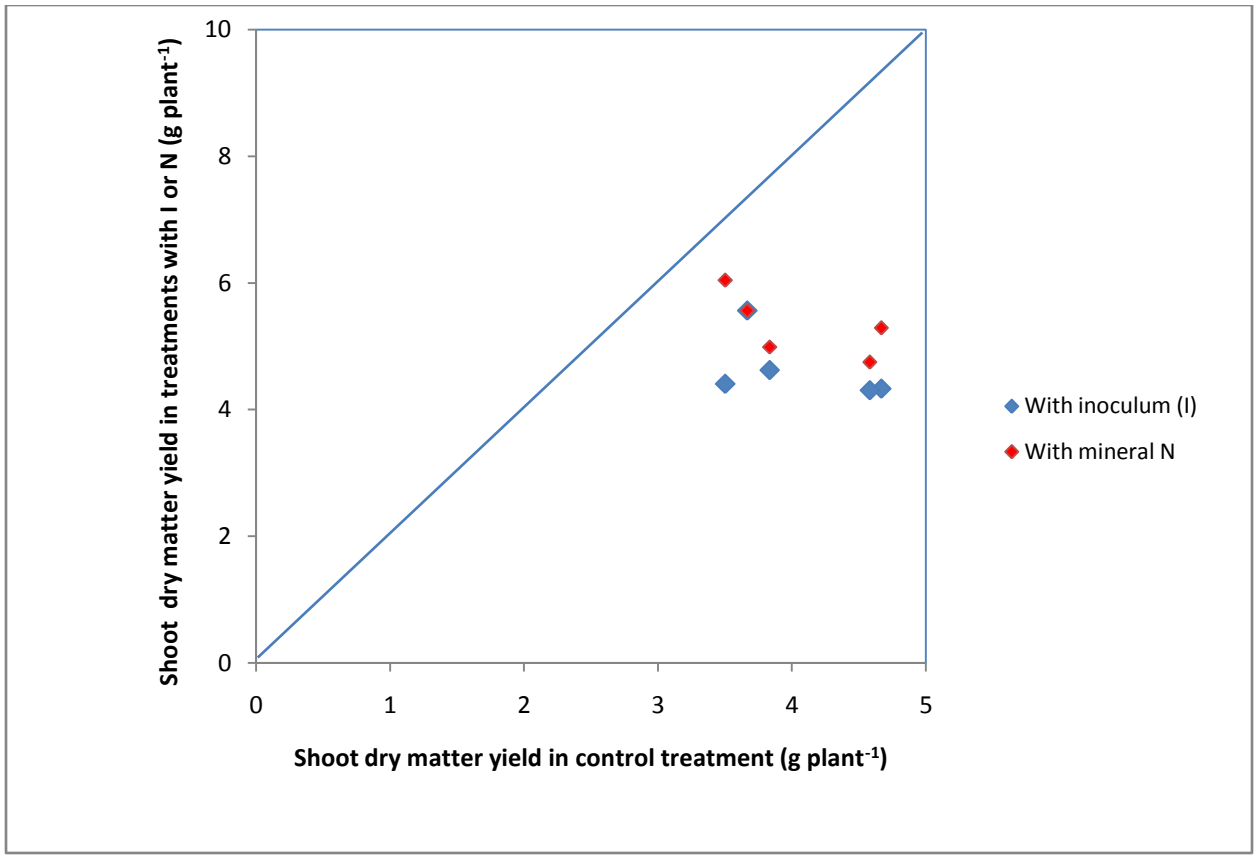


Figure 4.15. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on shoot dry matter yield in the southern Guinea savanna.

4.3.4. Total dry matter yield

The response of soybean to rhizobia inoculation in terms of total dry matter yield (TDMY) varies with location. Generally, in all the agro-ecological zones, the total dry matter yield for both the inoculated and mineral N treatments were high compared to control (Figure 4.16). The result shows that the highest TDMY were observed in some the plus N treatment. However, in some locations, TDMY was higher in inoculated treatments compared to mineral N was applied.

In the Sudan savanna, similar trend was observed as that of all the AEZs with some locations having higher response to inoculation and mineral N treatments relative to control treatment. However, the control treatment in some locations outperformed the inoculated and mineral N treatments (Figure 4.17).

In the northern Guinea savanna, there was less response to inoculation when compared to the Sudan savanna. Response to mineral nitrogen was also high with respect to control. However, in some locations, control treatments performed similarly to inoculated treatments as shown in Figure 4.18 where the points were below the critical line.

In the southern Guinea savanna, samples were collected from relatively few sites, which is not sufficient to provide information that will confidently allow for suitable recommendation. However, inoculation produced the highest TDMY.

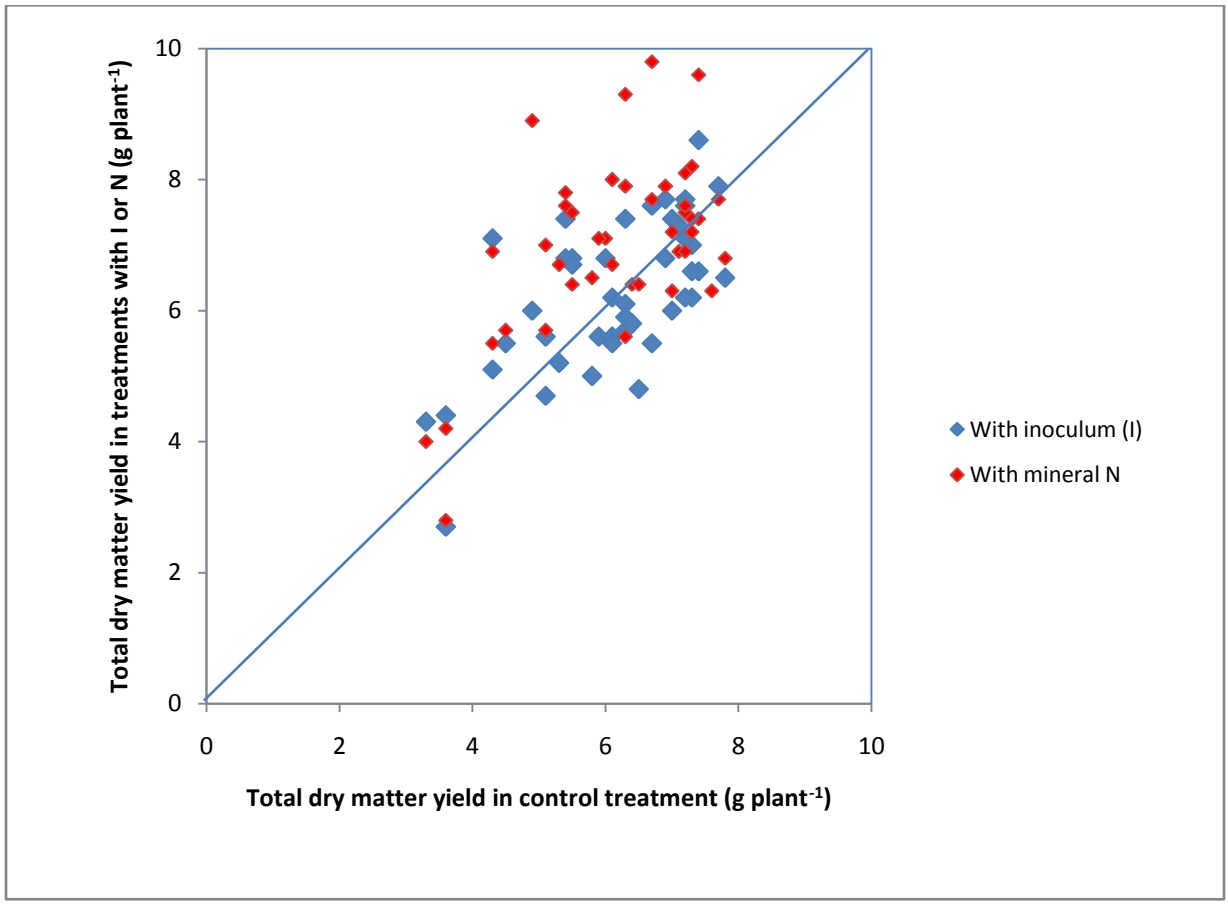


Figure 4.16. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on total dry matter yield in all the agro-ecological zones.

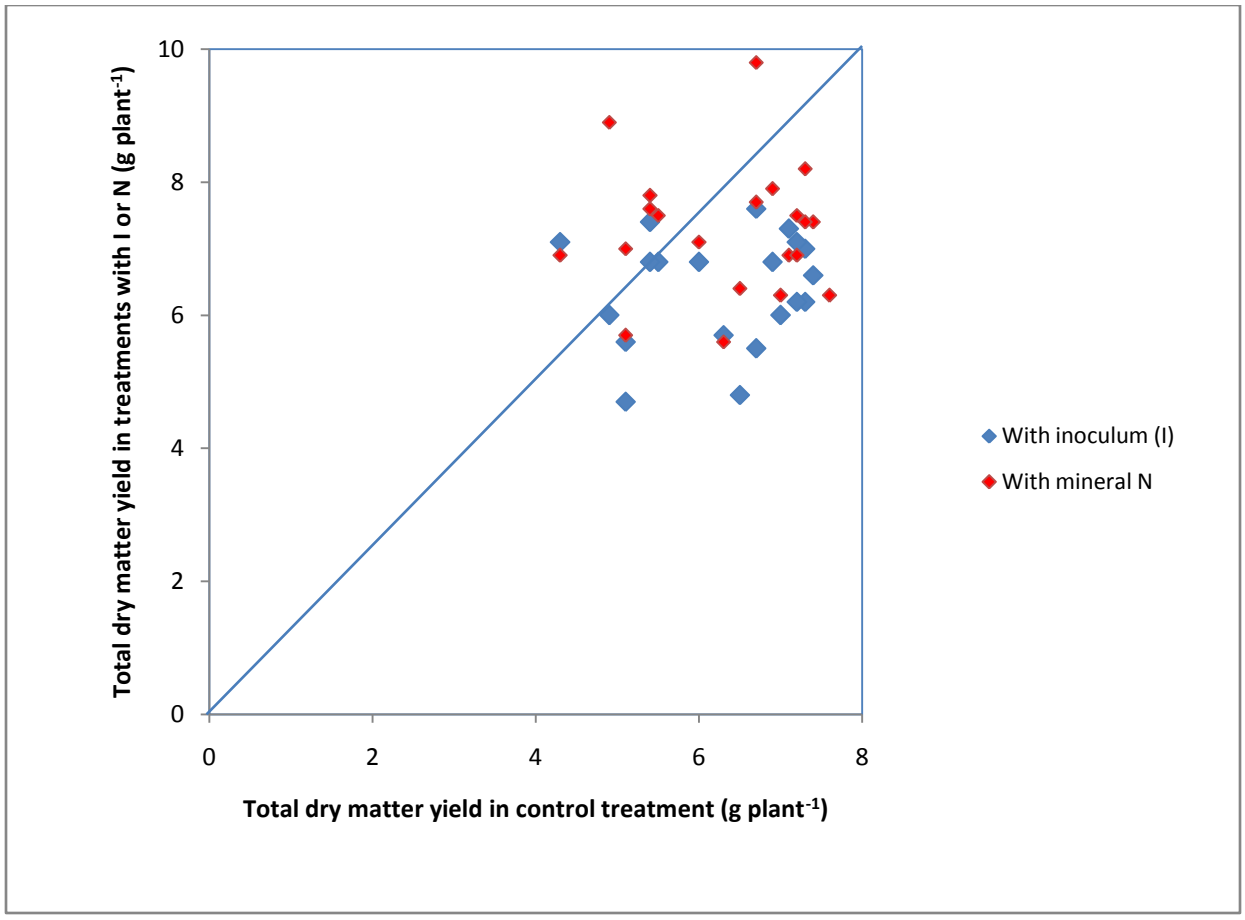


Figure 4.17. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on total dry matter yield in the Sudan savanna

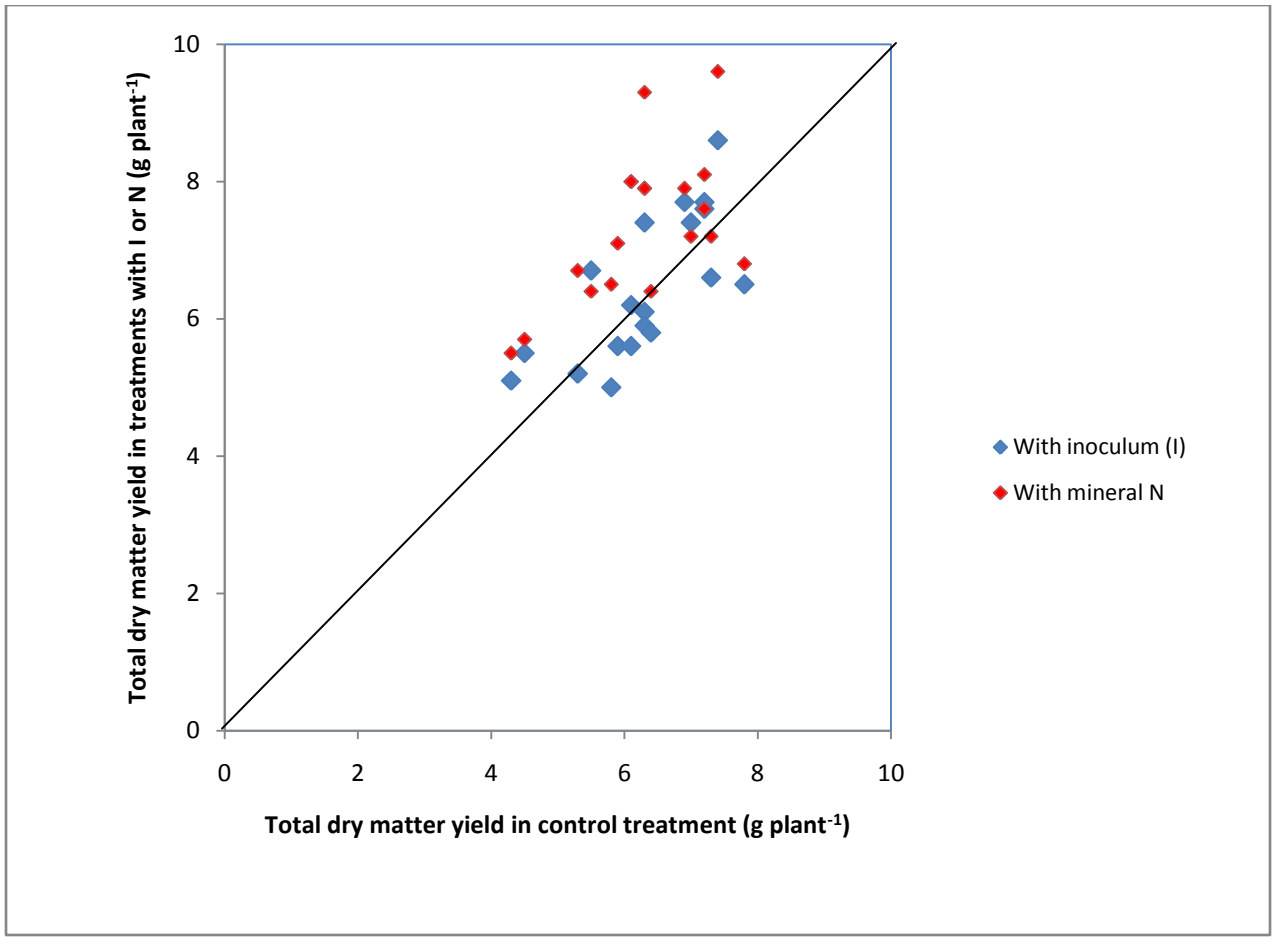


Figure 4.18. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application on total dry matter yield in the northern guinea savanna

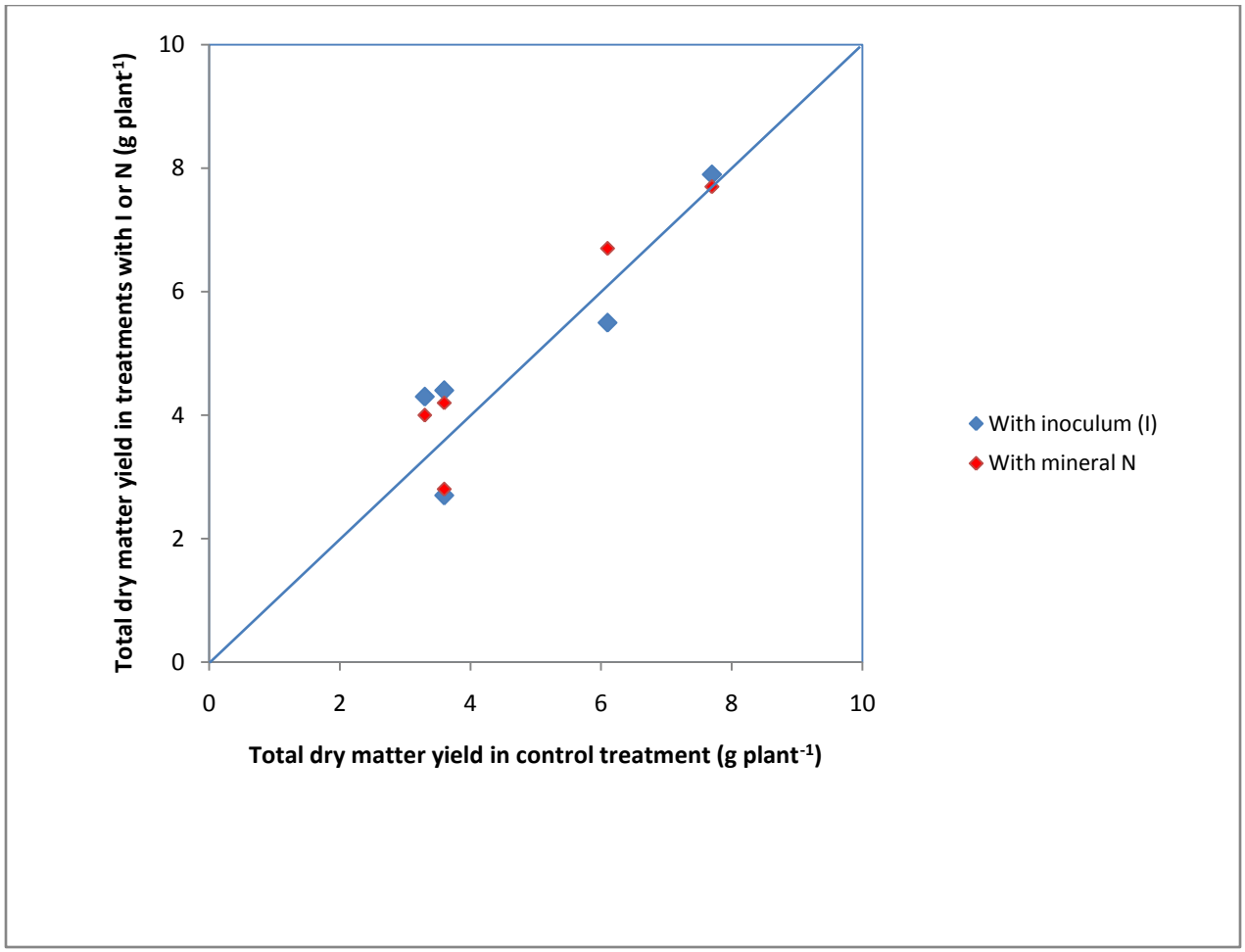


Figure 4.19. Effect of *Bradyrhizobium* inoculation (I) and mineral nitrogen (N) application total dry matter yield in the southern guinea savanna.

4.4. Isolation, Authentication and Characterization

4.4.1. Isolation and Authentication

A total of 350 strains were isolated on YMA medium incorporated with Congo red and Bromothymol blue. The presumptive isolates were authenticated as rhizobia by testing their capacity to form nodules on roots of legumes from which the isolates originally were obtained as described by Somasegaran and Hoben (1994) in screenhouse. Culture collection numbers were assigned to each of the strains isolated. For the purpose of this research, the strains were called 'SAMFIX'. Therefore SAMFIX 1-350 are being kept in the Soil Microbiology unit of the Department of Soil Science.

A. Reaction on Congo red (CR)

Variation in the intensity of dye absorption was observed among the rhizobia strains when plated on CR-YMA at 28°C. Most isolates did not absorb Congo red. However some isolates were able to absorb the dye weakly, some in the centre of the colonies and few absorbed the dye well. Based on this observation, the isolates were grouped into four, namely: non absorbant, partly absorbant, centre absorbant and fully absorbant. The summary of observations is shown in Table 4.2.

B. Reaction on Bromothymol blue (BTB)

Reaction on BTB also showed variation. Most isolates were acid producing, none were basic, while few were non reactive on BTB-YMA. The acid producers changed the medium from green to yellow while the non reactive isolates did not alter the pH, that is, the media retained its green colour even after 10 days of incubation. The summary of results is also shown in Table 4.2.

Table 4.2. Reaction of Rhizobia on BTB-YMA and CR-YMA.

	REACTION	NUMBER	PERCENTAGE (%)
CR-YMA	Non Absorbant	198	56.57
	Partly Absorbant	95	27.14
	Centre Absorbant	53	15.14
	Fully Absorbant	4	1.14
BTB-YMA	Acidic	331	94.57
	Non Reactive	19	5.43
	Basic	0	0

4.4.2. Characterization

All 350 strains isolated were characterized based on their cultural characteristics. A total number of 20 strains were characterized based on their physiological characteristics. Phenotypically all isolated strains had variation in colony morphology.

A. Morphological characterization

The cultural characteristics/colony morphology was determined which comprises of the colour, shape, size, elevation, margin, texture and opacity. The results of these observations are summarized in Table 4.3. From the study, most of the isolates were observed to be transient growers as colonies appeared within 24 hours of inoculation.

A total of 94% of the isolates were fast growers and 6% were intermediate growers. No slow growers were recorded in the course of the research. All isolated colonies were round in appearance. Eighty six percent (86%) of the colonies were milky white in appearance, 9.1% were clear (colorless) and 4.9 were yellow in colour. The results showed 29.4% large colonies, 15.1% medium sized colonies and 55.4% small colonies. Colony elevation showed 18.9% flat and 81.1 % raised colonies. 75.4 % of the isolates had regular margins, 21.4% were wavy in appearance while 3.1% were stringy or rib-like. The texture of the isolates showed that 40.6% were creamy, 53.1 % were mucoid while 6.3 % produced dry colonies. Some of the isolates had different reaction to light (opacity) with 15.7% being transparent, 78% being translucent and 6.3% being opaque.

Table 4.3. Summary of strains colony morphology

PROPERTIES	NUMBER OF STRAINS	PERCENTAGE (%)
GROWTH RATE		
Fast	329	94.0
Slow	0	0.0
Intermediate	21	6.0
TOTAL	350	100
SHAPE		
Round	350	100.0
Others	0	0.0
TOTAL	350	100
COLOUR		
Milky	301	86.0
Clear	32	9.1
Yellow	17	4.9
TOTAL	350	100
SIZE		
Large	103	29.4
Medium	53	15.1
Small	194	55.4
TOTAL	350	100
ELEVATION		
Flat	66	18.9
Raised	284	81.1
TOTAL	350	100
MARGIN		
Regular	264	75.4
Wavy	75	21.4
Stringy	11	3.1
TOTAL	350	100
TEXTURE/MUCOSITY		
Creamy	142	40.6
Mucous	186	53.1
Dry	22	6.3
TOTAL	350	100
OPACITY		
Transparent	55	15.7
Translucent	273	78.0
Opaque	22	6.3
TOTAL	350	100

B. Physiological characterization

The effect of extreme pH, extreme temperature and different salt levels on 20 strains were evaluated and the results are shown in Table 4.4.

(i) pH tolerance

The sensitivity of the isolates to extreme pHs was examined on YMA set at pH values of 4.0 and 8.5. SAMFIX, 7, 76, 53, 144, 23, 26, 279, 164, 177, 67, 109, 85, 285, and 292 were able to grow weakly at both pH 4.0 and 8.5. SAMFIX 238 and 13 did not grow at both pH 4.0 and 8.5 while SAMFIX 39, 270, 158 and 210 grew well at pH 4.0 but very weakly at pH 8.5.

(ii) Temperature tolerance

The isolates' tolerance to temperature was assessed at 8 and 35^{o}C on YMA. SAMFIX 7, 76, 53, 23, 26, 279, 158, 67, 109, 285, 292 were able to grow weakly at a temperature of 8^{o}C. SAMFIX 144, 39, 270, 164, 210, 177, 85 grew well at temperature of 8^{o}C but SAMFIX 238 and 13 did not grow at this temperature. At a temperature of 35^{o}C, only SAMFIX 39 and 210 grew well. SAMFIX 7, 76, 53, 144, 23, 270, 26, 279, 164, 158, 177, 67 grew very weakly while 238, 13, 109, 85, 285, 292 did not grow at all at this temperature.

(iii) Salt tolerance

Tolerance to sodium chloride (NaCl) was assessed by determining the growth on YMA medium supplemented with NaCl at a concentration of 1% and 3% (w/v). Most of the isolates were able to grow in the presence of 1% NaCl, although there was variation among the strains in the extent of growth.

At 1% NaCl concentration, there was weak growth observed for SAMFIX 7, 76, 53, 39, 270, 26, 164 and 67 but not at all at 3%. SAMFIX 13, 85 and 285 did not grow at all at 1% and 3% NaCl

concentration. SAMFIX 144, 238, 23, 279, 158, 210, 177 and 109 grew weakly at both concentrations while SAMFIX 292 grew well at 1% but weakly at 3%.

Table 4.4. Effect of extreme pH, temperature and salt concentration on Rhizobium growth.

Strains	pH		Temperature (°C)		Salt concentration (%)	
	4	8.5	8	35	1	3
SAMFIX 7	±	±	±	±	±	-
SAMFIX 76	±	±	±	±	±	-
SAMFIX 53	±	±	±	±	±	-
SAMFIX 144	±	±	+	±	±	±
SAMFIX 238	-	-	-	-	±	±
SAMFIX 23	±	±	±	±	±	±
SAMFIX 39	+	±	+	+	±	-
SAMFIX 270	+	±	+	±	±	-
SAMFIX 13	-	-	-	-	-	-
SAMFIX 26	±	±	±	±	±	-
SAMFIX 279	±	±	±	±	±	±
SAMFIX 164	±	±	+	±	±	-
SAMFIX 158	+	±	±	±	±	±
SAMFIX 210	+	±	+	+	±	±
SAMFIX 177	±	±	+	±	±	±
SAMFIX 67	±	±	±	±	±	-
SAMFIX 109	±	±	±	-	±	±
SAMFIX 85	±	±	+	-	-	-
SAMFIX 285	±	±	±	-	-	-
SAMFIX 292	±	±	±	-	+	±

(+) means positive growth, (±) means weak growth, (-) means no growth

CHAPTER FIVE

5.0. DISCUSSION

5.1. Soil Characteristics

The soils of the experimental areas are generally slightly acidic to moderately acidic. This is a common feature of savanna soils (Jones and wild, 1975). The pH value falls within the normal range of 5.5-7.0 reported to be optimum for the release of plant nutrients (Sharu *et al.,* 2013). The organic carbon contents were generally low for all the agro-ecologies. Jones and Wild (1975) attributed the low organic carbon of well drained savanna soils to the rapid humification and the low polymerization of humic compounds into stable complexes with clay. It may also be attributed to continuous cropping and bush burning (Esu, 1982). Low organic carbon (OC) of the savanna soils have been largely attributed to the rapid decomposition and mineralization of organic materials under high temperature (Sharu *et al.,* 2013). The total N of the soils for northern Guinea savanna are considered medium and for southern Guinea savanna and Sudan savanna high. The reason could be due to external inputs from inorganic nitrogen fertilizers. It could also be due to crop intensification because land is continuously cultivated in the NGS compared to the SGS and SS due to population density and market. Therefore, it is likely that more nitrogen is conserved in the SGS and SS. Available P was also low for all agro-ecologies. This may also be due to the decomposition of organic matter. The results of the exchangeable bases revealed that the dominant cations in the soils are Ca^{2+} and Mg^{2+}. The higher CEC in the Sudan savanna as compared to the northern Guinea savanna could account for the higher biomass observed in the Sudan savanna.

5.2. Relative Effectiveness Index

The high relative efficiency observed in some locations could be as a result of the interactions of the population of the native rhizobia in those locations. It is suggested that successful nitrogen fixation depends on the interaction between legume genotype, rhizobium strain and environment (Giller, 2001). Relative efficiency greater than 100% implies that the indigenous population of rhizobia was superior to the application of mineral N. The strains isolated from these location(s) have very high potential for use as inoculant. Hence it is not surprising that very few such cases occurred. Notable among such locations are BCH 2 (102.9%), GRK1 (R111.1%), GRK 4 (104.35%), GRK 5 (105.3%), WRA 1 (101.6%) in the Sudan savanna. In the northern Guinea savanna, there is IGB 2 (101.4%), SOB 4 (114.7%). In the southern Guinea savanna, there is KOL (128.6%). The higher inoculant response observed in the Sudan savanna could be as a result of high competitiveness of the introduced strains. The presence of large population density of compatible rhizobia does not preclude the possibility that responses to inoculation can be obtained if competitive and highly effective strains are introduced (Giller, 2001). Similarly, the population of indigenous rhizobia could have been very low in the Sudan savanna. In a study conducted by Aliyu *et al*. 2013 in Shanono which lies in the Sudan savanna, it was reported that the native rhizobia population was 1.10×10^2 which was quite low suggesting that the soils have little or no N fixing ability for soybean. Hence, introduction of a commercial inoculant would have resulted in high response. Low response to inoculation observed in some locations may have been caused by the presence of indigenous Bradyrhizobia that were more efficient than the introduced strain. On the other hand, high response to inoculation in other locations could be as a result of high effectiveness of the inoculant. Kumaga and Etu-Bonde (2000), demonstrated that

nodulation and N_2 fixation of promiscuous soybean may be increased by inoculation with effective Bradyrhizobia.

The negative response observed in certain locations could be as a result of the competitiveness of the indigenous rhizobia that were more efficient than the introduced strain. It was reported by Meade *et al*. (1985) that successful competition for nodule sites by native rhizobia is one reason for the failure to achieve a response to inoculation with elite rhizobial strains.

In the northern Guinea savanna, SOB 4 interestingly had the highest RE and the lowest IR. This suggests that the population of the indigenous rhizobia may have being highly competitive for nodule occupancy and highly effective in their nitrogen fixing ability. Therefore there will be no need for inoculation or mineral N application. It is generally argued that indigenous populations are highly adapted to their local soil environments and may form more effective symbioses than commercial inoculants isolated from a distant and un-related soil environment (Gandee *et al*., 1999). Similarly, this AEZ has a long history of soybean cultivation and inoculant use (Sanginga *et al*., 1995, 2003; Okogun *et al*., 2004; Yusuf *et al*., 2011). The previously introduced strains might have replaced the indigenous population, thus response to inoculation may be very minimal. This situation can be circumvented if superior strains are present in inoculants to be used in the zone.

5.3. Agronomic Responses

5.3.1. Effect of Rhizobium Inoculation and Mineral Nitrogen on Nodulation and Biomass Production

The total dry matter yield in all agro-ecological zones showed that there were responses for both the inoculated and mineral N treatments. The implication here is that, even though there were less points above the bench mark (1:1 line), some of the indigenous strains of rhizobia

outperformed the introduced commercial strain in some of the locations. The high response to mineral N generally observed in this study could also be achieved with highly effective strain(s), though not investigated in this study. This is because the control treatments also gave high response and strains from such locations have potential for effective symbiosis. The poor nodulation observed in some locations could be attributed to the presence of microorganisms antagonistic to rhizobia (Saha and Haque, 2005). Subba Rao (1999) reported that the viability of *R. leguminosarum* in natural soil was greatly affected by certain protozoa, fungi and bacteriophages. Also, successful symbiotic association requires the survival of rhizobia in sufficient number as free living bacteria in the soil ecosystem (Crozat *et al*., 1982). The Sudan savanna does not have long history of soybean cultivation, hence, it may not have compatible indigenous population of rhizobia. The inoculant could have contained more effective and competitive strains. This could be the reason for better response observed in this zone. It could also be as a result of other environmental factors affecting efficient symbiosis.

The low shoot dry weight observed for some of the locations could be as a result of ineffectiveness of the native rhizobial population and non-compatibility with the introduced strain. It is possible that most of the indigenous strains were highly competitive and infective but not effective. So even though they were able to nodulate the soybean, they could not effectively fix nitrogen, hence the low biomass. It was reported by Hahn (1986) that legumes cannot consistently recognize and exclude non-fixing rhizobia especially those that are closely related to their usual symbiotic partners. Similarly, it has been observed that response of legumes to inoculation depends largely on the number of already established rhizobia in the soil, the availability of soil nitrogen, and also management practices (Thies *et al*., 1991).

The lower nodule number in the plus N treatment in the southern Guinea savanna suggests that nodulation was suppressed by the addition of nitrogen. This is supported by the report of Eaglesham (1989) that increasing levels of mineral N in the rhizosphere inhibits soybean nodule formation and functioning.

Lack of response to inoculation observed in some locations could also be as a result of the number of populations of indigenous rhizobia in the soils of the study area. Monteleagre and Graham (1996), attributed lack of response to rhizobia inoculation to the presence of numerous, ineffective indigenous rhizobia. According to Mcloughlin and Dunican (1985), competition between rhizobia strains in the soil is a common phenomenon as the introduced inoculum strains compete with indigenous rhizobia for nodule sites.

High nodule number and weight does not always result in high plant biomass. It also does not always reflect the effectiveness of rhizobia because sometimes rhizobia are known to produce ineffective nodules which fix little or no nitrogen. This could be the reason why TDMY was low in some locations which had high nodule number and weight. It was expected that high nodule number for the inoculated plants will result in high biomass, but this was not the case, suggesting that the number of nodules alone did not reflect the effectiveness of the rhizobia introduced. It was reported by Okogun *et al.* (2004) that improved soybean variety TGx 1448-2E did not respond to inoculation in terms of nodule production in the Nigeria's moist savanna zone.

The current results on nodulation agree with the findings of Fening and Danso (2002) and Kumaga and Ofori (2004) that Bradyrhizobia numbers and effectiveness vary considerably among locations.

5.4. Morphological Characterization

The result of the morphological characterization shows the diversity of rhizobium strains in the three different agro-ecological zones. Most of the strains exhibited colony characteristics similar to rhizobium strains isolated from different legume crops in different parts of the world. This finding is in agreement with the work of Shetta *et al.* (2011) where they isolated strains from woody legumes that produced slimy/mucous, transparent to creamy colored colonies on YMA plates after 3 days of incubation at 28°C. Saha and Haque (2005) also reported colonies of *Rhizobium* on YMA that were circular, cream coloured, sticky, raised and translucent in nature. In a study reported by Kucuk *et al.* (2006), a high production of mucus was observed in 80% of the isolates; 23% were white and 70% opaque colonies. In another study, strains were isolated from the root nodules of species of wild vetch (*Vicia cracca*) collected from the different geographical and ecological areas of Turkey. Ten strains among 31 tested bacteria were found to have circular colonies with regular borders, flat in elevation, creamy in color, showing intermediate to high production of mucus (Adiguzel *et al.*, 2010).

Smita *et al.* (2011) isolated colonies from different areas in India that were whitish, creamy, yellowish and colorless in nature. They also isolated round colonies, raised and flat types with entire (regular) margins. The optical appearance of the colony of the rhizobia isolates were either semi translucent or translucent. Colony texture or consistency of the isolates showed variation with some isolates that were gummy and slimy/ mucus.

According to Fernanda *et al.* (2010), there was a small variation among strains isolated: most presented white coloring (97%), smooth colony edge (97%) and large quantity of mucus production (70%). Batista *et al*. (2007) noted an increasing tendency of *Bradyrhizobium* isolates

to produce mucus as a reflection of the adaptation to the acid soils of the Brazilian tropical savanna known as Cerrado.

The diversity of rhizobia may be influenced by the different chemical composition of the root exudates, as well as by the land use system, among other factors (Starin *et al.*, 1994). Due to their symbiotic relationship with legumes, it is suggested that rhizobia diversity is strictly related to the variety of legume species in the location, pointing evolutional adaptations of both symbiotic partners (Sprent, 1994; Fernanda *et al.*, 2010).

5.4.1. Growth Rate

Characterization of isolates based on growth rate did not fit into the traditional fast growing and slow growing types. It was observed that some of the isolates were intermediate growers while most were fast growers. This is in agreement with the work of Singh *et al.* (2008), where they observed fast growth of rhizobia isolated from the roots of *Trigonella foenumgraecum* (fenugreek).

Early reports of rhizobia associated with woody legumes described them as species that belong to the slow-growing group, but more recent reports have shown that this population includes a very diverse type of rhizobia including fast, intermediate and slow-growing bacteria (Shetta *et al.*, 2011).

5.4.2. Growth on Congo red

Variation in the intensity of dye absorption was observed among the rhizobial strains when plated on CR-YMA at 28°C.The uptake of Congo red varies among strains of *Rhizobium.* Congo red absorption does not distinguish rhizobia from other bacteria, but may be useful as a strain marker (Kneen and Larue, 1983).

Marked absorption of Congo red from CR-YMA at 26 to 28°C is generally considered a contraindication of Rhizobium as described by Dye (1980) and Vincent *et al.* (1979). But Kneen and Larue (1983) reported that if color on CR-YMA is used in enumeration, the number of rhizobia may be underestimated. Moreover, if only colonies not absorbing Congo red are selected when isolating rhizobia, culture collections of rhizobia will include only those with limited ability to take up the dye. Thus, the general rule that rhizobia fail to absorb Congo red will be perpetuated. Based on this, isolated colonies are classified according to their pattern of absorption.

5.4.3. Growth on BTB-YMA

The growth of rhizobia on YMA incorporated with BTB indicates if a particular strain is acid forming or basic forming. Most fast growing rhizobia are acid forming while slow growing rhizobia are alkali forming (Somasegaran and Hoben, 1994). The result of this characterization was in agreement with the work of several researchers (Saeki *et al*., 2005, Sharma *et al*., 2013 Shahzad *et al,* 2012) who have used YMA-BTB in categorizing indigenous soybean root nodulating rhizobia based on acid/alkali production. Kaur *et al.* (2012) also isolated Bradyrhizobia from soybean rhizosphere. The isolates when further tested on YMA plates containing BTB indicated that fast growing isolates produced yellow colonies due to acid production on the medium with high amount of mucus after 2 days of incubation.

5.5. Physiological Characterization

5.5.1. Effect of Extreme pH on Rhizobium Strains

The pH is an important parameter for the growth of rhizobia. Slight variation in pH of the medium might have significant effects on the growth of the organism (Singh *et al*, 2008; Deora and Singhal, 2010). The results obtained from this research which ranged from high to low pH

tolerant rhizobia are in agreement with previous studies of Kucuk *et al*. (2006) where they isolated rhizobia at a basic condition as high as pH 9. However, the study disagrees with the work of Sadowsky *et al.* (1983) where only the slow growers grew well at pH 4.5, whereas the fast growers did not grow at all at this pH. Instead some of the slow growers also grew well at pH 8.5. It also disagrees with the work of Shetta *et al.* (2011) where no growth was recorded at pH as low as 5. Also Deora and Singhal (2010), recorded no growth at pH 4.0 and 9.0. This result also shows the diversity of rhizobia in the different agro-ecologies. One of the most important factors that affects the efficiency of symbiosis between rhizobia and plants is the pH of the soil in which they interact (Garg and Sharma, 2013). Thus, these pH tolerant forms can be used to inoculate soils with high or low pH.

5.5.2. Effect of Temperature on Growth of Rhizobium Strains

Excellent growth of all isolates was observed at 27^{o}C and poor growth was recorded at 35^{o}C. This is in agreement with the report of Smita *et al*. (2011) where their isolates grew best at 27^{o}C. They also recorded poor to very poor growth at 37^{o}C. It implies therefore, from this study that the optimum growth temperature for most isolates was 27^{o}C. Although they grew weakly, the isolates to some extent were able to withstand high and low temperatures. However, since many other factors affect the competitiveness, establishment and efficiency of strains, *in vitro* selection of temperature tolerant rhizobia is not considered as a promising approach for field applications (Hungria and Vargas, 2000). The survey of such characteristics can be useful for improvement of inoculants by genetic engineering of strains with symbiotic nitrogen fixation (Shetta *et al.*, 2011).

5.5.3. Effect of Salinity on Growth of Rhizobium Strains

Salinity is one of the major factors restricting symbiotic nitrogen fixation. It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes (Kucuk *et al*., 2006; Shetta *et al*, 2011). It has been by proposed by Hashem *et al*. (1998) that salt stress may decrease the efficiency of the Rhizobium-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function.

Results of this study indicated that most strains were able to grow on 1% NaCl containing medium but few were able to grow on higher concentrations, thus showing that the isolates were sensitive to high salt concentrations. These results are in harmony with the findings of Hung *et al.* (2005) and Singh *et al*. (2008). Moreover, the soils of the Nigerian savanna are not known to have high salt concentration except in some patches where surface irrigation system is not properly designed to address drainage issues. It has been shown that increasing salt concentrations cause detrimental effect on rhizobium strains due to osmotic stress (Thrall *et al*., 2008; Garg and Sharma, 2013). Comparison of salt (NaCl) tolerance of rhizobial isolates in previous research work done by Tu (1981); Le Rudulier and Bouillard (1983); Bernard *et al*., (1986); Garg and Sharma (2013) reported different strains with different level of tolerance towards salt stress as in the present study where the isolates showed different rates of growth. The response of different concentration of NaCl on growth of rhizobia isolates as reported by Smita *et al* (2011) indicated that concentration of 1-3% NaCl were inhibitory to all isolates of the rhizobia as compared to control. However, El Sheikh and Wood (1989) and Zerhari (2000), found isolates that were salt tolerant and showed growth even at 5% salt (NaCl) concentration.

The difference between these previous studies indicate very wide variation in strains tolerance to salt which could be due to genetic make-up of the strains or evolution of adaptive mechanisms in response to environmental conditions.

CHAPTER SIX

6.0. SUMMARY AND CONCLUSION

In many soils, the population of rhizobia is not adequate to provide efficient symbiosis for nitrogen fixation to occur. The use of rhizobia inoculants has been shown to increase soil fertility and crop yields through biological nitrogen fixation. Therefore, screening rhizobia for effective nitrogen fixation would be the best way to maximize the use of natural methods of improving and maintaining soil fertility and sustainable crop yields. This study was conducted to assess the response of promiscuous soybean to inoculation in the Nigerian savanna and also to examine the relative efficiency of the indigenous rhizobia population on soybean biomass. The study also sought to characterize rhizobia isolates based on their appearance on yeast mannitol agar and tolerance to environmental stresses like pH, temperature and salt concentration.

The experimental soil samples were collected from forty-five (45) locations across the northern Guinea savanna, Southern Guinea savanna and Sudan savanna zones of Nigeria. The soils were used to conduct need to inoculate trials in the screenhouse using soybean genotype TGx1448-2E as a test crop. The results showed better response to inoculation in the Sudan savanna compared to the northern and southern Guinea savannas which was not surprising as response to inoculation is usually lower in areas where soybean has been extensively grown. Furthermore, high nodule number does not always indicate effectiveness because there are instances where high nodule number gave low biomass. In all the agro-ecologies assessed, the response to inoculation and mineral N application varies with respect to plant parameters. In locations where the control outperformed the inoculant and mineral N, strains from such locations have high

potential for use as inoculants to improve biological nitrogen fixation and crop yield. Hence further trials are recommended in order to improve their use as inoculants.

The results of the physiological characterization showed that some of the isolates were able to grow in a wide range of pH, salt concentration and temperature. It shows that the full benefit of biological nitrogen fixation can be achieved in diverse environmental conditions where other inputs may fail to produce desirable results. Thus, the present study can play a very big role in improvement of biological nitrogen fixation as symbioses between rhizobium and legumes provides a cheaper and usually more effective agronomic practice for ensuring an adequate supply of N. Therefore, these stress tolerant properties of some of the strains have enormous potential to be used as biofertilizers especially where adverse environmental conditions constrains the rhizobial populations within a locality. The results also showed that some strains of rhizobia are able to absorb Congo red. These strains were confirmed as rhizobia by authentication in the screenhouse. Reaction on Bromothymol blue showed 94.57% of the isolates to be acid forming while 5.43% did not react at all and no basic forming rhizobia was encountered during the course of this study.

In conclusion, the results show that there is variation in the response to inoculation of rhizobia in the various AEZs. This confirms the null hypothesis that response of promiscuous soybean to rhizobia inoculant may vary among the different agro-ecological zones. Although the isolates share similar characteristics in some instances and not in others, they appear to be closely related on the basis of their growth rate and their morphological characteristics. However, in order to obtain a better understanding of the diversity of rhizobia isolated during the course of this study, it is necessary to carry out molecular characterization of all isolates.

The study suggests adaptability of isolates to different ecological environments with some factors at stress levels. However, further research in the field, as well as their use as inoculants to improve growth and nitrogen fixation in legumes in soils of the Nigerian savanna needs to be carried out.

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APPENDIX 1

Coordinates for sampling sites

Agro-ecological zone	Site	Code	Latitude ^{O}N	Longitude ^{O}E	Elevation (m)
Sudan Savanna	Garko 1	GRK	11.643	8.810	5133.2
Sudan Savanna	Garko 2	GRK	11.679	8.812	4962.64
Sudan Savanna	Garko 3	GRK	11.528	8.775	5635.04
Sudan Savanna	Garko 4	GRK	11.590	8.938	5080.72
Sudan Savanna	Garko 5	GRK	11.583	8.926	5143.04
Sudan Savanna	Garko 6	GRK	11.637	8.881	4975.76
Sudan Savanna	Bunkure 1	BKR	11.669	8.628	4805.2
Sudan Savanna	Bunkure 2	BKR	11.577	8.517	5543.2
Sudan Savanna	Albasu 1	ALB	11.670	8.983	4956.08
Sudan Savanna	Albasu 2	ALB	11.673	9.133	4690.4
Sudan Savanna	Albasu 3	ALB	11.676	9.193	4723.2
Sudan Savanna	Minjibir 1	MJB	12.143	8.667	4710.08
Sudan Savanna	Bichi 1	BCH	12.228	8.245	5717.04
Sudan Savanna	Bichi 2	BCH	12.206	8.297	5615.36
Sudan Savanna	Bichi 3	BCH	12.192	8.305	5661.28
Sudan Savanna	Wudil 1	WDL	11.656	8.922	4906.88
Sudan Savanna	Wudil 2	WDL	11.748	8.820	4785.52
Sudan Savanna	Dawakin Kudu 1	DKD	11.852	8.587	5005.28
Sudan Savanna	Dawakin Kudu 2	DKD	11.835	8.720	4782.24
Sudan Savanna	Warawa 1	WRA	11.973	8.690	4736.32
Sudan Savanna	Warawa 2	WRA	11.873	8.728	4696.96
Northern Guinea Savanna	Giwa 1	GWA	11.278	7.382	6891.28
Northern Guinea Savanna	Giwa 2	GWA	11.246	7.337	6884.72
Northern Guinea Savanna	Giwa 3	GWA	11.335	7.386	6914.24
Northern Guinea Savanna	Giwa 4	GWA	11.349	7.388	6927.36
Northern Guinea Savanna	Giwa 5	GWA	11.335	7.386	6884.72
Northern Guinea Savanna	Soba 1	SOB	11.026	7.920	7022.48
Northern Guinea Savanna	Soba 2	SOB	11.027	7.919	7065.12

Coordinates for sampling sites (cont'd)

Agro-ecological zone	Site	Code	Latitude^{O} N	longitude ^{O}E	elevation (m)
Northern Guinea Savanna	Soba 3	SOB	11.032	7.933	7074.96
Northern Guinea Savanna	Soba 4	SOB	11.027	7.921	7019.2
Northern Guinea Savanna	Soba 5	SOB	11.032	7.933	7127.44
Northern Guinea Savanna	Soba 6	SOB	11.027	7.920	7045.44
Northern Guinea Savanna	Igabi 1	IGB	10.795	7.687	7294.72
Northern Guinea Savanna	Igabi 2	IGB	10.782	7.687	7137.28
Northern Guinea Savanna	Igabi 3	IGB	10.942	7.643	7363.6
Northern Guinea Savanna	Igabi 4	IGB	10.919	7.650	7412.8
Northern Guinea Savanna	Tudun Wada 1	TWD	11.243	8.384	6645.28
Northern Guinea Savanna	Tudun Wada 2	TWD	11.272	8.562	6064.72
Northern Guinea Savanna	Tudun Wada 3	TWD	11.305	8.005	5838.4
Northern Guinea Savanna	Tudun Wada 4	TWD	11.246	8.357	6619.04
Southern Guinea Savanna	Kachia 1	KCH	9.843	7.974	7944.16
Southern Guinea Savanna	Kachia 2	KCH	9.812	7.969	8006.48
Southern Guinea Savanna	Kachia 3	KCH	9.811	7.969	8009.76
Southern Guinea Savanna	Kachia 4	KCH	9.666	7.987	8081.92
Southern Guinea Savanna	Kachia 5	KCH	9.643	7.958	7904.8
Southern Guinea Savanna	Kachia 6	KCH	9.854	7.971	8019.6
Southern Guinea Savanna	Kolosok	KOL	9.843	8.173	8252.48
Southern Guinea Savanna	Zonkwa	ZKW	9.779	8.313	9676

APPENDIX 2.

Physicochemical properties of the experimental sites

AEZ	Location	Clay	Silt	Sand	Textural Class	pH (water, 1:2.5)	pH ($CaCl_2$, 1:2.5)	OC (g kg^{-1})	TN (g kg^{-1})	Bray-P (mg kg^{-1})	Ca	Mg	K	Na	CEC
NGS	Giwa 1	14	18	68	SANDY LOAM	7.4	6.4	4	2.1	1.75	6	3.33	0.17	0.35	10.6
NGS	Giwa 2	14	26	60	SANDY LOAM	7.5	6.3	11.2	2.1	12.25	6.8	4.7	0.21	0.3	12.8
NGS	Giwa 3	12	24	64	SANDY LOAM	7.2	5.8	5.4	1.8	6.13	7.2	3.1	0.14	0.26	11.2
NGS	Giwa 4	14	24	62	SANDY LOAM	6.8	5.3	5.4	2.5	3.5	7	3.2	0.18	0.3	11.7
NGS	Giwa 5	12	26	62	SANDY LOAM	6.5	5.1	4.8	2.5	3.5	9.8	2.5	0.21	0.3	13.2
NGS	Soba 1	18	22	60	SANDY LOAM	6.6	5.1	3.2	2.1	1.75	6.8	2.76	0.14	0.27	10.4
NGS	Soba 2	14	22	64	SANDY LOAM	6.3	5.1	4	2.8	8.75	5.6	0.86	0.2	0.7	7.4
NGS	Soba 3	16	28	56	SANDY LOAM	6.3	5	5	1.8	7	4.2	0.48	0.22	0.26	6.2
NGS	Soba 4	14	28	58	SANDY LOAM	6.3	5	2.4	1.4	5.25	4.8	0.38	0.24	0.28	11.8
NGS	Soba 5	12	22	66	SANDY LOAM	6.2	5	3.6	1.8	7	7.2	1.5	0.14	0.28	9.4
NGS	Soba 6	14	20	66	SANDY LOAM	6.1	5	4.8	1.4	6.13	8	4.2	0.15	0.27	12.6
NGS	Igabi 1	8	18	74	SANDY LOAM	6	5	4.6	2.5	10.5	5.6	1.92	0.18	0.66	9.4
NGS	Igabi 2	16	18	66	SANDY LOAM	6	5.1	3.8	2.1	7	8.6	3.4	0.15	0.74	12.9
NGS	Igabi 3	10	24	66	SANDY LOAM	6	5.1	5.6	1.4	6.1	5.8	2.2	0.21	0.33	9.2
NGS	Igabi 4	16	16	68	SANDY LOAM	5.9	5	2.4	1.1	8.75	4.8	3.5	0.11	0.66	9.6
NGS	Tudun Wada 1	14	18	68	SANDY LOAM	6.7	5.4	0.28	1.8	8.75	9.4	1.17	0.2	0.28	13
NGS	Tudun Wada 2	10	22	68	SANDY LOAM	6.6	5.3	0.18	1.4	5.25	11.6	4.83	0.15	0.29	17.2
NGS	Tudun Wada 3	8	8	84	LOAMY SAND	6.6	5.3	0.48	2.1	5.25	11.4	3.4	0.22	0.48	15.6
NGS	Tudun Wada 4	14	16	70	SANDY LOAM	5.7	5.4	0.47	1.1	5.25					
SGS	Kachia 1	18	10	72	SANDY LOAM	5.8	4.7	11.2	1.4	26.3	7.2	3.5	0.16	0.54	11.5
SGS	Kachia 3	16	16	68	SANDY LOAM	5.7	4.6	4	4.9	14	9.8	4.7	0.25	0.23	15.3
SGS	Kachia 4	16	6	78	SANDY LOAM	5.6	4.7	4.4	8.8	21	4.6	2.9	0.09	0.23	8.6
SGS	Kolosok								0						
SGS	Zonkwa	20	14	66	S CLAY LOAM	5.6	4.7	0.76	1.4	1.75					

AEZ-Agro-ecological zone, NGS-Northern Guinea Savanna, SGS- Southern Guinea Savanna, SS-Savanna

Physicochemical properties of the experimental sites (contd)

AEZ	Location	Clay	Silt	Sand	Textural Class	pH (water, 1:2.5)	pH ($CaCl_2$, 1:2.5)	OC (g kg^{-1})	TN (g kg^{-1})	Bray-P (mg kg^{-1})	Ca	Mg	K	Na	CEC
SS	Albasu 1	8	8	84	LOAMY SAND	7.3	5.4	2.4	5.3	1.75	8	0.84	0.06	0.75	11.2
SS	Albasu 2	8	2	90	SAND	7.2	5.4	0.6	6.3	8.75	6.2	2.83	0.11	0.43	10.6
SS	Albasu 3	8	2	90	SAND	7	6	3	6	12.3	8	3.33	0.12	0.26	11.9
SS	Minjibir 1	10	14	76	SANDY LOAM	6.8	5.4	2.6	5.6	31.5	6.8	3.1	0.38	0.38	10.7
SS	Bichi 1	8	4	88	SAND	6.3	5.3	0.4	1.4	42	4.4	2.06	0.14	0.25	9.8
SS	Bichi 2	8	4	88	LOAMY SAND	6.6	5.3	2.8	1.2	24.5	8.2	2.2	0.14	0.25	10.8
SS	Bichi 3	10	10	80	SANDY LOAM	6.6	4.9	2.4	1.4	7	8.2	2.8	0.16	0.3	12.5
SS	Bunkure 1	18	10	72	SANDY LOAM	5.9	5	2.6	6	14	10.6	4.2	0.2	0.31	15.5
SS	Bunkure 2	8	12	80	LOAMY SAND	5.8	5	0.4	4.2	12.3	9	2.17	0.27	0.29	12.4
SS	Warawa 1	12	4	84	LOAMY SAND	5.8	5	2.2	1.4	10.5	10	4.64	0.13	0.36	15.2
SS	Warawa 2	8	2	90	SAND	6.2	5.4	3.2	1.8	7	7.6	2.33	0.32	2.78	14.3
SS	Wudil 1	8	12	80	SANDY LOAM	6.2	4.8	2.8	1.8	13.13	5	0.86	0.1	0.31	7.3
SS	Wudil 2	8	6	86	LOAMY SAND	6.3	5.8	2.6	1.1	8.75	6.8	2	0.16	0.26	9.6
SS	Dawakin Kudu 1	10	10	80	SANDY LOAM	6.2	5.1	3.6	1.1	31.5	6.2	3.8	0.18	0.3	11.5
SS	Dawakin Kudu 2	10	4	86	LOAMY SAND	6.4	5.1	3	3.2	70	12	3.5	0.24	0.31	16.8
SS	Garko 1	14	16	70	LOAMY SAND	5.4	4.4	3.9	3.5	6.13					
SS	Garko 2	10	8	82	LOAMY SAND	5.7	4.8	1.4	2.8	14	9.6	3.67	0.13	0.26	14
SS	Garko 3	12	8	80	LOAMY SAND	5.7	4.9	2.6	5.6	14	7.2	3.7	0.17	0.18	13.8
SS	Garko 4	12	6	82	LOAMY SAND	5.7	5	3.4	6.7	21	7.8	4.1	0.4	0.33	12.8
SS	Garko 5	8	14	78	SANDY LOAM	6.1	5.4	2.9	1.8	29.75					
SS	Garko 6	10	14	76	SANDY LOAM	5.9	5.4	0.6	7.4	21.88	6.6	2.67	0.13	0.27	10.7

AEZ-Agro-ecological zone, NGS-Northern Guinea Savanna, SGS-Southern Guinea Savanna, SS-Sudan Savanna.

APPENDIX 3

Rating for soil data interpretation

1. Soil Depth (cm)

Very shallow	<25
shallow	25-50
Moderately shallow	50-100
Deep	100-150
Very deep	>150

2. Soil Reaction

Extreme acid	<45
Very strongly acid	4.5-50
Strongly acidic	5.1-5.5
Moderately acid	5.6-60
Slightly acid	6.1-6.5
Neutral	6.6-7.3
Slightly alkaline	7.4-7.8
Moderately alkaline	7.9-8.4

Strong alkaline	8.5-9.0
Very strongly alkaline	>9.0

3. Organic Carbon (g kg^{-1})

Low	<10
Medium	10-15
High	>15

4. Total Nitrogen (g kg^{-1})

Low	<1.5
Medium	1.5-2.0
High	>2.0

5. Available Phosphorus (Bray 1) (mg kg^{-1})

Low	<10
Medium	10-20
High	>20

6. Exchangeable Cations (cmol.kg^{-1})	**Ca**	**Mg**	**K**	**Na**
Low	0-2	0-0.3	0-0.15	0-0.1
Medium	2-5	0.3-1.0	0.15-0.3	0.1-0.3

High	>5	>10	>0.3	>0.3

7. Cation Exchange Capacity (cmol. kg^{-1})	**NH_4OAc**	**ECEC**
Low	<6	<4
Medium	6-12	4-10
High	>12	>10

8. Base Saturation (%)	**NH_4OAc**	**ECEC**
Low	<50	<70
Medium	50-80	70-90
High	>80	>90

9. Micronutrients (mg kg^{-1})	**Zn**	**Cu**	**Mn**	**Fe**
Low	<1	<0.2	<1	<4.5
Medium	1-2	0.2	1.0	-
High	>2	1.0	2-5	>4.5

Source: FMANR, 1990

APPENDIX 4

Relative efficiency and inoculant response in the experimental sites

Relative efficiency and inoculant response in the Sudan savanna

	Relative Efficiency (%)	Inoculant Response (%)
ALB 1	112.5	-9.523809524
ALB 2	73.33333333	23.63636364
ALB 3	62.31884058	65.11627907
BCH 1	69.23076923	25.92592593
BCH 2	102.8985507	2.816901408
BCH 3	72.85714286	9.803921569
BKR 1	55.05617978	22.44897959
BKR 2	84.50704225	13.33333333
DKD 1	71.05263158	37.03703704
DKD 2	89.18918919	12.12121212
GRK 1	111.1111111	-14.28571429
GRK 2	89.02439024	-4.109589041
GRK 3	98.64864865	-15.06849315
GRK 4	104.3478261	2.777777778
GRK 5	105.3333333	-10.12658228
GRK 6	87.34177215	-1.449275362
MJB	89.47368421	-7.843137255
WDL 1	82.89473684	90.47619048
WDL 2	71.42857143	21.81818182
WRA 1	101.5625	-26.15384615
WRA 2	68.36734694	13.43283582

Relative efficiency and inoculant response northern Guinea savanna

	Relative Efficiency (%)	Inoculant Response (%)
GWA 1	94.73684211	5.555555556
GWA 2	89.23076923	-13.79310345
GWA 3	78.18181818	18.60465116
GWA 4	78.94736842	22.22222222
GWA 5	85.9375	21.81818182
IGB 1	77.08333333	16.21621622
IGB 2	101.3888889	-9.589041096
IGB 3	67.74193548	-3.174603175
IGB 4	83.09859155	-5.084745763
SOB 1	97.22222222	5.714285714
SOB 2	79.74683544	17.46031746
SOB 3	70	-8.928571429
SOB 4	114.7058824	-16.66666667
SOB 5	79.74683544	-6.349206349
SOB 6	76.25	1.639344262
TWD 1	88.88888889	6.944444444
TWD 2	77.6119403	1.923076923
TWD 3	100	-9.375
TWD 4	87.34177215	11.5942029

Relative efficiency and inoculant southern Guinea savanna

	Relative Efficiency (%)	Inoculant Response (%)
KCH 1	100	2.597402597
KCH 3	85.71428571	-25
KCH 4	82.5	30.3030303
KOL	128.5714286	22.22222222
ZKW	91.04477612	-9.836065574