ISOLATION AND MOLECULAR CHARACTERIZATION OF INDIGENOUS BRADYRHIZOBIUM SPECIES AND ASSESSING THEIR CAPABILITY TO NODULATE AND FIX NITROGEN IN SOYBEAN (GLYCINE MAX)

BY

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BY

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DEPARTMENT OF MICROBIOLOGY,

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OCTOBER, 2017

DECLARATION

I declare that the work in this dissertation entitled, "Isolation and molecular characterization of indigenous *Bradyrhizobium* species and assessing their capability to nodulate and fix nitrogen in Soybean (Glycine max)" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

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Name of Student	Signature	Date

CERTIFICATION

This dissertation "ISOLATION AND MOLECULAR CHARACTERIZATION OF INDIGENOUS BRADYRHIZOBIUM SPECIES AND ASSESSING THEIR CAPABILITY TO NODULATE AND FIX NITROGEN IN SOYBEAN (GLYCINE MAX)" By IMRANA RUKAYYA (P13SCMC8045) meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents Alh. Haruna Imrana Yazid and Hajiya Fatima Imrana. I love you so much.

ABSTRACT

This study was carried out to isolate, characterize and assess the capacity of indigenous *Bradyrhizobium* spp. to nodulate and fix nitrogen in soybean (Glycine max). Six strains of *Bradyrhizobium* spp. were isolated from soil samples obtained from fields cultivated to soybean and cowpea using the plant trap method. The six isolates were characterized based on their colonial morphology, as well as biochemical and molecular characteristics. Similarity searches based on sequences on the genbank database showed that of the six isolates confirmed to be strains of *Bradyrhizobium* spp, four were identified as *Bradyrhizobium elkanii* with sequence similarity ranging from 93% to 99%, while the remaining two had no sequence similarities. All the six strains obtained were tested in inoculation trials in a screen house using soybean (TGx 1448-2E) as a test crop to assess the capacity of the isolates to form nodules. Thus, the total number, percentage effective nodules, nodule fresh and dry weights, as well as the fresh and dry biomass yield and nitrogen content were assessed. It was observed that *B. elkanii* (B64) formed significantly higher number of nodules per plant (70%) than all the other isolates tested and the standard inoculant. The same strain was also found to fix higher biological nitrogen (25.12%) but it was not statistically significant when compared with the standard inoculant (18.15%). There was a very significant positive correlation between the biological nitrogen fixed and the dry and fresh weight of soybean crop ($p<0.01$). It was therefore concluded that soils from fields with recent history of soybean cultivation harbor strains of *Bradyrhizobium* species that could be considered as candidates for the development of inoculants for enhanced soybean production in the Northern Guinea Savannah zone of Nigeria.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
BNF	Biological nitrogen fixation
C	Carbon
CR-YEMA	Congo red-Yeast extract mannitol agar
DMRT	Duncan multiple range test
DNA	Deoxyribonucleic acid
IITA	International Institute of Tropical Agriculture
K	Potassium
N	Nitrogen
NCBI	National Centre for Biotechnology Information
NFO	Nitrogen fixing Organisms
NaCI	Sodium Chloride
P	Phosphorus
PCR	Polymerase chain reaction
PCBN	Pentacholonitrobenzene
ppm	Parts per million
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
TGx	Tropical Glycine cross
YEMA	Yeast extract Mannitol agar
YEMB	Yeast extract Mannitol broth

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Soybean (*Glycine Max* [L] Merril) is currently an important cash crop that is widely cultivated in the northern Guinea and Sudan savanna zones of Nigeria (AMREC, 2007). It was first introduced into these agro-climatic zones in 1928 and it has since spread to other parts of the states (Agboola and Moses, 2015). According to Iwe, (2003), Nigeria is the largest producer of soybean in sub-Saharan Africa. Its annual production is estimated by the Ministry of Agriculture to be around 550,000 tons (Abubakar, 2015). Currently, the major soybean producing States in Nigeria are Benue, Kaduna, Taraba, Plateau and Niger, other states include: Delta, Ondo, Oyo, Kwara, and Kogi (Agboola and Moses, 2015). With increased demand for soybean both at home and abroad, this crop is steadily assuming an important position as a source of income for the Nigerian farmers (AMREC, 2007).

One of the major challenges to sustained increase in agricultural production in this part of the country is the low fertility status of most agricultural soils (Machido *et al.,* 2011). Reports in the literature suggest that most of the soils under cultivation for agricultural production in the savannah zone of Nigeria are inherently poor in their content of nitrogen, and available phosphorus (Yakubu *et al.,* 2010; Machido *et al.,* 2011; Laditi *et al.,* 2012). To ensure good growth and economic yield of soybean and other crops, farmers in the region rely heavily on application of inorganic nitrogenous fertilizers and organic manure. But this approach is engulfed with problems such as unavailability and high cost of inorganic nitrogenous fertilizers (AMREC, 2007). Falodun *et al.* (2010) also reported a significant decrease in soybean yield due to the use of organic fertilizers. This renders the practice unsustainable.

In many countries, the current approach to solving the problems posed by both scarcity of organic manure and high cost of nitrogen fertilizers required for soybean production, relies on

the long established understanding that, the soybean crop has the capacity to fix atmospheric N_2 in association with soil bacteria belonging to the genus *Bradyrhizobium* (Peoples *et al*., 1995). Earlier reports by Peoples *et al.* (1995) indicates that, soybean crop fixes from 54 to 300kg N/ha while in association with *Bradyrhizobium* and contributes more than 15kg N/ha to the soil on which soybean is cultivated. This and many other reports (Anuar *et al*., 1995; Unkovich *et al*., 1995; Iwe, 2003; Onwualu, 2007; Machido *et al.*, 2011; Abubakar, 2015) tend to suggest that, biological N_2-fixation could be the way out for Nigerian soybean farmers where N requirement for soybean production is concerned.

To avoid the need to inoculate soybean with *B. japonicum*, soybean breeders at the International Institute of Tropical Agriculture (IITA), Nigeria, developed new soybean genotypes for Africa, known as Tropical *Glycine* cross (TGx), which nodulate with *Bradyrhizobium* spp. populations indigenous to African soils, but inoculation in certain locations have shown nitrogen deficiency (Abaidoo *et al*., 2000).

The "state of the art" technology in this field employs direct inoculation of soybean seeds prior to sowing with strains of *Bradyrhizobium* spp. with the aim of improving nodulation and N_2-fixing capacity of the soybean crop (Ofori, 2016). Leguminous crops have been inoculated with strains of *Rhizobium* and *Bradyrhizobium* since the turn of the century in order to improve their growth in soils which do not contain appropriate species of these bacteria (Ilyas, 2009). This practice is in most cases recommended in soils that have either never or not been recently cropped to soybean (Abubakar, 2015). Unfortunately, however, some of the commercial inoculants available to soybean farmers in Nigeria are from foreign countries and thus, may not lead to much yield increase (Thies *et al*., 1991). This view lends support from reports of inoculation trials in the country involving the use of commercial inoculants (Yusuf *et al.*, 2013). Reasons advanced for the observed lack of positive response to seed inoculation on farmers' fields were given by Machido *et al.*, (2010) and they include:

inability of the inoculants strain to compete with indigenous strains present in the soil, inability of the inoculants strains to survive the extreme soil conditions typical of Nigerian soils, low viable counts of the inoculants strains in the imported commercial inoculants due to improper storage. Inhibitory effect of soluble components of seed coat on the inoculants strain was also a factor considered to hinder inoculation response (Deaker *et al*., 2004).

Given these constraints, it is conceivable that, application of imported commercial seed inoculants may not result in significant yield increases when applied in soils of the savanna region of this country (Yusuf *et al.*, 2013). There is, therefore the need to search for strains of *Bradyrhizobium* from the populations that are indigenous to soils of this ecological zone. Such strains are most likely to be well adapted to the physical and chemical as well as the biological constraints such as nutrient deficiency typical of soils in the region and still ensure higher rate of nodulation and nitrogen fixing capacity of soybean. Availability of locally formulated seed inoculants would provide a cheaper, more sustainable alternative to the imported products. Also, the quality of locally formulated inoculants would be better ensured. These are necessary prerequisites if the benefits of the seed inoculation technology are to be realized by the Nigerian soybean farmers.

1.2 Statement of Research Problems

Like all other crops cultivated on soils of the savanna zone of Nigeria, the yield of soybean crop depends on the availability of essential nutrients to the growing crop. Reports by Agboola and Moses, (2015), have shown that the current farming practices in the region depend on the application of organic manure and to a larger extent, on mineral nitrogenous fertilizers to enhance and/or sustain economic crop yield. This practice is engulfed with twin problems of scarcity and high cost of procurement (Sanginga, 2003). In addition to this, heavy use of N-fertilizer is both harmful to the environment and results in the depletion of fossil fuels (Bohlool *et al.,* 1991). Continuous depletion of nitrogen (N) from the soil pool by processes such as volatilization and leaching results in the decline of soil nitrogen reserves in agricultural soils (Abdullahi *et al,* 2013). The economic and environmental costs of the heavy or wrong use of inorganic nitrogen fertilizers in agriculture are a global concern and mandates that alternatives be urgently sought (Ladha *et al.,* 1998).

There are also problems of high cost of the commercial inoculants and the inability of the strains to survive the extreme soil conditions typical of Nigerian soils (Machido, *et al.,* 2010). There is variability in the effectiveness and population of indigenous bradyrhizobia in a given location (Fening and Danso, 2002) There is therefore, the need to search for strains of *Bradyrhizobium* from among the indigenous soil populations with all the desirable qualities required of candidates strains for the formulation of soybean seed inoculants.

1.3 Justification

Soybean is currently considered as an important crop in Nigeria both as a cash crop (AMREC, 2007) and as a cheap source of dietary protein for both animals and humans. There is therefore the need to find a sustainable means of boosting its production in the country.

Reliance on commercial inoculants as an alternative to chemical fertilizers to increase the yield of the crop on farmers' fields is engulfed with many problems and does not therefore always result in the expected yield increases. Hence, there is the need to look inward for strains of *Bradyrhizobium* that are better adapted and therefore more likely to be superior to imported commercial inoculants under conditions typical of soils of the savanna zone.

Even though TGx variety of soybean genotypes have been developed, they have however been reported to develop nitrogen deficiency symptoms, suggesting that the TGx soybean may require inoculation with Bradyrhizobia at some locations if high soybean yields are to be obtained (Abaidoo *et al.*, 2000).

The fact that the cultivation of soybean crop leads to economic yield on farmers' fields without inoculation with *Bradyrhizobium* and little or no fertilizer application; suggest that, these soils are populated with strains of *Bradyrhizobium* capable of nodulating and fixing nitrogen (Machido *et al.*, 2010). In addition, Abubakar, (2015) stated that increasing and extending the role of biofertilizers such as *Rhizobium* inoculants would reduce the need for chemical fertilizers and decrease adverse environmental effects. It is therefore important to improve crop productivity in an eco-friendly manner as 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).

1.4 Aim and Objectives

1.4.1 Aim

The aim of this research was to isolate and molecularly characterize indigenous *Bradyrhizobium* species and assess their capability to nodulate and fix nitrogen in soybean (Glycine max).

1.4.2 Objectives

The objectives of the study were to:

1. Determine the physical and chemical properties of the experimental soil sample.
2. Isolate and characterize strains of *Bradyrhizobium* species from the experimental field using morphological and biochemical tests and PCR amplification of 16S rRNA gene.
3. Assess the capacity of the isolates to nodulate and fix nitrogen in association with soybean crop.
4. Determine the biological nitrogen fixed by the isolates in the soybean crop.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Soybean Crop

Soybean is a leguminous vegetable of the pea family that grows in tropical, subtropical, and temperate climates (Uwaoma, 2015). Soybean was domesticated in the 11th century BC around northeast of China. It is believed that it might have been introduced to Africa in the 19th century by Chinese traders along the east coast of Africa (IITA, 2009). Soybean has been described in various ways, some call it the "miracle bean" or the "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. Compared to other protein rich foods such as meat, fish, and eggs, it is by far the cheapest (IITA, 2000; Dugje *et al.*, 2009).). It has therefore, tremendous potential to improve the nutritional status and welfare of resource-poor people particularly in a developing country like Nigeria (AMREC, 2007). Reports by Ofori, (2016) also indicate that soybean has some benefits over other legumes such as groundnut and cowpea due to its low susceptibility to pests and diseases, better storage quality and larger leaf biomass which benefits subsequent crops by way of soil fertility improvement.

Attempts to grow soybean in Nigeria were made as early as 1908 in Ibadan, Oyo State, but poor seed viability of the varieties introduced made it impossible to grow the crop commercially in the areas where it was first introduced (Agboola and Moses, 2015). However, successful cultivation of the crop was first achieved in Samaru, Zaria in 1928, and later in 1937, when the variety "Malayan" was introduced into the country, which was the earliest and most adaptable variety (Abubakar, 2015). According to reports by Okonmah, (2012) production is still concentrated in the Sudan and Guinea Savannah agro-ecological zones of Nigeria.

2.1.1 General characteristics

Like most crops, soybeans grow in distinct morphological stages as they develop from seeds into fully mature plants.

2.1.1.1 Germination

The first stage of growth is germination, a process that first becomes apparent as a seed's radicle emerges and it occurs within the first 48 hours under ideal growing conditions (Purcell *et al.*, 2014). According to this same source, the first photosynthetic structures is the cotyledons, developing from the hypocotyl and the first plant structure to emerge from the soil. These cotyledons both act as leaves and as a source of nutrients for the immature plant, providing the seedling nutrition for its first 7 to 10 days (Purcell *et al.*, 2014).

2.1.1.2 Maturation

The first true leaves develop as a pair of single blades. Subsequent to this first pair, mature nodes form compound leaves with three blades. Mature trifoliolate leaves, having three to four leaflets per leaf, are often between 6–15 cm (2.4–5.9 in) long and 2–7 cm (0.79–2.76 in) broad (Purcell *et al.*, 2000). Under ideal conditions, stem growth continues, producing new nodes every four days. Before flowering, roots can grow 1.9 cm (0.75 inch) per day. If rhizobia are present, root nodulation begins by the time the third node appears (Purcell *et al.*, 2014). Nodulation typically continues for 8 weeks before the symbiotic infection process stabilizes The final characteristics of a soybean plant are variable, with factors such as genetics, soil quality, and climate affecting its form; however, fully mature soybean plants are generally between 51-127 cm (20–50 in) in height and have rooting depths between 76–152 cm (30–60 in) (Bennett *et al., 2014).*

2.1.1.3 Flowering

Flowering is triggered by day length, often beginning once days become shorter than 12.8 hours (Purcell *et al.*, 2014). Soybeans form inconspicuous, self-fertile flowers which are

borne in the axil of the leaf and are white, pink or purple. Depending on the soybean variety, node growth may cease once flowering begins. Strains that continue nodal development after flowering are termed "indeterminates" and are best suited to climates with longer growing seasons. Often soybeans drop their leaves before the seeds are fully mature (Shurtleff *et al.*, 2015). The fruit is a hairy pod that grows in clusters of three to five, each pod is 3–8 cm long (1–3 in) and usually contains two to four (rarely more) seeds 5–11 mm in diameter but the soybean seeds come in a wide variety sizes and hull colors such black, brown, yellow, and green (Bennett *et al.*, 2014).

2.1.1.4 Seed resilience

The hull of the mature bean is hard, water-resistant, and protects the cotyledon and hypocotyl (or "germ") from damage. If the seed coat is cracked, the seed will not germinate. The scar, visible on the seed coat, is called the hilum (colors include black, brown, buff, gray and yellow) and at one end of the hilum is the micropyle, or small opening in the seed coat which can allow the absorption of water for sprouting. Remarkably, seeds such as soybeans containing very high levels of protein can undergo desiccation, yet survive and revive after water absorption.

Plate 1: Picture of root nodules on a Soybean crop from this study

Plate II: Picture of soybean growth stages (Purcell *et al.*, 2014).

2.1.2 Importance of Soybeans

Soybean is generally considered as a highly versatile grain which has about 365 applications in the formulation of both human food and animal feeds with other industrial uses as well (Omotayo *et al*., 2007). Soybean is a cheap source of quality protein that is superior to all other plant foods because it has good balance of the essential amino acids. Its seed has a similar protein content and fairly similar amino-acids with cow milk (Belewu and Belewu, 2007). The fats from soybean is the unsaturated type unlike saturated fats from animal origin and hence is good for patients with heart disease (Adegoke *et al.*, 2002; Fabiyi, 2006). Other than the high protein content, it also has good amount of calories and fat. It contains eight essential amino acids and is a rich source of polyunsaturated fatty acids (including the good fat, omega 3) and it is free of cholesterol (Food and Agriculture Organization, 1999). It also contains 19.5gms of fat, 21gms carbohydrate and provides 432 kcal per 100gms (William and Akiko, 2000). It is one of the best vegetarian food items as far as protein content is concerned (Fabiyi, 2006). Research has it that one kilogram of soybean contained as much protein as 2kg of boneless meat or 45cups of cow's milk or 5dozen of eggs. Soybean seed contains about 40% protein, 30% carbohydrates, 20% oil and 10% mineral (Osho and Dashiell, 1998).

According to Fabiyi, (2006), soybeans can be utilized in the liquid, powdery and curd forms for human consumption, the oil could be converted to margarine and salad oil. In most cases, soybean has found wide application in the reduction of malnutrition related problems. Owing to its nutritional value there is a growing demand for soy products such as soymilk, soy oil, soy cake, and soy cheese (Popoola, 2007). The medicinal value of soybean is extremely essential in building the bodys' immune system. It can be used as a nutritional supplement for pregnant women, lactating mothers and children (World Healthiest Foods, 2004). Soy food

has also been reported to provide significant, but not total protection against heart disease, high blood pressure, stroke, ulcer, menopause, diabetes and cancer (Fabiyi, 2006).

Recently, soybean has been found to be an industrially important crop used as anti-corrosion agent, bio-fuel (due to less or no nitrogen element in its oil), also as disinfectant, constituent of in pesticides, printing inks, paints, adhesives, antibiotics and cosmetics (Ngalamu, *et al*., 2012). Reports by Omojasola (2000), has shown that the most important domestic processing forms of soybeans in Nigeria are dadawa, soy milk, soy ogi and soy cheese (wara). The soy based products produced by commercial processors are soy oil, soy cake and meal, infant foods, instant foods, soy flour, soy gum and flax (Omueti et al., 2000). In addition, feed mills utilize between 8.5% -11% soy for poultry mash and between 18% - 49 % for poultry concentrates; instant food companies utilize between 20% – 80% soy depending on the products while infant food companies utilize 30 per cent soy in their products (Omotayo *et al.*, 2007).

At the household level, soybean serves as a good substitute for locust bean in the preparation of " daddawa'' (local condiment in soup preparation), when ground, it is used in place of melon in soup and is a good source of cheap protein (Papoola, 2007) Soybean has been used to fortify many traditional foods of different ethnic groups in Nigeria and they include soy-ogi, soy-vegetable soup, soy-gari, soy-akpu, soy-hatsi, soy-tuwo, soy ice cream, soy cheese among many others (Obatolu *et al.,* 2006). Soybean meal is the material remaining after solvent extraction of soybean flakes and oil (Onuorah, et al., 2007). It is used as a protein supplement in poultry feeds, hog and cattle feed. Soybean oil is an edible oil that can be refined to produce paints, varnishes, soap, lubricants, sealant and in pharmaceutical oil. In the traditional soybean growing areas of Nigeria, soybean is most commonly intercropped with cereal crops like maize, sorghum and millet to replenish soil nutrients. Soybean entered

Nigerian diets in an attempt to improve nutrient intake, especially the protein intake of the low-income populace (Obatolu *et al.,* 2006).

Poverty and malnutrition often afflict the same groups of people, so rates of malnutrition are used as indicators of poverty (Adewale, 2005). Inadequate protein in diet appears to be the greatest nutritional problem facing Nigerians. This is because most sources of animal protein are expensive and only few people can afford enough of them in the diet. When needs to alleviate poverty, malnutrition, and to improve the welfare of poor people are considered, issues relating to high quality protein food and greater income opportunities are of paramount importance. According to Dugje *et al*. (2009), it is believed that soybean production will increase as more farmers become aware of the potential of the crop, not only for cash and food but also for soil fertility improvement and striga control.

2.1.3 Soybean production in Nigeria

According to ACET (2011), Nigeria is the largest producer of the crop for human and livestock feeds in West and Central Africa and has great potentials for substituting soy oil for some imported vegetable Oils. The current domestic demand and home consumption have made the crop a versatile and multipurpose agricultural product that could be processed in almost 365 ways for human, livestock and industrial purposes (AMREC, 2007). This same source also reported that the hitherto idle mills across the country are now looking inwards, producing edible oils from soybeans, preventing inefficiency of vegetable oil processing facilities as well as preventing inadequate supply of the oils.

At present, the major soybean producing states in the country are Benue, Kaduna, Taraba, Plateau and Niger. Other growing areas include, Nasarawa, Kebbi, Kwara, Oyo, Jigawa, Borno, Bauchi, Lagos, Sokoto, Zamfara and FCT. The yield of soybean of 1,700 kg per hectare on research plots in Nigeria compared favourably with the United States (US) yields

of 2000 kg/ha and Brazil yields of 1,800 kg/ha. However, there is a gap between the yield on farmers' field and research plots. Like most other crops, the total output of the soya crop is invariably influenced by the farmer's environment, the genetic potential of the planting material and the farmer's management capacity (AMREC, 2007). Benue State is the major producer of soybean in Nigeria followed by Kaduna. The current expansion in the production of soybean in Nigeria has been attributed to many years of research from the mid-1960s through the 1980s when Scientists adopted a nationally-coordinated approach to Soybean research (ACET, 2011).

FAOSTAT (2009) revealed that there was a steady rise in the production of soya bean for the period from 2000 to 2008, with Nigeria being ranked tenth. World soya bean production rose from 159.8406 million metric tonnes in the year 2000 to 228.3696 million metric tonnes in 2008. Nigeria's contribution stands at 0.4290 million metric tonnes in 2000 and 0.5910 million metric tonnes in 2008 (Shalma, 2014).

2.1.4 Diseases of Soybean

Some of the common diseases are caused by fungi, bacteria, and viruses (Dugje *et al* 2009). They are spread by insect vectors and nematodes while others are spread by wind, splashing rain, or movement in soil. Symptoms of disease include plant damage caused by a pathogen and the reaction of plants to infection. Some diseases have characteristic symptoms and signs that are identifiable in the field. However, several soybean diseases can share common symptoms and are difficult to identify in the field. They are also a major cause of yield losses (Phipps *et al.*, 2010).

2.1.4.1 *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 leave. 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*. 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 (Abubakar, 2015).
- 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 crops may show a brown discoloration that begins at the soil line and extends up the stem. The brown, dead leave remain attached to the crop, 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: Small spots with dark reddish-brown margin. Old lesions have papery tan to white center. Spots usually develop in mid-season in young, upper leaves of plant. Older, fully expanded leaves or leaves that develop in dry weather may escape disease. Signs: Light gray to white spores of fungus are produced in moist, humid weather (Phipps *et al.,* 2010).

2.1.4.2 Viral 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 below.

- 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 disease affected 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 (pre flowering) and significant reduction in pods (Abubakar, 2015).

- 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 (Abubakar, 2015).
- 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 crops do not produce any pods (Dugje *et al.*, 2009).
- Charcoal rot (*Macrophomina phaseolina*): The symptoms include stunt and reddish brown to black discoloration of lower stem in seedlings. Taproot and lower stem of older plants also have reddish to brown stains. Sometimes black flecking under the bark and black streaking in wood of taproots. Black sclerotia of causal fungus in taproots (Phipps *et al.*, 2010).
- Bacterial pustule (*Xanthomonas campestris pv. glycines*): Symptoms begin as minute lesions with elevated centers. Pustules form in center of lesions mostly on lower leaf surface and can be confused with soybean rust (Phipps *et al.*, 2010).

2.1.5. Control of soybean diseases (Dugje *et al.,* 2009).

i. Sow in a good seedbed. Avoid poorly drained or compacted soil.
ii. Sow seeds treated with fungicides as mentioned earlier under 'seed dressing'.
iii. Rotate crops with maize to prevent the increase in inoculum levels in a field.
iv. 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.
v. 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.
vi. 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.
vii. Do not sow seeds obtained from mosaic-affected crops. Rouge (uprooting and destruction) symptomatic crops. This can reduce the incidence of insect-transmitted viruses.
viii. Eradicate the weeds and voluntary plants in the vicinity of the soybean farms.
ix. Treat seeds with systemic insecticides and apply one or two foliar sprays of insecticides to reduce the insect vector activity during pre-flowering stage (most vulnerable to virus infections) of the plant (Dugje *et al.,* 2009).

2.2 Legume-Rhizobium Symbiosis

All organisms capable of transforming atmospheric nitrogen gas into biologically available form of nitrogen, through the process of called biological nitrogen fixation (BNF), are in

general collectively referred to as nitrogen-fixing organisms (NFO) (Ahmad, *et al.,* 2012). Among the two most widely studied nitrogen-fixing groups are the asymbiotic and symbiotic groups. The symbiotic bacteria are capable of forming nitrogen-fixing organ nodules on leguminous plants have classically been named "Rhizobia." In the beginning, all bacteria able to nodulate legumes were included in a single genus, Rhizobium within the family Rhizobiaceae (Rivas *et al.,* 2009). This genus had four fast-growing species: *R. leguminosarum, R. phaseoli, R. trifolii* and *R. meliloti* and two slow-growing species: *R. japonicum* and *R. lupine*. Later, on the basis of infection data, *R. leguminosarum* was found as microsymbiont for Vicia, Pisum and Lens; *R. phaseoli* for Phaseolus; *R. trifolii* for Trifolium; and *R. meliloti* for Medicago. The slow-growing species, *R. lupini*, was found to nodulate Lupinus and *R. japonicum* mainly Glycine max (Zaidi *et al.*, 2012).

However, even after the role of Rhizobia was well established, this genus was less explored in terms of its diversity and functionality (Howieson and Dilworth, 2016). Recently, with the advent of some newer molecular techniques and interest of rhizobiologists in exploring them as microbial inoculants for raising the productivity of crops especially legumes, the identification of rhizobial species from various hosts and locations has received a renewed attention. As a result, currently, Rhizobia have been reported to include seven bacterial families, divided into 15 genera (Table 2.1). A recent major advance, is the discovery of nitrogen fixation in the ß-Proteobacteria. The regularity of these organisms in nodules of subtropical Mimosa (although not an indigenous species in Mexico) and in many herbaceous legumes of the South African fynbos is notable. These organisms grow very quickly and have almost certainly previously been ignored, possibly considered as contaminants by many rhizobiologists (Howieson and Dilworth, 2016).

2.3 Nitrogen-Fixing Bacteria

Nitrogen (N_2) is an element essential for the support of all forms of life and it is found in amino acids, proteins and many other organic compounds (Egamberdieva and Kucharova, 2008). It is well documented by Frank *et al.*, (2003) that biological nitrogen fixation is carried out only by prokaryotes, which may be symbiotic or free living in nature. Kuykendall *et al.*, (2005), reported that in the beginning of the century, an American microbiologist, Bergey, initiated the publication named Bergey's Manual in which bacteria were classified on basis of their phenotypic characteristics. Their ability to induce nodules in legumes was from this date the basic criteria for differentiation of rhizobia and their taxonomy reached lower development than that of other soil related bacteria whose taxonomy was much more developed just due to their inability to form plant nodules (Rivas *et al.*, 2009).

Biological nitrogen fixation mediated by nitrogenase enzymes is a process important to the biological activity of soil. Nitrogenase activity in soil depends on ecological conditions in association with the specific nitrogen fixation capabilities of certain micro rganisms and plant genotypes under various climatic conditions (Simon, 2003). The nitrogen fixing activity of free-living, non-photosynthetic aerobic bacteria is strongly dependent on favourable moisture conditions, oxygen concentration and a supply of organic C substrates (Matthew, 2008). Nitrogen-fixing organisms are generally active in plant root zone soil (Egamberdieva and Kucharova, 2008). Before nitrogen can be incorporated into biological molecules, N_2 must be chemically reduced to the equivalent of ammonia. The biological reduction of nitrogen is catalyzed by a multimeric enzyme complex, nitrogenase (Frank *et al.*, 2003). Bacteria known to fix atmospheric nitrogen are collectively called ‘‘*Rhizobia*’’ and they are famous for their ability to induce nodules on the roots (and occasionally, stems) of legume plants. This symbiosis provides the bacteria with an exclusive niche and in return, the plants obtain a

‘‘bacteroid’’ form fix atmospheric nitrogen and the personalized nitrogen source (Andrew *et al.,* 2007). They occur in the so - called free - living forms e.g. aerobic Azotobacter, anaerobic Clostridia or in symbiosis with certain higher plants e.g. *Rhizobia* with legumes. The potential for biological nitrogen fixation is increased greatly by the fact that there is a close relationship between plants and nitrogen Prokaryotes (Bagali, 2012). Nitrogen fixing prokaryotes are able to make completely useful associations with plants: from loose associations to intercellular symbioses. There exists associative symbioses in which nitrogen fixing prokaryotes (e.g. *Azospirillum* and *Azotobacter species*) have been found to occur in rhizosphere of different plants such as sugarcane, maize, wheat, rice, grasses and others (Affourtit *et al.*, 2001).

2.3.1 *Rhizobium*

Rhizobium are involved in a symbiotic interaction with leguminous plants to form N-fixing nodules. Rhizobia have also been found to be capable of colonizing roots of non-legumes as efficiently as they colonize their legume hosts (Noshin, 2009). These organisms are characteristically able to invade the roots hairs of temperate-zone and some tropical-zone leguminous plants and incite production of nodules (Gonzalez *et al.,* 2005). Rhizobia have great potential to nitrogen fixers. They are rods, cocci, gram negative; colonies are circular, convex, semitranslucent, raised and mucilaginous, usually 2-4mm in diameter within 3-5 days on yeast mannitol-mineral salt agar media, commonly pleomorphic under adverse conditions (Holt *et al*., 1994).

2.3.2 *Azotobacter*

Azotobacter is an obligate aerobe, although it can grow under low oxygen concentration. The ecological distribution of this bacterium is a complicated subject and is related with diverse factors which determine the presence or absence of this organism in a specific soil

(Doroshenko *et al*., 2007). Bacteria of the genus *Azotobacter* are a well-known example of so-called associative nitrogen fixers, which are widespread in the soils of tropical, subtropical and temperate regions. These bacteria develop in close relationships with the roots of various wild and agricultural plants (Rawia *et al*., 2009).

2.3.3 *Azospirillum*

Azospirillum is considered as an important rhizobacterial genus involved in improvement of plant growth or crop yield worldwide (Bashan *et al*., 2004). Bacteria of the genus *Azospirillum* are associative nitrogen (N)-fixing rhizobacteria that are found in close association with plant roots. Genus *Azospirillum* is known for many years as plant growth promoting rhizobacteria (Okon, 1994; Okon and Vanderleyden, 1997).They are able to exert beneficial effects on plant growth and yield of many agronomic crops under a variety of environmental and soil conditions. *Azospirillum* is a much studied diazotroph, especially the species *lipoferum* and *brasilense*, which have been shown to infect a number of cereal plants including wheat, maize and sorghum (Dobbelaere *et al*., 2001). They were isolated from the rhizosphere of many grasses and cereals all over the world, in tropical as well as in temperate climates (Dobereiner *et al*.,, 1997). Both in greenhouse and in field trials, *Azospirillum* was shown to exert beneficial effects on plant growth and crop yields (Noshin, 2009).

2.3.4 *Cyanobacteria*

Cyanobacteria are important for global nitrogen cycle (Rodrigo *et al*., 2007). N_2-fixing *Cyanobacteria or* blue green algae are among the most widespread and important N fixers on Earth and they are considered to be the major N_2 fixers in freshwater and marine systems and terrestrial environments (Peter *et al*., 2002). *Cyanobacteria* or blue green algae are a diverse group of prokaryotes that often form complex associations with bacteria and green algae in

structures known as cyanobacterial mats and because of these structures, they have been used as biofertilizer in modern agriculture (Rodrigo *et al*., 2007).

2.3.5 Bradyrhizobium

The genus is rod shaped (0.5-0.9 µm x1.2-3.0 µm), commonly pleomorphic, non-spore forming, Gram negative, motile by polar or sub polar flagellum, aerobic, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor. Optimal temperature 25-30ºC, while turbidity develops only after 3-4 days in agitated broth. Growth on carbohydrate media is usually accompanied by extracellular polysaccharides, slime production particularly with glycerol, gluconate and mannitol. Rhizobial taxonomy has progressed notably during last 10 years, mainly due to the characterization of new host isolates and introduction of 16S RNA sequencing. Genus *Bradyrhizobium* with a single species, *B. japonicum,* was proposed for symbiont of soybean. Later, Hollis *et al.*, (1981) separated *B. japonicum* into three DNA homology groups with species *B. elkanii* for one group and *Bradyrhizobium* comprising extra slow growing glycine isolates, retaining the name *B. japonicum* for slow-growing isolates of *G. max.*

Symbiotic nitrogen fixation in agriculture can be attributed mainly to legumes, the plants in the Leguminosae contains more than 200 genera and 20000 species, which range from small plants such as clover to the large plants, *Acacia* species. Approximately 90% of them fix nitrogen from the atmosphere with *Rhizobiaceae,* either with *Azorhizobium, Bradyrhizobium, and Rhizobium* or with *Sinorhizbium* in root nodules. Symbiotic nitrogen fixation (SNF) resulting from mutual beneficial interaction between soybean and soil nodule bacteria provides a significant role of N fertilization. Soybean depends on its symbionts for a large part of its nitrogen requirement and effective growth with dry matter production (Deshmukh, 2013).

Table 2.1: The currently described rhizobia and the number of species in each genus (Howieson and Dilworth, 2016).

Family	Genus	Numbers of described species
α-Proteobacteria		
Bradyrhizobiaceae	*Bradyrhizobium*	15
Brucellaceae	*Ochrobactrum*	2
Hyphomicrobiaceae	*Azorhizobium*	3
	Devosia	1
Methylobacteriaceae	*Methylobacterium*	1
	Microvirga	3
Phyllobacteriaceae	*Phyllobacterium*	1
	Aminobacter	1
	Mesorhizobium	29
Rhizobiaceae	*Rhizobium*	43
	Neorhizobium	3
	Sinorhizobium/Ensifer	13
	Shinella	1
ß-Proteobacteria		
Burkholderiaceae	*Burkholderia*	6
	Cupriavidus	2

(Howieson and Dilworth, 2016)

2.3.5.1 Diversity *of Bradyrhizobium*

Bradyrhizobium species are encountered in Africa, Asia, North and South America, Europe, and Australia. They nodulate a wide diversity of legumes in the Mimosoideae, Caesalpinoideae, and in the Papilionoideae. Many native tropical legumes from the Amazons,

Africa, and Central America, but also legumes in Mediterranean and temperate areas (Parker, 2001) are nodulated by *Bradyrhizobium*. Photosynthetic *Bradyrhizobium* strains have been isolated from stem and root nodules of *Aeschynomene*. More recently, *Bradyrhizobium* strains have been found inside rice plants in Asia (Engelhard *et al.,* 2000) and Africa (Chaintreuil *et al*., 2000).

A characteristic of the genus is its slow growth and alkali production in some media. *B. liaoningense* was described as a particularly slow grower (Xu *et al.,* 1995), a characteristic shared with some isolates of *Acacia* and *Phaseolus lunatus* or a number of Caesalpinoidea isolates from Brazil (Moreira, 2000). In *Bradyrhizobium* there are strains tolerant to high temperatures, desiccation, acidity and strains that fix nitrogen as free-living bacteria under low levels of oxygen. The size of the genome of *B.japonicum* is 8.7 Million base pairs (Mb), whereas the size of other species is unknown. In Africa, native *Bradyrhizobium* strains were recruited to nodulate adapted soybean cultivars, eliminating the need for inoculation (Abaidoo *et al*., 2000). Soybean symbionts used as inoculants in Brazil were identified as *B. elkanii* (Rumjanek *et al.*, 1993) or *B. japonicum* (Boddey and Hungria, 1997). Both *B. japonicum* and *B. elkanii* have been recovered from soybean nodules in the USA, in Paraguay (Chen *et al*., 2000), and in Japan (Minamisawa *et al*., 1992). *B. japonicum* strains with different interstrain nodulation competitive abilities that belong to different serogroups were recognized.

Few species have been described in *Bradyrhizobium,* and three of them correspond to soybean symbionts despite the fact that *Bradyrhizobium* strains are symbionts of a wide range of legumes. These symbionts were ascribed to “cowpea” bradyrhizobia in the past. Recently, novel bradyrhizobia groups have been reported. Legumes from the Canary Islands in Spain are also nodulated by *Bradyrhizobium* spp. (Vinuesa *et al*., 1998).

2.3.5.2 Methods of identifying *Bradyrhizobium* species.

Several phenotypic and genotypic methods are being used to classify bacterial isolates to the species and subspecies level (Kiess and Eberle, 2012). *Bradyrhizobium* have various characteristics which are important in distinguishing them from other organisms. There are tests which utilize these characteristics and help determine whether contaminants are present (Abubakar 2015). Some of the methods are described below:

- Gram stain: This is a stain which divides all bacteria into two classes; they either react positively or negatively. Rhizobia in general 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 (Abubakar, 2015).
- Growth on yeast mannitol agar: Growth on YMA is very slow for *Bradyrhizobium*. Fast growers show moderate to excellent growth after three days while the slow growers show moderate growth after seven days to ten days (Abubakar, 2015).
- Growth on YMA with Congo red: *Bradyrhizobium* absorb very little of the red dye while some contaminants absorb the dye strongly (Sharma *et al.,* 2010).
- Bromothymol Blue (BTB) Test**:** This test helps to segregate alkali & acid producing strains. *Bradyrhizobium* strains are very slow growers and produce an alkali reaction. The reaction is observed after inoculation on plates of YEMA supplemented with 10% bromothymol blue. Alkaline range gives green colour & in acidic range it turns yellow (Rao and Ansari, 2013).
- Molecular identification:

Although, phenotypic methods play a key role in identification, genotypic techniques are more authenticated, reliable and useful for identification and diversity studies of bacterial isolates (Emerson, 2008). Phenotypic methods rely on morphological tests, biochemical tests, carbohydrate metabolism and enzyme production tests (Holt *et al.*, 1994), fatty acid analysis, intrinsic antibody resistance (Howieson and Dilworth, 2016), fluorescent antibody technique and polyacrylamide gel electrophoresis of total proteins (Prakash, 2012) Genotypic methods include: Polymerase Chain Reaction (PCR), Polymerase Chain Reaction-Randomly Amplified Polymorphic DNA (PCR-RAPD) (Lin *et al*. 1996), (Rep-PCR), Nucleic acid (DNA-RNA, DNA-DNA) hybridization (Vandamme *et al*. 1996), Two primer-Polymerase chain reaction (TP-PCR) (Rivas *et al*., 2001), low molecular weight-ribonucleic acid (LMW-RNA) profiles and restriction fragment length polymorphism (RFLP) (Noshin, 2009).

Currently, the preferred phylogenetic marker used in microbial ecology is 16S rRNA sequence (Tringe and Hugenholtz, 2008). The 16S-rRNA is a highly conserved molecule and its sequence pattern is used to indicate phylogenetic relationship between two bacterial species (Coenye and Vandamme, 2003). Highly conserved portions carry the information of early evolutionary events and the degree of divergence of present day rRNA sequences gives an estimation of their phylogenetic distances. (De Mandal *et al.*, 2015). These 16S rDNA sequence analyses support the well-established subdivision of rhizobia into species and genera (Noshin, 2009).

2.4 Nitrogen Fixation in Soybean

Rhizobia in general require plant hosts, they cannot independently fix nitrogen. Soybean crops get nitrogen from bacteria while the crop supplies carbohydrates, protein and sufficient oxygen so as not to interfere with fixation process. Nodules that fix nitrogen, are pink or red

inside when they are healthy. The significance of rhizobia forming root nodules and growth enhancement in soybean was widely studied by many scientists in the recent past (Saeki *et al.,* 2006). Until 1980, *Bradyrhizobium japonicum* was considered the sole symbiont of soybean (Jordan, 1982). However, the isolation of rhizobia from new areas around the world and the availability of molecular techniques allowed researchers to identify and/or classify, already known rhizobia, as new symbionts of soybean (Appunu *et al*., 2008). There is a wide array of responses of soybean to rhizobium (Lo`pez-Bellido *et al*., 2005). While some cultivars are fully incompatible with rhizobia, others might exclude or restrict nodulation by bacteria belonging to certain serogroups of *Bradyrhizobium japonicum* (Van *et al*., 2007).

Alternatively, soybean cultivars might be highly promiscuous like TGx African soybean cultivars (Vanlauwe *et al*., 2003). However, Sanginga *et al*. (2000) noted that promiscuous soybean is incapable of nodulating effectively with indigenous rhizobia in all locations in the moist savanna zone of Nigeria. However, it is important to note that even promiscuously nodulating soybeans (that often do not require inoculation), developed and cultivated in some parts of Africa, sometimes respond to inoculation (Lichtfouse *et al*., 2011).

Soybean can obtain up to 80% of its total nitrogen requirement from biological nitrogen fixation (Salvagiotti *et al.,* 2008). Sanginga et al., 2003 reports that some soybean varieties can biologically fix 44 to 103 kg N ha^{-1} annually. However, the quantity of biologically fixed nitrogen can be reduced if the crop is supplied with starter nitrogen above 50 kg N/ha and or if soil available N is far below 10 $kgha^{-1}$ (Van Kessel and Hartley, 2000). Other nutrients influencing biological nitrogen fixation include: P, Ca, Mg, and Zn (Hungria and Vargas, 2000). Inoculation of soybean with rhizobia in areas with low or ineffective native rhizobia is also reported to increase biological nitrogen fixation (Abaidoo *et al.,* 2007). Inoculated late and medium maturing soybean cultivars exhibit increased nitrogen content and dry matter in

seed and vegetative parts (stem and leaves), nitrogen harvest index and seed yield (Sogut, 2006).

The amount of nitrogen fixed is primarily controlled by four principal factors: the effectiveness of rhizobia-host plant symbiosis, the ability of the host plant to accumulate nitrogen, the amount of available soil nitrogen and environmental constraints to N_2 fixation (Van Kessel and Hartley, 2000). The soil environment is influenced by a combination of factors including acidity (leading to toxicities of Al and Fe), salinity, alkalinity (including high concentrations of Ca and boron) soil temperature, moisture, fertility (including nutrient deficiencies), and soil structure (Hungria and Vargas, 2000). Legumes should have effective root rhizosphere associations for effective N_2 fixation. Successful inoculant strains must be able to rapidly colonize the soil and tolerate environmental stresses, as well as compete with other soil micro-organisms (Slattery *et al.,* 2001).

2.5 Measurement of biological nitrogen fixation

Measurement of biological nitrogen fixation is critical as it enables establishment of the amount of nitrogen fixed by different legumes and their potential on improving soil fertility (Omondia, 2013). Several methods are being used such as the nitrogen balance method, nitrogen difference method, ureides method, ^{15}N isotope technique and acetylene reduction method, (Unkovich *et al.,* 2008). The N balance method estimates N_2 fixation on an area basis, i.e. kg N/ha), while the N difference method can be used for either single plants or an area (Giller and Merckx 2003. The ^{15}N isotope technique and ureide methods provide estimates of the percentage of total N of the plant or crop that is derived from N fixation (%Ndfa). Finally, the acetylene reduction technique assays the activity of nitrogenase catalysing N_2 fixation (Howieson and Dilworth, 2016).

2.6 Significance of Biological Nitrogen Fixation

The atmosphere is a nearly homogeneous mixture of gases, the most plentifully is nitrogen 78.1% (Garrison, 2006). About 96% of the N taken up by the crop has been measured as nitrogen derived from atmosphere (Lo`pez-Bellido *et al*., 2005). Biological nitrogen fixation involves conversion of atmospheric nitrogen (N) to ammonium, a form of N that can be utilized by plants (Vessey *et al*., 2005). The nature of biological nitrogen fixation is that the di nitrogenase catalyzes the reaction, splitting triple-bond inert atmospheric nitrogen (N_2) into ammonia molecule (Cheng, 2008).

Biological nitrogen fixation is regarded as a renewable resource for sustainable agriculture as it helps to reduce nitrogen requirements from fertilizer and thus increases economic returns to producers (Walley *et al*., 2007). Furthermore, it plays a key role in the assessment of rhizobial diversity, contributes to worldwide knowledge of biodiversity of soil microorganisms and to the establishment of long-term strategies aimed at increasing contributions of legume-fixed N to agriculture. The fixation of nitrogen by legumes has the potential to contribute greatly to more economically viable and environmentally friendly agriculture (Odair *et al*., 2006). It has been estimated that 80% – 90% of the nitrogen available to plants in natural ecosystems originate from biological nitrogen fixation (Rascio and Rocca, 2008).

Biological nitrogen fixation contributes to the replenishment of soil N, and reduces the need for industrial N fertilizers (Larnier *et al.,* 2005). It offers an economically attractive and ecologically sound means of reducing external N input (Yadvinder-Singh *et al.,* 2004). In recent years, agricultural systems have changed to improve environmental quality and avoid environmental degradation (Waswa, 2013). One of the most promising techniques to avoid environmental degradation is the use of inoculants composed of diazotrophic bacteria as an alternative use of nitrogen fertilizers (Roesch *et al*., 2007; Shridhar, 2012).

2.7 Mechanism of Biological Nitrogen Fixation

Biological nitrogen fixation is catalyzed by a two-component nitrogenase complex (Yan *et al*., 2010). Rhizobia is able to synthesize the enzyme nitrogenase, which catalyzes the conversion of N_2 to two molecules of ammonia (NH_3) (Tsvetkova *et al*., 2003). As indicated by Burris, (2001), the reduction of one nitrogen molecule by nitrogenase requires the energy of at least 16 ATP, or 2 ATP for each electron involved in the reduction. The best known Biological Nitrogen Fixation (BNF) system occurs between legumes and rhizobium bacteria (Carvalho *et al*., 2011). This symbiotic association accounts for the development of a specific organ, the symbiotic root-nodule, whose primary function is nitrogen fixation and the root nodules make a crucial contribution to the nitrogen content of the soil playing a key role in agricultural practices (Alla *et al*., 2010; Abubakar, 2015).

Perception of legume root exudates triggers the production of rhizobial Nod factors which are recognized by compatible plant receptors leading to the formation of root nodules, in which differentiated bacteria (bacteroids) fix atmospheric nitrogen (Oldroyd and Downie, 2008). In the nodule, maintenance of nitrogenase activity is subject to a delicate equilibrium. Firstly, a high rate of oxygen respiration is necessary to supply the energy demands of the Nitrogen reduction process (Sanchez *et al*., 2011), but oxygen also irreversibly inactivates the nitrogenase complex. These conflicting demands are reconciled by control of oxygen flux through a diffusion barrier in the nodule cortex and by the plant oxygen carrier, legheamoglobin, which is present exclusively in the nodule (Minchin *et al*., 2008).

In addition to fixing nitrogen, Burris (2001), reported that some rhizobia species are able to grow under low oxygen conditions using nitrate as electron acceptor to support respiration in a process known as denitrification by which bacteria reduce sequentially nitrate (NO_3) or nitrite (NO_2) to Nitrogen (N_2). Nitrate is reduced to nitrite by either a membrane-bound or a

periplasmic nitrate reductase, and nitrite reductases catalyse the reduction of nitrite to nitric oxide (NO). Nitric oxide is further reduced to nitrous oxide (N_2O) by nitric oxide reductases and, finally O_2 is converted to N_2 by the nitrous oxide reductase enzyme (Van Spanning *et al*., 2007). The significance of denitrification in rhizobia-legume symbiosis can be appreciated when oxygen concentration in soils decreases during environmental stress such as flooding of the roots, which causes hypoxia. Under these conditions, denitrifying activity could work as a mechanism to generate ATP for survival of rhizobia in the soil and also to maintain nodule functioning (Sanchez *et al*., 2011).

2.8 Factors Affecting Nitrogen Fixation

Environmental factors influence all aspects of nodulation and symbiotic N-fixation, in some cases reducing rhizobial survival and diversity in soil; in others affecting nodulation or nitrogen fixation and even growth of the host. Factors that are important are discussed below:

Phosphorous (P) fertilization is the major mineral nutrient yield determinant among legume crops (Chaudhary *et al*., 2008). Phosphorus deficiency is a major constraint of effective nitrogen fixation because phosphorus is an important nutrient in the process of nodulation and nitrogen fixation (World Bank, 2006). The high requirement for P in legumes is consistent with the involvement of P in the high rates of energy transfer that must take place in the nodule. Under P shortage conditions, legumes may lose the distinct advantage of an unlimited source of symbiotic N (Sulieman *et al*., 2008). The more the supply of phosphorus, the more abundant are the nodules (Gowariker *et al*., 2009). Phosphorus is a key structural and functional element in the plant: its efficiency has effects on plant morphology but also on its metabolism (Ofori, 2016). Thus, a decrease in phosphorus requirement mainly reflects on reducing the leaf area, shoot dry matter and phosphorus content in shoot and root. However,

root growth has inverse relations with P because under deficiency, root growth is stimulated as a strategy to improve the phosphorus nutrition (Ahmed, 2007).

Drought, as defined in agronomy, is a temporary or durable change in the plant water status, affecting its functioning, and it is related to a decrease in soil water content (Katerji *et al*., 2011). Drought is by far the most important environmental factor contributing to crop yield loss, including soybean (*Glycine max*.) where symbiotic fixation of atmospheric nitrogen (N_2) is sensitive to even modest soil water deficits **(**Sinclair *et al*., 2007). Drought-related inhibition of biological nitrogen fixation (BNF) seriously limits legume yield in many arid and semiarid regions of the world. Decline of N_2 fixation with soil drying causes yield reductions due to inadequate N for protein production, which is a critical seed product **(**Sinclair *et al*., 2007). Several studies have shown that drought stress reduces nitrogen fixation in leguminous species (Waswa, 2013).

Among the most common effect of soil salinity include growth inhibition by Na^+ and Cl^- (Waswa, 2013). Elevated Na^+ in soil solution inhibits the uptake of other nutrients (e.g., P, K, Fe, Cu, and Zn) directly by interfering with various transporters in the root plasma membrane (Giri *et al*., 2007). Salinated soil has very little nitrogen and therefore not suitable for cultivation of most plants. An appropriate solution to this situation would be the cultivation of plants that are able to fix nitrogen through biological nitrogen fixation (Chen *et al*., 2000). Most leguminous plants, however, are sensitive to even low levels of salinity (Waswa, 2013).

Increasing salt concentrations may have a detrimental effect on soil microbial populations as well, either due to direct toxicity or through osmotic stress (Yadav *et al*., 2010). For example, most rhizobia are sensitive to moderate and higher levels of salinity during both the free-living stage and the symbiotic process. However, both legume growth and the process of nodule formation are more sensitive to salinity than are rhizobia (Singleton *et al*., 1982;

Zahran, 1999). For instance, *Sinorhizobium meliloti* tolerated up to 300 mM NaCl, while nodulation and nitrogen fixation of its host (*M. sativa*) was inhibited at about 100 mM salt (Graham and Vance, 2000).

Optimum temperature for nitrogen fixation and soybean growth, range from 25 to 30°C. In this temperature range, the bacteria can begin actively fixing nitrogen within seven days of forming nodules. A continually cool root zone temperature can significantly delay the onset of nitrogen fixation compared to an optimum soil temperature (Abendroth *et al*., 2006).

The optimum soil pH is between six and seven. A soil pH significantly outside this range (less than five or greater than 8) is detrimental because it disrupts the communication process leading to root hair infection, thereby limiting nodule development. Soil pH also affects the amount of nitrogen fixed. For example, in a very acidic soil (pH 4), nitrogen fixation can be reduced up to 30 percent (Abendroth *et al*., 2006). Apparent effect of soil pH and exchangeable acidity on the relative dominance of rhizobial types have been reported by Bala and Giller, (2007).

2.9 The need to Inoculate Soybean Seed

Until 1980, *Bradyrhizobium japonicum* was considered the sole symbiont of soybean (Jordan, 1982). However, the isolation of rhizobia from new areas around the world and the availability of molecular techniques allowed researchers to identify or classify, already known rhizobia, as new symbionts of soybean. There is a wide array of responses of soybean to rhizobium. While some cultivars are fully incompatible with rhizobia, others might exclude or restrict nodulation by bacteria belonging to certain serogroups of *Bradyrhizobium japonicum* (Van *et al*., 2007). Alternatively, soybean cultivars might be highly promiscuous like TGx African soybean cultivars (Vanlauwe *et al*., 2003). However, Sanginga *et al*. (2000) noted that promiscuous soybean is incapable of nodulating effectively with indigenous

rhizobia in all locations in the moist savanna zone of Nigeria. A study carried out by Osunde *et al.,* (2003) at five sites in the moist savanna region of Nigeria showed that promiscuous soybean varieties responded to inoculation. However, 'Magoye' an exceptionally promiscuous line released in Zambia in 1981, nodulates readily in all soils of Southern Africa where it has been tested and rarely responds to inoculation in Zambia and Zimbabwe (Mpepereki *et al*., 2000).

Despite the apparent potentials of the crop legumes as contributors to soil-N, very little conscious efforts have been made towards enhancing their performance. There is therefore the need for the development and application of Rhizobium seed inoculants from the existing populations present in soils found in this region. This advocacy is founded on the understanding that, doing that would provide a simple means of maximizing both their yield as crops and their contribution to soil-N (Machido *et al.,* 2010).

2.10 Prospects of inoculant production in Nigeria

Legume inoculation is an established agricultural practice used since the end of the last century (Abdullahi *et al.,* 2013). It has contributed to increased N_2 fixation and yield of legume crops in situations where the natural N_2 fixation is deficient, thereby increasing yield and adding nitrogen to the soil. Biological nitrogen fixation is economical and environmentally suitable hence it improves the condition of soils (Deaker *et al.,* 2011).

Microbial inoculants are becoming more available as sustainable alternatives to nitrogen fertilizers (Agboola and Moses, 2015). The benefits, production and utilization of rhizobia inoculants for legume improvement has been recognized for a very long time in developed countries, but still developing in sub-Saharan African countries, including Nigeria (Abdullahi *et al.,* 2013). Australian agriculture, for instance, has this recognition for more than 100

years, which includes substantial increases in legume nodulation, grain and biomass yield, nitrogen fixation and post-crop soil nitrate levels (Isaac, 2013).

BIOFIX is a commercial Rhizobium inoculant produced at MEA Ltd Nakuru, Kenya to meet specific need of most legumes crops such as beans, soybeans. One of the problems affecting farmers' use of BIOFIX is its quality. Poor quality control in the production process as well as problems associated with its transportation and storage negatively affects the viability of inoculants (Odame, 1997). A series of inoculation trials have been conducted on farmers'field by N_2Africa program to improve legume crop yields and soil fertility through the use of commercial inoculants (Isaac, 2013). The benefit of increased knowledge of symbiotic nitrogen fixation is the inoculation of legumes by selected rhizobia, a practice that allows producers to introduce rhizobia into the soil-plant ecosystem (Ben Reban *et al.*, 2006). The use of inoculants in other countries has increased legumes production. According to Abdullahi *et al.*, (2013) positive responses of these soybean cultivars to inoculants have been reported in Africa with yield increases of up to179%. Biofix that was used in this study was provided by the Department of Soil Science at Institute of Agricultural Research (IAR), Ahmadu Bello University, Zaria.

In Nigeria IITA and N_2 Africa are exploring commercial inoculant production through its recently established Business Incubation Platform (BIP) and through this, a pilot product has been produced called Nodumax (Woomer and Mahmadi, 2014). NoduMax is a peat-based carrier intended for soybean crops, containing elite Bradyrhizobia. It is packaged in 100 g quantities intended for 10 kg to 20 kg of soybean seed along with gum arabic and instructions in four languages, English, Hausa, Ibo and Yoruba. Currently only inoculants for soybean are under development, but additional products, including a granular formulation for groundnut

are planned. It is inexpensive, costing under 500 Naira per 100g package, enough to inoculate 10 kg to 15 kg of seed and one hectare requires about five or six packages (IITA, 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Soil sample collection and handling

Soil samples were collected from fields previously cropped with soybean, cowpea and maize at Institute of Agricultural Research (IAR) of Ahmadu Bello University, Zaria. From five (5) different points in each field, 2 kg of soil sample was taken from 0-15 cm soil depth. This was done along a Z-plane on each field (Ofori, 2016). Samples were packaged into clean polythene bags labeled appropriately and transported to the laboratory. The five (5) sub-samples were bulked together and mixed thoroughly to obtain one composite sample. The soil sample were air dried, crushed and passed through a 2 mm or 0.5 mm mesh sieve (for physical and chemical analysis) and 4 mm mesh (for planting). Clear polythene bags were used to store sub samples for physical and chemical analysis and the remaining bulk sample was kept for screen house studies (Machido, 2010).

Samples of river sand were also collected from river bed sites using a hand trowel, stored in clean polythene bags as described earlier by Machido, (2010), and transported to the laboratory at Department of Microbiology, Ahmadu Bello University, Zaria. The river sand collected was bulked and then sieved through a 4 mm mesh sieve, dried under the sun and sterilized by autoclaving at 121°C temperature for 30 mins.

3.1 Determination of Physical and chemical Properties of Soil Samples

3.1.1 Soil texture

This was determined by the Bouyoucos hydrometer method (Ofori, 2016). Fourty (40) g soil was weighed into 250 mL beaker and oven dried at 105°C over night. The sample was placed in a desiccator to cool, after which the oven dry weight was taken. A 100 ml of dispersing agent sodium hexa-metaphosphate was added to the soil. It was then placed on a hot plate and heated until the first sign of boiling was observed. The content of the beaker was weighed into a shaking cap and fitted to a shaker and shaken for 5 minutes. The sample was sieved

through a 50 µm mesh sieve into a 1.0 L cylinder. The sand portion was dried and further separated using graded sieves of varying sizes into coarse, medium, and fine sand. These were weighed and their weights recorded. The 1.0 L measuring cylinder containing the dispersed sample was placed on a vibration-less bench and then filled to the mark. It was covered with a watch glass and allowed to stand overnight. The hydrometer method was used to determine the silt and the clay contents. The cylinder with its content was agitated to allow the particles to be in suspension. It was then placed on the bench and hydrometer readings taken at 40 seconds and 6 hours interval. At each hydrometer reading, the temperature was also taken.

The percent sand, silt and clay were calculated as follows:

% Clay = corrected hydrometer reading at 6 hours x 100/weight of sample

% Silt = corrected hydrometer reading at 40 seconds x 100/weight of sample - % clay.

% Sand = 100 % - % silt - % clay

The various portions were expressed in percentage and the textural triangle was used to determine the soil textural class (Ofori, 2016).

3.1.2 Determination of soil pH

Soil pH was measured in a 1:2.5 soil: water ratio and 0.01M $CaCl_2$ using a glass electrode pH meter. Ten (10) g of soil was weighed into 50 mL shaking bottle and 25 mL of deionised water was added to the soil. The soil-water suspension solution was stirred thoroughly for 30 minutes after which the suspension was allowed to stand for 20 minutes. The pH was read by immersing the electrode into the upper part of the soil suspension and the pH value recorded (Waswa, 2013).

3.1.3 Determination of soil nitrogen

This parameter was determined following the Kjeldhal procedure described by Bremner (1965). One grams (1 g) of the soil sample was weighed using an electronic top loading balance and placed in dry 500 ml micro-Kjeldhal flasks. To this few drops of water was added and the flask was swirled for 5 minutes, after which the flask was allowed to stand for 30 minutes. This was followed by addition of 5 g of Khjeldahl catalyst (a mixture of 500 g $NaSO_4$, 50 g of $CuSO_4$ and 0.5 g of selenium grinded to fine powder) and 20 ml of concentrated H_2SO_4 into the flasks and it was subjected to heating on the digestion stand until all the water was removed and frothing has ceased. At this point the heat was increased until the digest has cleared. The digest was then gently boiled for 5hours. The flask was then allowed to cool and little water was added slowly with shaking while cooling under a cold water tap. The digest was then transferred into a 100 ml conical flasks for distillation.

To determine the ammonium-nitrogen liberated in the digest, the flask was held at angle followed by the addition of a teaspoonful of pumice and 10 mL of 10 N NaOH solution was poured down the neck of the flask. The distillation process then commenced immediately and the distillate was collected in a 100 mL Erlenmeyer flask containing 20 ml of 2% boric acid indicator. Distillation was continued until 50 mL of the distillate was collected. The ammonium-N in the distillate was determined by titration with 0.01 N H_2SO_4. The end point was attained and titration discontinued when the colour changed from green to purple. The titre was then recorded and the ammonium-N calculated (Machido, 2010).

3.1.4 Determination of water holding capacity of soil samples

Hundred grams (100g) of each of the soil sample was weighed in triplicate metal cans with perforated bottoms. The samples were flooded with distilled water until water was dripping through the perforations at the bottom of the cans. The cans were then allowed to stand overnight to bring the soil to field capacity. The weight of the can and the wet soil were then

determined and recorded as W_1. The can plus the wet soil were transferred into an oven at 105ºC until it reached a constant weight and weighed to obtain the weight of can plus dry soil and recorded as W_2. The water holding capacity of the soil samples was calculated using this formula;

$$WHC\ (\%) = \frac{W_1 - W_2}{100} \times 100$$

The results obtained for the three replicates of the soil samples was then added up and divided by three to obtain the average WHC of the soil (Machido 2010)

3.1.5 Determination of organic carbon

Ten grams of the soil sampled was grounded and passed through 0.5mm sieve. Samples were placed in 500ml wide mouth Erlenmeyer flask. To this, 10 ml of 1 N dry potassium dichromate ($K_2Cr_2O_7$) solution was added and the flask was swirled gently to disperse the soil in the solution. This was followed immediately by the addition of 20 ml of concentrated H_2SO_4 and the flask was swirled gently to mix the soil and the reagent, then vigorously for at least 1 minute. The flask was then kept on asbestos sheet for 30 minutes followed by addition of 200 ml of water. The suspensions was then filtered and 3 drops of 0-phenanthrolien indicator was added to the filtered solutions and titrated with 0.5 M $FeSO_2$. The end point was noted when the solution changed to dark green colour. The organic-carbon content of the soil samples was then calculated using the formula (Machido 2010).

$$OC\% = \frac{(\text{meq. } K_2Cr_2O_7 - \text{meq.}FeSO_2\ ) \text{ X } 0.003 \text{ X } 100}{\text{gram of dry soil}} \times f$$

Where f= correction factor = 1.33 (Machido 2010)

3.2.6 Determination of exchangeable bases

The soil contents of exchangeable Na, K, Mg and Ca was estimated following the ammonium acetate (NH_4OAc) saturation method (Abubakar, 2015). This was carried out by leaching 10g of soil sample using 250ml 1 N NH_4OAc. The quantity of Mg^{2+} and Ca^{2+} ions was determined using atomic absorption spectrophotometer. The Na^+ and K^+ was determined by flame emission photometry.

3.2.7 Determination of available phosphorus

Available phosphorus was determined by Bray 1 Method. Five (5) g of the soil sample was weighed into 100 ml extraction bottle with 35 ml of Bray 1 solution (0.03*M* NH4F and 0.025*M* HCl) was added. The bottle was placed in a Stuart (United Kingdom) reciprocal shaker and shaken for 10 minutes after which it was filtered through a Whatman No. 42 filter paper. A 5 ml aliquot of the filtrate was pipetted into 25 ml flask and 10 ml colouring reagent (ammonium paramolybdate) was added followed by a pinch of ascorbic acid. The contents were well-mixed and allowed to stand for 15 minutes to develop a blue colour. The colour intensity was measured using a spectrophotometer at wavelength of 660 nm. The available phosphorus was extrapolated from a standard curve (Ofori, 2016)

Calculation: $P\ (mg\ kg^{-1}) = \frac{35 \times 15 \times (a-b) \times mcf}{s}$

where: a = mg P L^{-1} in the sample extract

b = mg P L^{-1} in the blank

mcf = moisture correction factor

35 = volume of extracting solution

15 = final volume of sample solution

s = sample weight in grams.

3.2.8 Determination of cation exchange capacity

The sodium acetate saturation method was used as described by Chapman (1965). Four gram (4g) of the soil samples was placed in a 50ml round bottom narrow centrifuge tubes. To this, 33 mL of 1 N sodium acetate (NaOAc) was then added and the tubes were stoppered and agitated on a mechanical shaker for 5 minutes the tubes were then centrifuged to obtain a clear supernatant liquid which was decanted. This exercise was repeated 3 times. The soil samples were then washed in the same manner with 33 mL portions of 99% isopropyl alcohol. The soil was leached with acidified sodium chloride gradually to a volume of 250 ml. Fifty (50) ml of 2% boric acid with a few drops of indicator was measured into a 250 ml conical flask. The acidified NaCl was poured in a 500 ml kjeldhal flask, to this 10 ml of 1 N NaOH was added and distilled. The distillate was collected (150 ml) and titrated with 0.1 N HCL. Cation exchange capacity was calculated by the summation of the exchangeable cations (K^+, Na^+, Ca^{2+} and Mg^{2+}) and total exchangeable acidity (Agboola and Moses, 2015).

3.3 Isolation of *Bradyrhizobium* species

3.3.1 Plant Trap Experiment

Three (3) plastic pots (13cm x 8cm) were perforated at the bottom. A Whatman filter paper was placed at the bottom of each plastic pot to prevent too much water from draining out and 1 kg of the soil sampled was weighed into each pot. Single super phosphate and muriate of potash were mixed with the samples in each pot at the rate of 15.9 mg per kg (P_2O_5) and 11.4 mg per kg (K_2O) per hectare (IITA, 2009). The treated soil was moistened until the water started draining out and then left to stay overnight. Five (5) Seeds were sown into each pot at a depth of 2cm. The crops were watered daily with the appropriate volume of water and allowed to grow for a period of 6 weeks (Plate I). Crops were harvested by washing the soil under slow running tap water to minimize loss of root nodules (Machido 2010). The nodules formed on the roots of the trap plants were harvested for the isolation of *Bradyrhizobium* sp.

Plate III: Experimental set-up for the plant trap.

3.3.2 Preparation of *Bradyrhizobium japonicum* selective medium (BJSM)

Bradyrhizobium japonicum selective medium (BJSM) was prepared as described by Tong and Sadowsky (1994). It is a mineral salts medium supplemented with 0.1% yeast extract, 0.1% L-Arabinose and 0.1% sodium gluconate. The medium was further supplemented with 83ug of $CoCl_2$, 88ug $ZnCl_2$ per ml, and 1.0 µg of BG per ml (Appendix I) which serve as the selecting agents for *Bradyrhizobium sp.*

3.3.3 Surface sterilization of nodules.

Healthy nodules were detached from the roots. The nodules were thoroughly washed with distilled water to remove all traces of soil, and then immersed in 95% ethanol for 30 secs to break the surface tension and to remove air bubbles before soaking in a 5.25% sodium hypochlorite solution for 4 min to surface sterilize them. Thereafter, the nodules were rinsed six times with sterile water to remove all traces of the sodium hypochlorite and crushed

between blunt-tipped forceps and the nodule contents mixed with a drop of sterile water (Mungai and Karubiu, 2000).

3.3.4 Inoculation and incubation

A loopful of the nodule suspension was aseptically streaked onto plates of BJSM. The plates were incubated at 28^{o}C aerobically for 10 days. Colonies that were small, pure white and grew after 10 days were selected and subcultured onto fresh (Yeast extract mannitol agar) YEMA slants for further characterization.

3.4 Identification of Isolates.

3.4.1 Microscopic identification

The isolates obtained were microscopically identified by preparing smears of bacterial culture on glass slides. The smears were air-dried; heat fixed and Gram stained. This was done by flooding the smears with crystal violet for one minute followed by washing with distilled water. The smears were then flooded with iodine solution for one minute and decolorized with 95% ethanol for 30secs followed by washing with distilled water and counterstaining with safranin. The slides were washed with distilled water, air dried and observed under light microscope at ×100 magnification using oil immersion lens (Abubakar, 2015).

3.4.2 Growth on YEMA with Congo-red

This was carried out to check the purity of the isolates. Stock solution of Congo red was prepared by dissolving 0.25g of Congo red in 100ml of sterile distilled water. From stock solution, 10ml was added to a liter of YEMA and autoclaved. Loop full of test isolates were streaked on the medium and covered with aluminium foil to a dark condition and incubated at 28±2ºC for 3 to 7 days to detect Congo red absorption by the colonies (Sharma *et al.,* 2010).

3.4.3 Growth on Yeast extract mannitol agar (YEMA)

Morphological characteristics of all the isolates were investigated after an incubation of 3 to 7 days at 28°C on yeast extract mannitol agar plate (appendix II), individual colonies were characterized based on their shape, texture and colour (Aneja, 2003).

3.4.4 Bromothymol Blue (BTB) Test

The isolates were streaked on YEMA-BTB agar plates. YEMA-BTB agar was made by adding 5 ml of (0.5% BTB in ethanol) to 1 litre of YEMA medium. The plates were incubated at 28±2ºC for 2-10 days. The change in color of medium was observed. The isolates were classified as slow growers (medium turns blue) or fast growers (medium turns yellow) on their reaction on YEMA supplemented with BTB (Rao and Ansari, 2013).

3.4.5 Carbohydrate utilization

Bradyrhizobium japonicum has the ability to form acid slowly from the utilization of xylose. Yeast extract agar slants were prepared but in this case mannitol was substituted with xylose. To detect colour change phenol red (pH indicator) was added and a loopfull of the isolates were streaked on the slants and incubated at 28ºC for 7-10days. Acid formation was indicated by a change in colour from orange (pH 7) to yellow (below 6.4) due to the formation of organic acid (lactic acid) that indicated positive test while a red colour indicates alkali production (Kaur *et al.*, 2012).

3.4.6 Motility Test

The motility medium was inoculated with the isolates by making a fine stab with a needle to a depth of about 1-2cm short of the bottom of the tube and incubated at 28ºC for 48-72hrs. The line of inoculation was observed for growth. Cloudy growth indicates motility while growth restricted to the line of inoculation indicates a non-motile organism (Ilyas, 2009).

3.5 Molecular Characterization

3.5.1 DNA Extraction

Isolates were inoculated in yeast extract mannitol broth (YEMB) for 4 days on a shaker. Isolation of genomic DNA was conducted using the method described by Kasova-Wade *et al* (2003). Two hundred micro litre (200 µl) of the isolate was transferred into a 1.5 ml tube, to this 400 µl of lysis buffer and proteinase K was also added. The tube was placed on a heat block for 1 hr at 60°C. Phenol chloroform (400 µl) was added and vortexed briefly and later spun at 13000 rpm for 10 mins to separate the phases. The upper layer was carefully removed and transferred to another 1.5 ml tube using a pipette. Four hundred micro litre of chloroform (400 µl) was added to the recovered layer, vortexed briefly and spun at 13000 rpm for five minutes. The upper layer was removed carefully and transferred into another tube. Equal volumes of ethanol (100%) and 20 µl of 3M sodium acetate was added, mixed properly and incubated overnight at -20 °C. The ethanol was removed and the mixture was washed again with 400 µl of 70% ethanol, spun for 5 minutes and the ethanol was removed again. The washing was repeated twice. The DNA was dried out by leaving the tube open for 3-10mins and the resuspended in 50 µl of sterile water (Kasova-Wade *et al.*, 2003).

3.5.2 Polymerase chain reaction

The forward primer (5'-GGACTACAGGGTATCTAAT-3) and reverse primer (5'-AGAGTTTGATCCTGG-3') were used to amplify the 16S rRNA gene of *Bradyrhizobium*. PCR amplification reaction was performed in a 25µl volume containing: 6µl of the template DNA, 5µl Flexi buffer, 2.5µl MgCl, 0.5µl dNTPs, 0.5µl of each of the forward and reverse primers, 0.5µl Taq polymerase and 9µl nuclease free water. Amplifications was carried out in an Eppendorf Master cycler Gradient apparatus (Applied Biosystems, USA) with an initial denaturation at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 1min, annealing at 52°C for 1min, extension at 72°C for 1 min and a final extension at 72°C for 5

min. PCR amplified products were then separated by electrophoresis on a 1% agarose gel and viewed under UV light (Wilson *et al.,* 1990). The amplicons were purified with PCR purification kit and sequenced with DNA sequencing system at a commercial lab (DNA labs Kaduna, Nigeria). The resulting sequences were subjected to similarity searches on the NCBI data library (http://blast.ncbi.nlm.nih.gov/Blast.cgi, 2016) to determine the identity of the isolates.

3.6 Assessment of the ability of the Isolates to Form Nodules

The pure isolates were subcultured on yeast exctract manittol (YEMA) agar slants and stored at a refrigerating temperature (4°C) until required.

3.6.1 Sand culture

Assessment for nodule formation was carried out for each isolate that had been confirmed. Plastic cups (7cm x 3.75cm) were surface sterilized and filled with sterilized sand. Surface sterilization of the seed was carried out by exposing it to 2% solution of sodium hypochlorite, followed by rinsing 5-6 times with sterile distilled water. The seeds of soybean were then allowed to germinate on a large petri dish containing sterilized moist filter paper and incubated at 28°C. The germinated seeds were selected and transferred by using sterile forceps into the sterile sand at a depth of 1cm after four days. One surface sterilized seed was sown per cup. Nitrogen free mineral nutrient solution with a solution of trace elements was prepared and supplied to the crops (Appendix III). Three replicates were used for each treatment including the commercial inoculant (BIOFIX) which was supplied by Department of Soil Science (IAR) and also three replicates of positive control (Nitrogen added) and negative control (nitrogen free) treatments were used as controls. The treatments were arranged in a completely randomized design were fertilized with 20 ml Broughton and Dilworth N-free medium at The Department of Microbiology A.B.U. Zaria. (Solomon, 2016).

3.6.2 Preparation of inoculum and inoculation of root nodules

The medium Yeast extract manitol broth (YEMB) was prepared (Appendix II). Pure YEMB of colonies of *Bradyrhizobium* spp from soybean under growth on solid medium slant was then transferred into the broth and incubated at room temperature (28°C) on a rotary shaker for 3 days. The concentration of the bacterial inoculum was adjusted to 10^8 CFU per ml using 0.5 McFarland standard. 1 ml of the inoculum was applied to the root zone of the crops after sowing (Hassen *et al.,* 2014). Each plastic cup was labeled appropriately and all treatments were monitored for a period of 6 weeks.

3.6.3 Examination of sand culture for nodule formation

Soyabean crops were removed by washing the soil under running tap water to minimize loss of roots nodules. The roots were then carefully removed from the sand, rinsed with a gentle stream of water and the nodules removed (Machido, 2010). Nodulation efficiency was evaluated by enumerating the number and assessing the dry weight of viable nodules for each isolates at six (6) weeks after sowing. The percentage of root nodules formed was then calculated for each isolate by counting the number of nodules formed per isolate, dividing it by the total number of nodules formed and multiplying by 100 (Machido, 2010) To test the nitrogen fixation activity, fresh nodules per plant were cut open with razor blade and the colour observed. Red or pink colours were recorded for nitrogen fixing nodules while whitish colours were from non-nitrogen fixing species. These colorations reflect the colour of legheamoglobine which is an indication within a root nodule that N-fixation is taking place (Linderman, 2008). The nodules and shoots were oven dried at 60°C for 72hrs and then weighed to determine shoot and nodule dry weight (Mungai and Karubiu, 2000).

3.7 Determination of Nitrogen Content of Experimental Soybean crop

The dried soybean crop (shoots) were grounded and passed through a 40 mesh screen and the N-content in the crop material was determined using the micro-kjeldhal method (Machido, 2010). The biological nitrogen was determined by using the N difference method (Howiensen and Dilworth, 2016).

3.8 Data analysis

Data collected on nodulation and plant biomass were analysed using analysis of variance (ANOVA). Where the F-ratios were found to be significant, the Duncan Multiple Range Test (DMRT) was used to separate the means of treatments.

CHAPTER FOUR

4.0 RESULTS

4.1 Physical and chemical properties of the soil

Results of physical and chemical analysis revealed the soil used for the study was sandy loamy in texture and moderately acidic (Table 4.1). Although the total nitrogen and organic carbon was found to be low (FMANR, 1990), the pH, exchangeable bases (Na, Ca, Mg, K,), available phosphorus and cation exchange capacity are within the normal range that supports the growth and survival of *Bradyrhizobium* species (Table 4.1).

4.2 Occurrence and identification of *Bradyrhizobium species* in the soil Examined

A total of 29 isolates presumptily identified to be *Bradyrhizobium* spp were obtained based on their cultural and microscopic morphologies (Table 4.2a and Table 4.2b). Of this, 18, 7 and 4 were recovered from soil samples from fields cultivated with Soybean, Cowpea and maize respectively (Table 4.3). However, these numbers declined to 5, 1 and 0 following further biochemical tests (Table 4.3). Five (5) of the isolates confirmed to be *Bradyrhizobium* spp were obtained from soil samples from fields cultivated with soybeans and only 1 from field cultivated with cowpea the previous year. No *Bradyrhizobium sp* were detected in soil sample from field cultivated with maize (Table 4.3)

The identity of all the six (6) isolates were confirmed by PCR amplification of the 16S rRNA gene with the expected amplicon size of 789bp (Plate I). Of the isolates thus identified, 4 were found to be *Bradyrhizobium elkanii* with closest sequence similarity of 93% for B36, 95% for B64 and 99% for B29 and B45 respectively based on GenBank database. However, no corresponding close match was found for B1 and B55 in the Genbank database (Table 4.4).

Table 4.1: Physical and chemical properties of soil

Test parameters	Test Values

Particle size distribution (%)	
Clay	10
Silt	30
Sand	60
Textural class	Sandy loam
pH(H_2O)1:2	6.50
pH($CaCl_2$) 1:2	5.40
Water holding capacity (%)	27.2
Organic Carbon (%)	0.65
Total Nitrogen (%)	0.06
Available P (ppm)	10.50
Exchangeable bases (cmol/kg)	
Ca	3.26
Mg	0.48
K	0.25
Na	0.35
Cation exchange capacity (Cmol/kg)	5.63

Table 4.2a: Identification and characterization of *Bradyrhizobium* isolates.

Isolates	1	2	3	4	5	6	7	Inference
B1	-	+	Weak	White, Buttery slightly raised	Alkaline	S	+	*Bradyrhizobium spp*
B2	-	+	Strong	Slightly slimy, very scanty	Alkaline	F	++	NB
B3	-	+	Strong	Highly raised, mucoid, creamy	Acid	S	++	NB
B4	-	+	Weak	Glistening white, slightly raised	Acid	F	+	NB
B6	-	+	Strong	Mucoid, scanty Transparent	Alkaline	F	++	NB
B7	-	+	Strong	Very slimy,slightly raised	Acid	F	+	NB
B28	-	+	Strong	Semi translucent very slimy	Acid	F	++	NB
B29	-	+	NA	White, slightly buttery,raised	Alkaline	S	+	*Bradyrhizobium spp*
B30	-	+	NA	Very slimy, raised, semi translucent	Acid	F	++	NB
B31	-	+	Weak	Translucent, raised, very slimy	Acid	F	++	NB
B34	-	+	Strong	Slightly raised, slimy	Alkaline	F	+	NB
B36	-	+	Weak	White, buttery, slightly raised	Alkaline	S	+	*Bradyrhizobium spp*
B37	-	+	NA	Slightly slimy, slightly raised, white	Acid	F	++	NB
B40	-	+	Strong	Slimy, Glistening, transparent,	Acid	F	++	NB
B41	-	+	Weak	Watery, transparent, very slimy	Acid	F	+	NB
B43	-	+	Strong	Slimy, transparent, very scanty	Alkaline	S	++	NB
B44	-	+	Weak	Slimy, scanty, transparent	Acid	F	+	NB
B45	-	+	Weak	Slightly raised, white, buttery	Alkaline	S	+	*Bradyrhizobium spp*
B54	-	+	NA	Slimy, raised, very scanty	Acid	F	++	NB
B55	-	+	NA	Buttery transparent, slightly raised	Alkaline	S	+	*Bradyrhizobium spp*

1-Gram reaction, 2-Motility, 3-absorbtion of congo red 4-Growth on YEMA. 5-Growth on YEMA BTB, 6-Growth rate, 7-Xylose utilization. NA-No Absorbtion, NB- Not *Bradyrhizobium spp.* S-slow, F-Fast, ++: fast xylose utilization, +:slow xylose utilization

Table 4.2b: Identification and characterisation of *Bradyrhizobium* isolates.

Isolate number	1	2	3	4	5	6	7	Inference
B56	-	+	NA	Transparent, slightly slimy, raised	Alkaline	F	++	NB
B57	-	+	Weak	White, slightly raised, very little gum	Alkaline	S	++	NB
B59	-	+	Strong	Slightly raised, opaque, mucoid	Acid	S	+	NB
B60	-	+	NA	Very slimy, translucent slightly raised	Alkaline	F	+	NB
B61	-	+	Weak	Slimy, transparent, raised	Acid	F	++	NB
B62	-	+	Strong	Mucoid, opaque, highly raised	Acid	S	+	NB
B64	-	+	Weak	White, buttery, slightly raised	Alkaline	S	+	*Bradyrhizobium spp*
B65	-	+	Strong	Very slimy, Transparent, raised	Acid	F	++	NB

1-Gram reaction, 2-Motility, 3-absorbtion of congo red 4-Growth on YEMA. 5-Growth on YEMA BTB, 6-Growth rate, 7-Xylose utilization, NA-No Absorbtion, NB- Not *Bradyrhizobium spp,* S-slow, F-Fast, ++: fast xylose utilization, +:slow xylose utilization

Table 4.3: Occurrence of *Bradyrhizobium* species in soil samples from Research fields of the Institute for Agricultural Research, Ahmadu Bello University Zaria.

No of isolates identified as *Bradyrhizobium sp* based on:	**Category of fields sampled**		
	Soybean	Cowpea	Maize
Cultural and microscopic characteristics Of Isolates	18	7	4
Biochemical characteristics of isolates	5	1	0
PCR Amplification of 16S rRNA gene	5	1	0
Percentage occurrence of *Bradyrhizobium* species in the soils examined	27.8	14.3	0

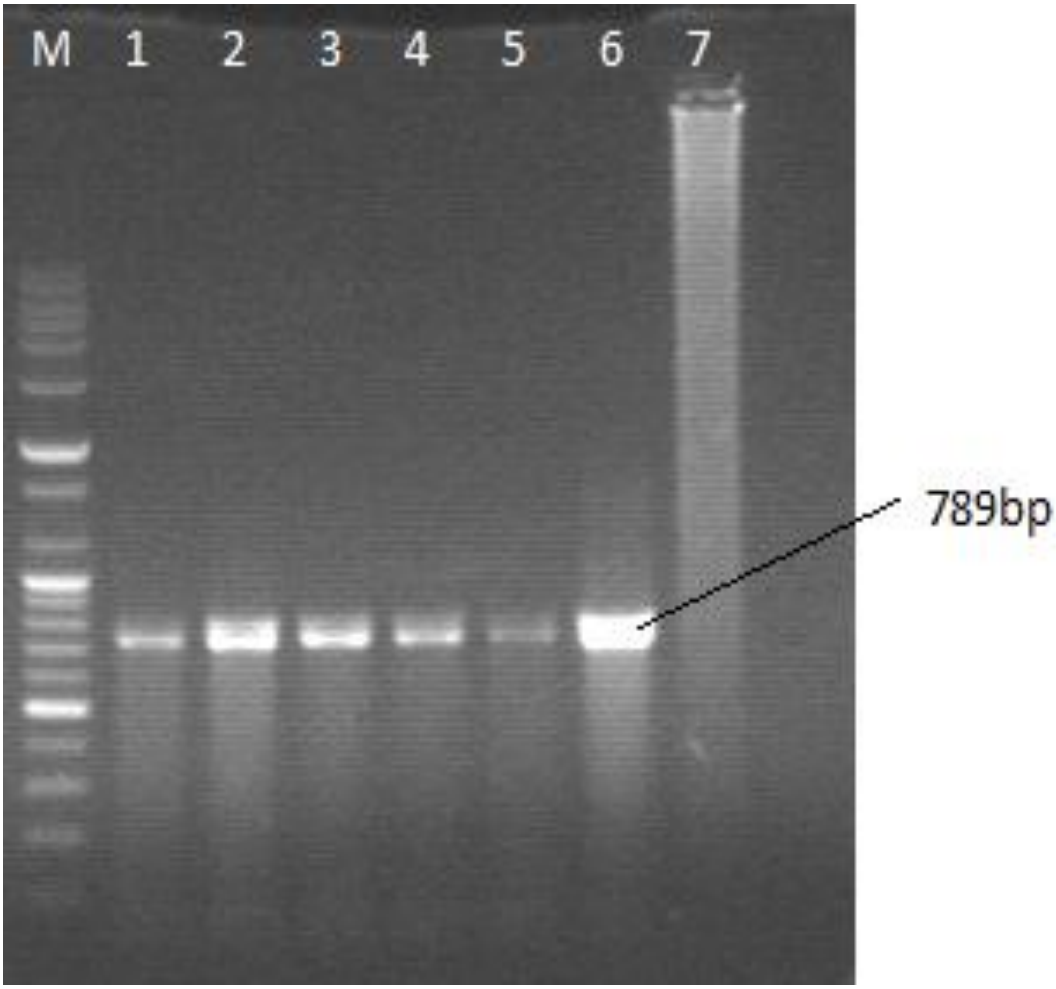

Plate I: Amplicon of 16S rRNA gene of *Bradyrhizobium spp*. at 789bp.

Key:

M: Molecular Marker (789bp)

Lane 1: Isolate B1 (isolate from soybean field).

Lane 2: Isolate B29 (isolate from soybean field).

Lane 3: Isolate B36 (isolate from soybean field).

Lane 4: Isolate B45 (isolate from soybean field).

Lane 5: Isolate B55 (isolate from cowpea field).

Lane 6: Isolate B64 (isolate from soybean field).

Lane 7: Negative control (Premix with sterile water).

Table 4.4: Sequence similarities of 16S rRNA gene sequences from *Bradyrhizobium* spp isolated from soils based on Genbank database.

Isolate number	Closest sequence match in Genbank	E-value	Maximum identification (%)	Accession number
B29	*Bradyrhizobium elkanii*	0.0	99	KX396582.1
B36	*Bradyrhizobium elkanii*	0.0	93	AF417553.1
B45	*Bradyrhizobium elkanii*	0.0	99	KX396580.1
B64	*Bradyrhizobium elkanii*	0.0	95	LC167476.1
B1	*Bradyrhizobium* specie	ND	ND	ND
B55	*Bradyrhizobium* specie	ND	ND	ND

ND = Not Determined (No close match in Genbank database)

4.3 Capacity of the Isolates to form Nodules in roots of Soybean

The four indigenous strains of *Bradyrhizobium elkanii* and two unspeciated strains of *Bradyrhizobium species* isolated demonstrated the ability to form nodules in association with soybean (Glycine max L.)(Plates II to VII) as was demonstrated by the standard inoculants (Plate VIII). It was also observed that, *B. elkanii* (B64), formed significantly higher number of nodules/plant than all the tested isolates and the standard inoculants (Table 4.5). It was also noted that, *B. elkanii* isolates (B36) and (B45) performed comparably with the standard inoculants in terms of number of nodules formed/plant (Table 4.5). This was followed by Isolates (B1) and (B29) with isolate (B55) producing the least number of nodules in association with Soybean but it was not statistically significant when compared with isolate (B29).

Similarly, *B. elkanii* (B64) produced significantly ($P \leq 0.05$) higher proportion of effective nodules which exhibited significantly ($P \leq 0.05$) higher fresh and dry weight compared to all the other experimental and standard inoculants (Table 4.5). It was also noted that, all the other inoculants tested produced significantly higher percentage of effective nodules compared to isolate (B55) (Table 4.5).

4.4 Effects of seed inoculation with the isolates on nitrogen content of soybean crops

Results of inoculation trial revealed soybean crops inoculated with *B. elkanii* (B64) to have significantly higher nitrogen content ($p \leq 0.05$) compared to crops inoculated with all the other test inoculants (Table 4.6). However, it was also observed that crops inoculated with isolates B1, B29, B36, B45 and B55 had significantly lower nitrogen contents compared to those inoculated with the standard inoculant (Table 4.6). The amount of Biological nitrogen fixed was found to be significantly higher in isolate (B64) and the standard inoculant used. There was no statistical difference between the other test isolates ($p \leq 0.05$) (Table 4.6).

4.5 Correlation analysis between shoot biomass yield and biological nitrogen fixation.

The biological nitrogen fixed by the *Bradyrhizobium* isolates was determined to have a highly significant positive correlation when compared with the fresh weight (0.577) and dry weight (0.804) of the soybean crop ($p<0.01$) (Table 4.7).

(a)

(b)

(c)

(d)

Plate Va: Photos of nodules formed by *Bradyrhizobium* spp. isolated from fields in IAR.

(a): Nodules formed by isolate B29

(b): Nodules formed by isolate B1

(c): Nodules formed by isolate B36

(d): Nodules formed by isolate B55

(e)

(f)

(g)

(h)

Plate Vb: Photos of nodules formed by *Bradyrhizobium* spp. isolated from fields in IAR.

(e): Nodules formed by isolate B64

(f): Nodules formed by isolate B45

(g): Nodules formed by BIOFIX

(h): Negative control (nodules)

Table 4.5: Effects of Isolates of *Bradyrhizobium* species on the nodulation of soybean

Isolates tested	Nodule related parameters assessed/plant			
	Mean no of nodules formed**	Effective nodules formed (%)	Nodule fresh weight (mg)**	Nodule dry weight (mg)**
***Bradyrhizobium sp* (B1)**	7.0^{c}	56.0^{bc}	213.33^{c}	26.33^{c}
***B. elkanii* (B29)**	6.0^{cd}	60.0^{b}	250.00^{b}	29.67^{b}
***B. elkanii* (B36)**	9.0^{b}	45.0^{c}	193.33^{cd}	23.00^{cd}
***B. elkanii* (B45)**	8.0^{b}	53.0^{c}	186.67^{bc}	30.33^{b}
***Bradyrhizobium sp* (B55)**	5.0^{d}	35.0^{d}	133.33^{d}	13.67^{d}
***B. elkanii* (B64)**	12.0^{a}	70.0^{a}	350.00^{a}	42.00^{a}
BIOFIX	9.0^{b}	60.0^{b}	200.00^{cd}	39.67^{a}

**:Means (3 replicates) in a column followed by same letter(s) are not significantly different at $p \leq 0.05$ level using Duncan Multiple Range Test.

Table 4.6: Effects of inoculation on nitrogen content, fresh and dry weights of soybean

Treatments Tested	Nitrogen content of soybeans (%) **	Fresh weight of shoot (g/plant)**	Dry weight of shoots (g/plant)**	Biological nitrogen fixed (mg/plant)**
***Bradyrhizobium sp* (B1)**	2.26^{c}	2.20^{ab}	0.54^{ab}	6.38^{b}
***B. elkanii* (B29)**	2.35^{c}	2.07^{b}	0.41^{bc}	5.17^{b}
***B. elkanii* (B36)**	1.92^{c}	2.23^{ab}	0.38^{bc}	3.27^{b}
***B. elkanii* (B45)**	2.35^{c}	2.17^{ab}	0.47^{ab}	6.15^{b}
***Bradyrhizobium sp* (B55)**	2.18^{c}	1.90^{b}	0.20^{c}	2.28^{b}
***B. elkanii* (B64)**	4.98^{a}	2.67^{a}	0.64^{a}	25.12^{a}
BIOFIX	4.30^{b}	2.40^{ab}	0.54^{ab}	18.15^{a}
Positive control (+KNO_3)	4.50^{ab}	2.07^{b}	0.44^{ab}	-
Neagative control (-KNO_3)	1.05^{d}	1.40^{d}	0.20^{c}	-

**:Means (3 replicates) in a column followed by same letter(s) are not significantly different at $p \leq 0.05$ level using Duncan Multiple Range Test.

Table 4.7: Correlation analysis between shoot biomass yield and biological nitrogen fixation.

Parameters	Shoot fresh weight	Shoot dry weight
Shoot fresh weight		
Shoot dry weight	0.628**	
Biological nitrogen fixed	0.577**	0.804**

**: Correlation is significant at $p<0.01$.

CHAPTER FIVE

5.0 DISCUSSION

The physical and chemical properties of the soil obtained in this study shows that it is low in total N content and organic carbon according to (FMANR 1990) (Table 4.1). A feature common to soils found in the semi-arid region of this country, is their low content of organic carbon, total nitrogen and phosphorous, as a consequence these soils are inherently low in fertility (Machido *et al.,* 2011). However, all the other physical and chemical parameters were within the optimum range for the growth and survival of *Bradyrhizobium* spp. This is similar with the work of Muhammad (2010) who also reported a low carbon and N content in soil samples from Minna, Niger state. It is also in line with the findings of Yusuf *et al.,* (2013) who reported soils with sandy loamy texture and very low levels of carbon and nitrogen in both Sudan and Southern Savannah zone of Nigeria. Abubakar (2015), 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. The soil texture of sandy loamy recorded in this study increases soil moisture loss through evaporation and deep percolation (Omondi, 2013). This explains the low water content of the soil in this study. Low organic carbon of the savanna soils have also been largely attributed to the rapid decomposition and mineralization of organic materials under high temperature (Sharu *et al.,* 2013).

The low fertility status of these soils remain one of the major constraints to production of both food and cash crops in the zone (Machido *et al.,* 2011). However reports by Mungai and Karubui (2006) indicated that soil fertility based on organic C, total N and exchangeable K vary with geographical location even within the savanna zone of Northern Nigeria. The growth and survival of *Bradyrhizobium* sp in soil is strongly dependant on the physical and chemical properties of the soil. The moderately acidic pH of 6.5 recorded for the soil used in

this study tend to suggest that soil pH does not threaten the survival of indigenous population of Rhizobia in general and *Bradyrhizobium* in particular (Howieson and Dilworth, 2016).

Twenty nine (29) suspected *Bradyrhizobium* strains were isolated from BJSM showing a characteristic feature of very small whitish colonies on the medium. Growth was very slow appearing only after 10 days of incubation. However, several fungi, Gram-negative bacterial colonies; that were small, compact and coloured grew on the medium this could be due to the unavailability of pentachloronitrobenzene in the medium which would have suppressed their growth (Tong and Sadowsky, 1994). All the 29 isolates were motile and gram negative but when transferred to YEMA for cultural identification, 11 of the isolates were found to grow within a period of 4-5 days while 18 grew from 2-3 days. This finding contradicts the findings of Rao and Ansari, (2013) where they recorded growth characteristics from 7-10 days this is probably because growth rates are generally faster from subcultures, presumably because the bacterial metabolism for growth on artificial media has already been expressed (Howieson and Dilworth, 2016).

Furthermore, twenty nine (29) isolates exhibited different colonial morphology ranging from buttery to mucoid/slimy and from slightly raised to highly raised colonies (Table 4.2). Some colonies were also opaque while others were translucent. Such colonial morphology were also observed in the Bradyrhizobia isolated in the study by of Rao and Ansari (2013) where majority of the slow growers were either opaque or translucent and slightly raised. The twenty nine suspected *Bradyrhizobuim* isolates were further tested on YEMA plates containing Congo red to detect the presence of contaminants. This is to distinguish the isolates from *Agrobacterium.* Only six of the isolates showed identical characteristics of *Bradyrhizobium* species i.e: did not (or slightly) absorb congo red, could utilize xylose slowly and were alkaline on YEMA- BTB. Similarly, Gachande and Khansole (2011) isolated *Bradyrhizobium japonicum* colonies, which were circular in shape with whitish pink color on

yeast extract mannitol agar supplemented with congo-red (CR-YEMA) medium. Also, the use of YMA-BTB medium for categorizing indigenous soybean root nodulating fast and slow growing rhizobia based on acid/alkali production was also carried out by Saeki *et al.,* (2006) in Vietnam and Sharma *et al.,* (2010) in India. It is also in line with the findings of Abubakar (2015) where Rhizobia isolated and characterized in Nigeria varied in their growth rate, growth on YEMA-BTB and CR-YEMA medium. Utilization of xylose differed among the isolates which was also reported in the works of Rao and Ansari (2013). The low presence of *Bradyrhizobium* spp in fields cultivated with cowpea and maize indicates that only fields that had recent cultivation of soybean can harbor the species.

More recently, molecular based methods have been widely used for bacterial identification as well as study of functional capabilities of microbial communities in soils. Four of the isolates were found to be closely related to species of *Bradyrhizobium elkanii*. It was reported that the Bradyrhizobia which nodulate TGx soybeans are unique populations within the *Bradyrhizobium* spp and it was also established that the Bradyrhizobia populations nodulating the local soybean cultivars grown in Nigeria were not strains of *B. japonicum* and are highly diverse (Abaidoo *et al.,* 2000). No sequence of close similarity was found for two of the *Bradyrhizobium* isolates (B1 and B55). This could be because the nucleotide sequences were too short and therefore could not be matched in the NCBI gene bank. This is in line with the findings of Abaidoo *et al.* (2000) where some of the isolates were closely related to *B. japonicum* and *B. elkanii* while others were related to *Bradyrhizobium* spp. that nodulate a wide range of tropical legume species.

Six of the isolates confirmed as *Bradyrhizobium elkanii* (B29, B45, B36 and B64) and *Bradyrhizobium* spp. (B1 and B55) were authenticated in this study and all of them were able to nodulate soybean crop. Brockwell *et al.* (1991) suggested that an isolate cannot properly be regarded as a Rhizobia until its identity had been confirmed through plant infection test on

an appropriate host. *B. elkanii* (B64) had the highest number of nodules but number of nodules alone did not reflect the effectiveness of the isolates because some ineffective isolates produce high number of nodules meaning nitrogen fixation was very little as seen in *Bradyrhizobium elkanii* (B36). Abd El-Maksoud and Keyser (2010), reported that great number of nodules can be formed by a strain fixing little or no nitrogen. Further suggestions were made that rhizobia that fix little or no nitrogen could exhibit parasitic behavior (Waswa, 2013). The greater soybean nodule biomass recorded could also be attributed to the large size of nodules formed, which is similar to the findings reported in cowpea by Ngakou *et al.* (2007).

The biological nitrogen fixed was found to be significantly higher in *B. elkanii* (B64) and the standard inoculant (BIOFIX). This shows the good performance of *B. elkanii* (B64) since it performed comparably to the standard inoculant. Although it is generally argued that indigenous populations are highly adapted to their local soil environments and may form more effective symbioses than commercial inoculants (Abubakar and Yusuf, 2016). The effectiveness and efficiency of nitrogen fixation by strains used resulted in the higher shoot biomass as demonstrated by the positive and highly significant correlation between the biological nitrogen fixed and the fresh and dry weight of plant shoot (r =0.628 and 0.804) (Table 4.7). This shows that the biological nitrogen fixed influenced the shoot fresh and dry weight.

Isolate *Bradyrhizobium elkanii* B64 good performance could be because that the strain was most compatible, best competitor for nodule occupancy and most efficient in nitrogen fixation than the other isolates (Muhammad, 2010). It is as a result of intimate mutualistic relationship between the root nodule bacteria and the host plant (Kaur *et al*., 2012). This result supports the findings of Solaiman and Rabbani (2004) who reported isolates having significantly increased dry weight of shoot per plant compared to a positive control. Shoot

dry weight and total nitrogen content are usually highly correlated; thus it is used routinely as an indicator of relative effectiveness (Vincent, 1970).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The conclusion that could be drawn from this study include: the physical and chemical properties of the soil used for the isolation of *Bradyrhizobium* species in this study was found to be suitable for the growth and survival of *Bradyrhizobium* spp except the total nitrogen and organic carbon which was recorded to be low.

Six (6) *Bradyrhizobium species* were isolated and characterized based on morphological, biochemical and molecular studies. Four of the isolates (B29, B45, B36 and B64) were confirmed to be *Bradyrhizobium elkanii* while two isolates (B1 and B55) were confirmed as *Bradyrhizobium* spp.

All the isolates had the ability to nodulate and fix nitrogen in association with soybean crop. Isolate B64 and the standard inoculant formed significantly higher in terms of effective and number of nodules/plant than all the tested isolates. It was also noted that, isolate (B55) had the significantly lower percentage of effective nodules compared to the other test isolate.

Isolate *B. elkanii* (B64) and the standard inoculant (BIOFIX) fixed significantly higher biological nitrogen ($p \leq 0.05$) compared to crops inoculated with all the other test isolates. It was also observed that crops inoculated with isolates B1, B29, B36, B45 and B55 had significantly lower nitrogen contents ($p \leq 0.05$) compared to isolate B64.

6.2 RECOMMENDATIONS

1. *Bradyrhizobium elkanii* isolate (B64) can be used as a candidate for the production of seed inoculants.

2. Further inoculation trials should be done on the field with the effective isolates to ascertain the competitiveness of the strains.

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APPENDICES

Appendix I: *Bradyrhizobium japonicum* selective medium (BJSM)

	Compound	Quantity/L
1	Na_2HPO_4	125mg
2	Na_2SO_4	250mg
3	NH_4Cl	320mg
4	$MgSO_4.7H_2O$	120mg
5	$CaCl_2$	10mg
6	$FeCl_3$	4mg
7	$CoCl_2$	88ug
8	$ZnCl_2$	83ug
9	Brilliant Green	1ug
10	Yeast agar	1g
11	Agar	20g
12	L-Arabinose	1g
13	Sodium gluconate	1g
14	pH =	6.8

Appendix II: Yeast extract mannitol agar

	Compound	Quantity/L
1	Mannitol	10g
2	Yeast extract	0.5g
3	$MgSO_4.7H_2O$	0.2g
4	K_2HPO_4	0.5g
5	Nacl	0.1g
6	Agar	15g

Appendix III: Plant nutrient solution

	Compound	Quantity(g/L)
1	$Cacl_2.H_2O$	294.1
2	KH_2PO_4	136.1
3	$MgSO_4.7H_2O$	123.3
	$FeCl_2$	100.0
	K_2SO_4	87
	$MnSO_4$	0.338
4	Trace elements	
	H_3BO_3	2.8
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4$	0.1
	$NaMoO_4$	0.1
	pH	6.5

Appendix IV: A healthy pink effective nodule