Shendi University

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RHIZOBIUM FROM WILD LEGUMES:

DIVERSITY, COMPTABILITY AND NITROGEN FIXATION OF WILD LEGMES RHIZOBIA

BY

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بسم الله الرحمن الرحيم

قال تعالى:

﴿فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ ۗ وَلَا تَعْجَلْ بِالْقُرْآنِ مِن قَبْلِ أَن يُقْضَىٰ إِلَيْكَ وَحْيُهُ ۖ وَقُل رَّبِّ زِدْنِي عِلْمًا ﴾

صدق الله العظيم

الآية (114) سورة طه

Dedication

To my Parents

To my husband

To my kids

Acknowledgement

Above all I would like to thank the almighty Allah who enable me finish my work in his will.

I would like to express my gratitude and appreciation to my supervisor Prof. Tagelsir Hassan Mohamed Ahmed for his invaluable suggestions, critical comments, assistance and supervision starting from problem identification up to finalization of my thesis.

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Contents

List of Tables

List of Figures

Abstract

This study aims at isolation, identification and preservation of rhizobia isolated from wild legumes and further to test their ability to fix nitrogen with their homologous crop legumes.

A field survey was carried out during 2012 in some parts of Gezira States and Shendi area for the purpose of collecting samples of wild legumes for isolation and identification of their associated rhizobia, *Rhizobium* strains were isolated using YEMA media and presumptive test were carried out .Seedlings of some leguminous plants representing the main cross-inoculation groups were inoculated with isolates from the wild legumes.

The result showed that all collected wild legumes were found to bear nodules on their roots. Some isolates were found to be fast-growing and acid producing rhizobia while others were found to be slow-grwoing and alkaline. The cross inoculation classification of the isolates showed that isolates from *Phaseolus trilobus* plants can be grouped with *Rhizobium leguminosarum* bv.*Viceae* and *Rhizobium* spp. Isolates from *Sesbania sesban* and *Indigofera articulata* can be grouped with *Rhizobium leguminosarum* bv. *Viceae, Rhizobium leguminosarum* bv. *Phaseoli* and *Rhizobium* spp.

None of the isolates can be grouped with *Rhizobium leguminosarum* bv. *Trifoleae, Bradyrhizobium lupinus* or *Bradyrhizobium japonicum.* It was also noticed that all isolates formed nodules on roots of *Vigna unguiculata* and they are belonging to the miscellaneous cowpea group.

PCR experiment was done. Nine isolates were amplified using 4 different Operon RAPD primers.

All of the RAPD primers gave amplification products and they were all reproducible. The most relative isolates were SH1 and ENRRI23 with 73% similarity; while the most distant were ENRRI21 and TAL380 with similarity percentage of 26%.

المستخلص

يهدف هذا البحث لدراسة عينات من بكتريا الرايزوبيا بعد عزلها من جذور البقوليات البرية ومن ثم دراسة قدرتها على تثبيت النيتروجين مع نظيراتها من بكتريا الرايزوبيا في البقوليات المحصولية مثل اللوبيا والترمس والحمص والفول والفاصوليا, نظرا لتميز البقوليات البرية بصفات وراثية جيدة كالقدرة على تحمل درجات الحرارة العالية وملوحة التربة والجفاف فضلا عن تثبيت النيتروجين الجوي.

أجريت الدراسة في العام 2012 في منطقة شندي بالسودان علما بأنها قد أجريت قبلها دراسة في منطقة الجزيرة بالسودان.

تم عزل عينات بكتيريا الرايزوبيا بأستخدام مستخلص الخميرة YEMA ثم أجريت إختبارات كيميائية و كيموحيوية على عينات البكتيريا المعزولة من جذور البقوليات البرية حيث تم تحضيرها و من ثم لقحت نبتات من البقوليات المحصولية أعلاه تمت زراعتها سلفا في حرم جامعة شندي و بعد ثلاثة أسابيع من عملية التلقيح إتضح أن هنالك تكون لعقد بكتيرية في عينات الممحاصيل البرية التي لقحت بعزلات بكتيريا البقوليات البرية.

أوضحت الإختبارات الكيميائية أن معظم العينات كانت سريعة النمو و منتجة للأحماض بينما كانت بعضها بطيئة النمو و منتجة للقلوي.

أجريت أختبارات PCR التي أكدت و دعمت النتائج السابقة و قد أكدت هذه الإختبارات على أن العينتين الأكثر تقاربا من حيث الصفات الوراثية هما العينتين SH1 و EN23 بنسبة تقارب 72% بينما كانت العينتان الأقل تشابها هما العينتان EN21 و TAL380 بنسبة تقارب 26%.

Chapter One

INTRODUCTION

Legumes are the most important source of dietary protein, especially in developing areas of the world like Sudan. Although legumes only account for some 9% of the combined world dry matter yield (cereals and legumes), they constitute as much as 24% of total protein yield of these crops because of their high protein content (Anon, 1992). The important food leguminous crops produced and consumed in the Sudan are the cool season legumes (Faba bean, common bean, pea, chickpea and lentil), their production concentrated in the Northern and River Nile states.

Faba bean *(Vicia faba* L.*)* is one of the major legume crops cultivated in the Northern and the River Nile states, produced in an average area of 3500 ha, with an average yield of 1.8 t/ha, in the middle of the nineties of the last century. Faba bean is the main food for most of the middle and north Sudan population. There are some factors affecting the growing area and the annual production (Ahmed, 1996). In spite of the variation of the locally annual growing area of faba bean in Sudan, the production is not enough for the local consumption (Ali *et al*.,1982).

Rhizobia are common a group of small, rod-shaped, Gram-negative bacteri that collectively have the ability to produce nodules on the roots and stems of leguminous plants. The taxonomy of rhizobia was based on the rate of growth of isolates on laboratory media and their selective interaction with their plant hosts. It was soon established that no strain could nodulate all plants, but that each could nodulate some legumes though not others (Long,

1989). This led to the concept of cross-inoculation groups, with organisms grouped according to the hosts they nodulated. Environmental factors that limit growth and competitiveness of rhizobia

include low or high pH, high temperature, low moisture nutrient deficiency, mineral toxicity and salinity (Dowling and Broughton, 1986; Zahran, 1999). Drought and salinity also adversely affect the nitrogen fixation capacity of rhizobia, resulting in lower productivity of legumes (Miller and wood, 1996).

The majority of rhizobia from wild legumes have a wide host range (Zahran, 2000). Wild legumes are known to be nodulated naturally by indigenous rhizobia inhabiting the soil. These rhizobia are expected to be adapted to environmental conditions like their symbiont plants. Accordingly, these rhizobia can serve as adaptive inoculant rhizobia if they prove to be highly effective in nitrogen fixation with their homologous crops legumes nitrogen-fixing symbiosis between plants of the family Leguminosae and their specific microsymbionts, collectively named rhizobia, was widely discussed.

A great deal of *Rhizobium* research has been traditionally devoted to cultivated species, for which the rhizobial microsymbionts have been thoroughly characterized and described (Thies et al., 2001). In contrast, little attention has been dedicated to the root-nodule symbionts of truly wild legumes, intended as those whose ecology is for the most part independent of human action or carry-over. Most research on *Rhizobium* populations focuses on cultivated crops, often growing outside the natural range of their wild ancestors (Demezas et al. 1995).

Although there have been some surveys of ‘wild’ rhizobia, there has been little systematic comparison of the symbionts of wild and cultivated legumes, nor between populations in cultivated and uncultivated soils (Mutch, et al., 2004).

However, soils containing indigenous rhizobia are also problematic as they create a barrier to the establishment of introduced efficient inoculants in nodules of target host plants. There is evidence of widespread sub-optimal efficiency of native strains with legumes. But it is generally argued that indigenous populations are highly adapted to their local soil environments and may form more effective symbioses than commercial inoculants isolated from a distant and un-related soil environment (Gandee *et al.*, 1999). Thus, selection of indigenous strains with high nitrogen-fixing capacity and adapted to a range of environmental conditions at a specific site, is an important strategy to maximize legume production (Sajjad *et al.*, 2008).

Molecular techniques based on the polymerase chain reaction(PCR) are very convenient for characterization, because they are rapid, simple and discriminative. PCR has been found useful for rhizobial strain discriminative. PCR has found useful for rhizobial strain differentiation using GC. Rich Oligonucleotide primers. It has been possible to differentiate strains belonging to different colony morphology variants from standard strains which differ in symbiotic nitrogen fixation. Random amplified polymorphic DNA (RAPD) can be used in the present study to assess the genetic diversity of *Rhizobium leguminsoarum bv.viceae* strains from various districts area.

This present study was carried out to fill a gap in genetic and phenotypic diversity of *Rhizobium leguminosarum bv. viceae* research in the Sudan.

The objectives of this study were: isolation, identification and preservation of rhizobia isolated from wild legumes, and further to test their ability to fix nitrogen with their homologous crops.

Chapter two

Literature Review

2.1 Legumes:

Legumes are also referred to as pulses includ all forms of beans and peas from the family *Fabaceae* (Leguminosae). They are angiosperms, belonging to the phylum Rosales. The family Leguminosaea was separated into three sub-families. *Minosoideae, Saesalinoideae* and *Papiliodeae*. The family comprises about 650 genera and 20000 species including numerous cultivated plants (Wild, 1993). Only about 20 species all belonging to the sub-family *Papiliodeae* are used for human consumption(Griganac and Wery, 1989).

Legumes are used for a wide variety of purposes such as human nutrition and animal feed. Grain legumes are common food throughout the world. They are second only to cereals as a source of human and animal food. The importance of legumes as a food lies primarily in their high protein content. the percentage of protein runs well above twice or three times the level found in cereals and significantly more than the level of root crops (Bressani, 1973).

Legumes are grown agriculturally, primarily their grain seed (e.g. Beans and Lentil or generally pulses), for livestock forage and silage and as soil-enhancing green manure. Legumes are notable in that most of the have symbiotic nitrogen-fixing bacteria in structure called root nodules.

Most dry legumes contain 20-25% protein, groundnut have slightly higher content of about 27% , soybean contains about 35% and faba bean contains

about 28-31% protein (Ali et al., 1982). Legumes ptovide in terms of human nutrition:22% protein; 32% fat and 7% carbohydrated. In terms of livestock nutrition they provide: 38% protein;16% lipids and 5% carbohydrates (Grignac and Wery, 1989).Through combinations with other foods, legumes can constitute a cheap source of high value dietary protein for both human and animals. It balances the deficiencies of the basically cereal-based diet (Dart and Krantz, 1976).

It has been observed that legumes have beneficial on yield of cereals grown on the same soil (Nambiar et al., 1982). The residual effect is usually attributed to the enrichment of the soil with nitrogen fixation by legume-*Rhizobium* symbiosis and through nitrogen-rich root and nodule materials after shedding (Poth et al., 1986). The beneficial effect of legumes have been recognized before the principles of crop rotation were established (Herridge., 1982).

Legumes are important component of the agricultural systems because of the nitrogen provided by their bacterial symbionts, the rhizobia (O'Hara et al., 2003). Successful of spread of legume crops depends critically on the availability of compatible rhizobia. The use of European *Lotus correculatus* as a forage legume in New Zealand and Asian *Cicer arietinum* (Chickpea) as a grain legume in Australia required the introduction of the corresponding symbionts. The soils lacked appropriate indigenous rhizobia and few or no nodules formed in the absence of rhizobia (Howieson et al., 2000).

2.2 Wild legumes:

Wild legumes (herb or tree) are widely distributed in arid regions and actively contributed to soil fertility in these environments. N_2 fixing activity and tolerance to drastic conditions may be higher in wild legumes than in crop legumes. The majority of rhizobia from wild legumes have a wide host range (Zahran, 2000). Wild legumes are known to be nodulated naturally by indigenous rhizobia inhabiting the soil. These rhizobia are expected to be adapted to environmental conditions like their symbiont plants. Accordingly, these rizobia can serve as adaptive inoculant rhizobia if they prove to be highly effective in nitrogen fixation with their homologous crop legumes).

Nitrogen-fixing symbiosis between plants of the family Leguminosae and their specific microsymbionts, collectively named rhizobia, was widely discussed. A great deal of *Rhizobium* research has been traditionally devoted to cultivated species, for which the rhizobial microsymbionts have been thoroughly characterized and described (Thies et al., 2001). In contrast, little attention has been dedicated to the root-nodule symbionts of truly wild legumes, intended as those whose ecology is for the most part independent of human action or carry-over. Most research on *Rhizobium* populations focuses on cultivated crops, often growing outside the natural range of their wild ancestors (Demezas et al ., 1995). Although there have been some surveys of 'wild' rhizobia, there has been little systematic comparison of the symbionts of wild and cultivated legumes, nor between populations in cultivated and uncultivated soils (Mutch, et al., 2004).

2.3 The Root Nodule Bacteria:

Bacteria in the genus *Rhizobium* are facultative symbionts, living in the soil as part of the normal flora. They are gram negative rods, aerobic, motile and on-spore forming with one polar or sub-polar flagellum or 2-6 peritichous flagella and that are pleomorphic under adverse growth conditions (Postgate, 1998). They are diverse group that are similer not only so much by biochemical characteristics, DNA base ratio or morphology as by their ability to form nodules on some legumes (Weaver and Frederick, 1982). Rhizobia are classically defined as symbiotic bacteria capable of eliciting and invading root and stem tissue forming nodules on leguminous plants where they undertake symbiotic nitrogen fixation (Sahgal and Johri, 2003**).**

2.4 Taxonomy of Rhizobia:

The taxonomy of root nodule bacteria has changed considerably over the last 20 years. The first time isolated a bacterium from root nodule of legume in 1888 by Martinus Willem Beijerinck (Beijernck, 1888) and named it *Bacillus radicicola,* but then reclassified into the genus *Rhizobium*.

Historically the classification of rhizobia was made by virtue of their ability to nodulate group of plants of the family *Leguminosae*. Early in the study of the legume-*Rhizobium* association, it was noted that certain plants showed preference to certain rhizobia and vice versa. Legume species naturally susceptible to nodulation by a certain kind of *Rhizobium* constitute a " cross inoculation group ". Strains capable of nodulating the plant of one of these groups were considered a species regardless whether nitrogen fixation occurs or not (FAO). Based on specificity of symbiotic plant range of

bacterial species, Sahgal and Johri, (2003) recognized six species in the genus *Rhizobium* viz. *R. japonicum* (*Lathyrus*, *Lens, Pisum* and *Vicia*), *R. upine* (Lupinus), *R. Meliloti* (*Melilotus, Medicago, Trigonella*) *R. phaseoli* (*Phaseolus*) and *R. Trfolii* (*Trifolium*) based on their host range and certain morphological and physiological properties. This classification on the bases of the legume nodulated, referred to as cross inoculation group, represents many difficulties for soil microbiologists, but herein lies some importance to agronomists (Somasegran and Hoben, 1994).

Based on their growth rate ,rhizobia were grouped as fast growers with mean doubling time of 2-4 hours and slow growers with mean doubling time 0f 6-8 hours, but were still placed in the genus *Rhizobium* till Jordan (1984) coined the new genus *Bradyrhizobium* for isolates from *Glycine max*. which is the slow grower. The fast growers are acid producers and the slow growers are base producers. Then a third genus, *Azorizobium*, fast growing with only one plant species identified as a host which is *Sesabana rostrata*.The genus *Azorhizobium* includes strains that are very distinct from other rhizobia in many characteristics and *A. caulinodans* is the only species characterized up to now nodulating the roots and stems of *Sesbania rostrata* (Xu *et al*., 1995).

A fourth genus, *Sinorhizobium*, has been introduced. *Sinorhizobium* includes *S. fredii*, *S. meliloti*, *S. teranga*, *S. saheli* and *S. fredii* comprised of fast-growing strains nodulating soybean, although strains of this species are also able to nodulate and fix nitrogen on various legumes (Krishnan and Pueppke, 1994). *S. meliloti* was isolated from alfalfa, while *S. teranga* and *S. saheli* isolated from various tree legumes such as *Sesbania* and *Acacia*

species (De Lajudie *et al*., 1994). *S. medicae* members are able to nodulate various alfalfa species but show a different host range than *S. meliloti* strains (Rome *et al*., 1996).

Recently, *Photorhizobium*, *Mesorhizobium* and *Allorhizobium* has been described as new genera with only one host plant for each (De Lajudie *et al*., 1998). Although the micro symbionts of plants other than crop species have been neglected for a long time, efforts have been undertaken to analyze rhizobia associated with economically les important leguminous plants such as nitrogen fixing trees (Mcinroy *et al*., 1999). Furthermore the diversity of rhizobia occurring on native shrubby legumes in Southeastern Australia was recently investigated revealing a respectable diversity among the isolated strains (Lafay and Burdon, 1998).

2.5 Rizobial diversity:

The Leguminosae family includes many species, but only a minor portion, mostly representing by crops of agricultural interest, has been examined , thus the knowledge available on the biological diversity of interactions between legumes and microbes is still very small. As a result of their nitrogen-fixing capacity, legumes can colonize nitrogen-deficient soils enhancing their fertility, which makes them optimal candidates for revegetation programs. However, despite their economic and environmental importance, only a small proportion of existing legume species and their rhizobial associates have been investigated. So far, most of these studies have been focused on herbaceous species of agronomic interest. Notwithstanding, the past few years have witnessed a surge in rhizobial

biodiversity studies performed on wild shrubby and woody legumes in different ecosystems worldwide including semiarid Mediterranean regions. In the Mediterranean ecosystems, shrubby and woody legumes are an essential part of many revegetation projects owing to their ecological benefits such as improving soil fertility, preventing soil erosion, and contributing to graze animal nutrition.

For years, a limited number of bacterial species were believed to be nitrogen fixers (Postgate, 1981), but in the last 30 years, nitrogen fixation has been found to be a property with species in most of the phyla of bacteria and also in Methanogenic archae. Symbiotic nitrogen fixing property within nodules of vascular plants is found in two phylogenetically unrelated major groups of bacteria: rhizobia (Alpha- proteo-bacteria), associating essentially with leguminous plants belonging to one super family of angiosperms (Fabaceae), and Frankia (in Actinobacteria) with a broader spectrum of plants from eight families (Huss-Danell, 1997 ; Vessey et al., 2004). Besides this, cyanobacteria is another important group of nitrogen fixing bacteria found in association with a large variety of higher and lower variety of plants, fungi and algae (Meeks and Elhai, 2002).

The diversity of rhizobia in a particular soil may be influenced by the method of its isolation. Several arrays of techniques are used for detecting and describing rhizobial diversity. These are host range, comparative growth in culture, intrinsic antibiotic resistance, tolerance in pH, and salt. These are among the most common methods that are considered as phenotypic characters and are used primarily to study rhizobial diversity (Maatallaah *et al.*, 2002). The size, shape, color and texture of colonies and the ability to

alter the pH of the media are generally stable characteristics useful in defining strains of isolates. Colonies could be discrete, round varying from flat to domed and even conical on agar surface. Moreover; they may be white, opaque, and milky and watery translucent (Ahmad *et al*., 1984).

Tolerance to acid or alkali, resistance to antibiotics, and tolerance to salinity, were used by many researchers to determine a wide physiological diversity among tested isolates (Ahmad *et al*., 1984 and Maatallah *et al.*, 2002). The diversity of rhizobia associated with beans has been studied with emphasis on those of Mesoamerican origin (Bernal and Graham 2001). Andrade et al. (2002) did a long term study on the diversity of phaseolus-nodulating rhizobial populations in acid soils in Brazil using molecular methods, the authors hypothesized that the abundance and diversity of rhizobial species and of strain types within the species would decrease with increasing soil acidity stress. Thus, they are best regarded as preliminary screening methods for relative screening methods.

Antibiotics are defined as a group of natural microbial products or synthetic chemical compounds that inhibit the growth of and even kill bacteria. Natural antibiotics are produced by both bacteria or fungi and act by blocking some essential cell processes in other bacteria. Some studies have been evaluated on the effect of different antibiotics on *Rhizobium* bacteria growth (Rennie and Dubetz, 1984).

Modern molecular tools which are more preferred to phenotypic methods can also be used to evaluate the specificity of the stains (Thies *et al*., 2001). Some of these molecular tools employed for rhizobial diversity identification

includes: sequence comparison of 16S rRNA genes and genetic finger printing (Sahgal and Johri, 2003), plasmid profiling (Broughton *et al.*, 1987), restriction fragment length polymorphism (RFLP) (Odee *et al.*, 2002), polymerase chain reaction based techniques (PCR) (Richardson *et al*., 1998), PCR-RFLP of 16S r RNA genes (Sahgal and Johri, 2003). A similar study in Argentina by characterizing their isolates using nifH- PCR of genes coding for 16S rRNA and nodC as well as REP fingerprinting These phenotypic methods provide a valuable insight into rhizobial population structure and strain diversity.

2.6 Rhizobia - legume symbiosis:

The interaction between rhizobia and legumes results in a formation of highly specialized structures called nodules. On the basis of morphological, anatomical, and histological differences, legumes nodules are divided in to two main separate types: determinate and in-determinate nodules. In-determinate nodules retain their meristematic activity, while determinate nodules do not (Prell and Poole, 2006). The latter appears on legume plants of tropical climate like soybean, bean and cowpea plants, whereas in-determinate nodules are found in more temperate climate legumes like chick pea, pea, alfalfa, and clover.

Development of a *Rhizobium*-plant symbiosis involves a highly coordinated exchange of signals between the host plant and the bacterial symbiont which leads to a gradual and coordinated differentiation and adjustment of physiology and metabolism in both partners. The infection process is triggered by plant root exudates, flavonoids, to which the rhizobia respond

by induction of nodulation genes (nod genes). The nod-ABC genes are present in almost all rhizobia and are required for the synthesis of the lipochitooligo saccharide backbone that can be modified by various chemical groups. Detection of nod factors by the host plant induces major developmental changes such as cortical cell division and root hair formation which are required for the entry of rhizobia into the host (Brencic and Winans, 2005).

The next step is the binding of rhizobia to host root hairs. The weak Ca_2 dependant binding is mediated by a bacterial protein called rhicadhesin and followed by a tight binding with cellulose fibrils also synthesized by the bacteria. Host lectins have also been shown to play roles in rhizobial adhesion. The tip of a root hair, to which rhizobia are bound, curls back on itself, trapping the bacteria and forming the infection thread (Gage, 2004).
Probably, a localized degradation of root hair wall occurs at the site of the infection. After bacteria entered a root hair, they begin to travel along an infection thread toward a developing nodule. The initiation and extension of the infection thread depends on the production of specific extracellular polysaccharides (EPS) by the bacteria. Afterwards, the bacteria are differentiated into bacteroids which differ from normal bacteria in size, form, and cell wall composition. Typically, cyclic glucanes, nitrogenase, and specific terminal oxidase are synthesized. Within the nodule, the plant supplies rhizobia with a carbon source in the form of dicarboxylic acids, which are then metabolized via the tricarboxylic acid cycle in the generation of ATP. In return, nitrogenase in the bacteroids catalyzes N_2 to ammonium that can be used as nitrogen source for the host plant (Lodwig *et al*., 2003).

The conditions in the nodules must be micro-aerofilic for nitrogen fixation to take place because of the sensitivity of the nitrogenase to oxygen. Oxygen concentration is the major signal controlling the expression of nif (coding nitrogenase) and fix genes (coding membrane- bound cytochrome oxidase). The central zone of nodules is protected by a layer of internal cortical cells bonded with glyco-proteins. Nodules also synthesize large amounts of leg hemoglobin that binds oxygen and holds the oxygen concentration levels to approximately 25nm, and finally, the bacteroids produce the specific terminal oxidase cbb3 with high affinity to oxygen (Brencic and Winans, 2005).

2.7 Biological nitrogen fixation:

Nitrogen is a crucial mineral element required for the sustenance of life; it is the soil nutrient element needed in greatest quantity by crops. It comprises up to 80% of the atmosphere, yet in nature very little of this nitrogen is available in mineral from and plants are unable to make use of atmospheric nitrogen.

Biological nitrogen fixation is the process whereby atmospheric nitrogen is reduced to ammonia in the presence of nitrogenase enzyme. Nitrogenase is a biological catalyst found naturally only in certain micro organisms such as the symbiotic *Rhizobium* and Frankia , or the free – living *Azospirillum* and *Azotobacter* (Mulongoy, 1992). The nitrogenase complex consists of tow proteins , highly conserved in sequence and structure throughout nitrogen fixing bacteria. The protein containing the site of substrate reduction is nitrogenase molybdenum – iron protein , and the obligate electron donor to

molybdenum – iron protein is nitrogenase iron protenin, also known as dinitrogenase reductase (Dean and Jacobsen, 1992).

Nitrogen fixation is second only to photosynthesis, in terms of importance for the growth and development of plants. It is estimated, on a global scale, that biological nitrogen fixation may reach 175 million metric tons of nitrogen fixed per year, approximately 90 million tons from agricultural areas and 50 million tons from undeveloped land and forests. The transformations of nitrogen are not exclusively biological. The total nitrogen fixation by non- biological processes (Bezdicek and Kennedy, 1988). Nitrogen in the soil originates from decomposing organic matter and from nitrogen introduced by lightning. Lightning probably accounts for approximately 10% of the world's supply of fixed nitrogen, but this is generally insufficient for extensive crop production (Zahran, 1999).

Soil nitrogen deficiency may result in poor yields or failed crops and has traditionally been overcome by applying fertilizers, but this is expensive and harmful to environment. The annual worldwide expenditure for nitrogen fertilizer currently exceeds 20 billion us dollars (Hardy, 1997). The production of nitrogen fertilizer requires high – energy inputs and makes use of natural gas. These fossil fuels comprise 70% -90% of the cost of producing nitrogen fertilizers and as they become ever scarcer; their cost will continue to increase.

Environmental concerns for using nitrogen fertilizer include changes in the global nitrogen cycle, loss of nitrous oxides to the atmosphere, acid rain, nitrate pollution of ground water and induced leaching of soil nutrients

(Kinzig and Socolow, 1994; Vilousek *et al.,* 1997). The input of nitrogen through biological nitrogen fixation allows for increased soil fertility, which helps to maintain soil nitrogen reserves, thus eliminating the need for nitrogenous fertilizers (Graham and Vance, 2000).

Biological nitrogen fixation can play a key role in land remediation. An examination of the history of biological nitrogen fixation shows that interest generally has focused on the symbiotic system of leguminous plants and rhizobia, because these associations have the greatest quantitative impact on the nitrogen cycle. A tremendous potential for contribution of fixed nitrogen to soil ecosystems exists among the legumes (Brockwell *et al.,* 1995 ; Peoples *et al.,* 1995a). Successful *Rhizobium*- legume symbioses will definitely increase the incorporation of biological nitrogen into soil ecosystems. *Rhizobium*- legume symbioses are the primary source of fixed nitrogen in land based systems, and can provide well over half of the biological source of fixed nitrogen (Tate, 1995). Atmospheric N_2 fixed symbiotically by the association between *Rhizobium* species and legumes represents a renewable source of N_2 for agriculture (Peoples *et al.,* 1995b).

2.8 Ecological factors that affect BNF

The marked influence of environment on symbiotic nitrogen fixation has been known for a long time. The delicate balance between the host plant and the micro-symbiont is disturbed by adverse environmental conditions which have otherwise no effect on plant growth. Quite obviously, environmental factors affect the growth of both plants and bacteria. (Dowling and Broughton, 1986).

Some of the factors affecting biological nitrogen fixation are soil acidity, drought, salinity, high temperature and nutrient deficiencies (Giller, 2003; Hungria and Vergas, 2000).

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity (Bordeleau and Prevost, 1994; Correa and Barneix, 1997). Acid soil conditions pose problems for the plant, the bacteria and the symbiosis (Giller and Wilson., 1991). Legume and their rhizobia exhibit varied response to acidity. Some rhizobial species can tolerate acidity better than others, and tolerance may vary among strains within a species (Vargas and Graham., 1998).

The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 (Jordan., 1984) and a relatively few rhizobia grow well at pH less than 5.0 (Graham *et al.*, 1994). Soil acidity constrain symbiotic N_2 fixation in tropical and temperature soils, limiting *Rhzobium* survival and persistence in soils and reducing nodulation (Graham *et al.*, 1982).

Rhizobia are mesophiles and most have a poor growth at temperature below 10^oC or above 37^oC (Graham, 1992). Although responses to temperature are strain s dependent, rhizobia are found to tolerate between 4-42.4^oC (Moawad and Beck, 1991). For most rhizobia, the optimum temperature range for growth in culture is 28^oC to 31^oC (Bordeleau and Prevost, 1994).

Salt stress is one of the major environmental stresses adversely affecting legume production in arid and semi-arid regions (Bernstein and Ogata, 1966). High soil salinity can deleteriously affect symbiotic association

between legume and *Rhizobium* by osmotic stress and ionic toxicity and imbalance (Sprent, 1972; Aparicio-Tejo and Sanchez-Diaz**,** 1982).

Successful *Rhizobium*-legume symbiosis under salt stress requires the isolation and development of salt tolerant rhizobial inoculant (Zahran, 1991). The modification of rhizobial cells by water stress will eventually leads to a reduction in infection and nodulation of legumes (Zahran, 1999).

2.9 Genetic diversity of *Rhizobium*

The development of molecular microbiology has changed the process of characterizing, comparing and indentifying bacteria. DNA fingerprinting techniques are a means of visualizing DNA polymorphism between samples as they involve the display of a set of DNA fragments from a specific DNA sample.

DNA fingerprints may be used as a tool for determining the identity of a specific DNA sample or to assess the relatedness between sample. Rapid DNA typing methods such as the techniques that target the whole genome (RAPD, rib typing, AFLP. PFGE and rep- PCR) and individual genes (PCR-RFLP, ARDRA and sequencing) enable the differentiation between species and strains of the same species.

The choice of fingerprinting technique is dependant both on the organism to be studied and the application of the study. The criteria for evaluating which DNA fingerprinting technique to use include type ability, reproducibility, discriminatory power and ease of interpretation. Type ability is the ability of a method to provide readable results for each isolate analyzed.

Reproducibility measures the ability of a technique to yield the same result when replicate assays are performed on the same isolate. Discriminatory powers are the ability of a typing method to distinguish different strain. Ease of performance includes both performance of the technique and interpretation of the results (Coenye *et al.*, 2002). An identification technique should be insensitive to previous manipulation of the strains and the technique difficulty, cost, and time to obtain a result must also be evaluated in assessing the value of a particular typing method (Olive and Bean, 1999).

2.9.1 PCR- based locus- specific RFLP

Restriction fragment length polymorphism (RFLP) makes use of restriction end nucleases that cut the DNA at specific sites, generating numerous smaller DNA fragments that result in an illegible smear when viewed by gel electrophoresis.

To overcome this problem, the PCR- RFLP technique is used; oligonucleotide primers are use to first amplify specific regions of the genome, combined with RFLP restriction enzyme digestion of these amplification products to generate fingerprint patterns. The discriminatory power of locus- specific PCR is generally not as good as that of other methods, due primarily to the limited region of the genome that can be examined.

2.9.2 Amplified rDNA restriction analysis (ARDRA)

Amplified rDNA restriction analysis (ARDRA) is the name of the technique when PCR- RFLP targets the ribosomal DNA (16 s or 23 s rDNA or parts of

both genes, with or without the intergenic spacer region (ITS). The rDNA is amplified by using universal primers located in the conserved regions of the rRNA genes and then digested with a combination of restriction enzymes.

ARDRA is a rapid method, less demanding than direct sequencing or hybridididization with specific probes. It is able to distinguish between closely related species. (Koeleman *et al.*,1998) found ARDRA to the differentiation of many bacterial genera, including several species of rhizobia; *Rhizobium galegae* and rhizobia nodulating Acacia trees in morocco. (Mhamdi et al., 2002) use 16 s RFLP to determine the identity of rhizobia nodulating *phaseolus vulgaris* in Tunisian soil.

2.9.3 DNA fingerprinting techniques utilizing the whole genome

2.9.3.1 Randomly amplified DNA fingerprinting (AP- PCR, RAPD, DAF)

Short arbitrary sequences are used as primers in the generation of genomic fingerprints for samples where little is known of the target sequences to be amplified. Arbitrarily primed PCR fingerprinting (AP- PCR) makes use primers of 20 bases. Randomly amplified polymorphic DNA (RAPD) analysis has random primers of 9-10 bases (Williams *et al.*, 1990) while DNA amplification fingerprinting (DAF) can use primers as short as 5 bases, but typically 8-10 bases (Caetano- Anolles *et al.*, 1991). AP- PCR fingerprinting and RAPD both involve the amplification of random DNA segments by PCR, using single primers of arbitrary nucleotide sequences in order to reveal differences as DNA polymorphisms or fingerprints. The resulting DNA fingerprints are determined by nucleotide sequence of the primer and the nature of the DNA template. These techniques are generally

applicable to a wide variety of organisms as a result of the universal primers and are species or sub- species specific. DAF differs from AP-PCR and RAPD in its reaction conditions, separation and detection systems. DAF is able to detect a large number of amplification products and profiles can be tailored to vary in complexity. RAPD has been used successfully to distinguish bacterial strains among diverse species including *Heliobacteria pylori* and *Staphlococcus aureus* (Saulnier *et al.*, 1993).

2.9.3.2 Pulsed- field gel electrophoresis (PFGE)

For PFGE, bacterial isolates are grown either in broth or on solid media, combined with molten agarose and poured into small moulds. The results are agarose plugs containing the whole bacteria. The embedded bacteria are subjected to *in situ* detergent- enzyme lysis and digestion with an infrequently cutting restriction enzyme. The digested bacterial plugs are then inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. The pulsed field allows clear separation of very large molecular length DNA fragments ranging from 10 to 800 kb (Olive and Bean, 1999). The gels are stained with a fluorescent dye such as ethidium bromide and then the electrophoretic patterns can be visualized. Gel results can be photographed, and the data can be stored and data analysis can be accomplished by using any of a number of commercially available software packages available. PFGE is highly discriminatory, with moderate running and set- up costs. One of the factors that has limited the use of PFGE however, is the time involved in completing the analysis. While the procedural steps are straightforward, the time needed to complete the procedure can be two to three day. This can reduce the laboratories ability to analyze large numbers

of samples (Olive and Bean, 1999). PFGE has been used for the typing of *pseudomonas aeruginosa* and *staphylococcus aurous* (Saulnier *et al*., 1993).

2.9.3.3 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a selective restriction fragment amplification technique, which produces highly complex DNA profiles . The technique comprises three steps: digestion of total cellular DNA with one or more restriction fragments; selective amplification of some of these fragments with two PCR primers, which have corresponding adaptor and restriction site- specific sequences; and electrophoretic separation on a gel matrix. The restriction fragments are selected by performing restriction using tow restriction enzymes, ideally a rare and a frequent cutter. This would yield DNA fragments with two different types of sticky ends to which adapters are ligated to provide primer- binding sites for amplification. The selective amplification is achieved by using two different primers consisting of the same sequence as the adaptors with extra selective nucleotide next to the restriction site of the enzyme. Under the stringent primer annealing conditions used, only the fragment in which the selective nucleotides completely match the primer extensions are amplified. This results in an array of fragments, which are detected by denaturizing polyacrylamide gel electrophoresis. The choice of restriction enzymes, the number and base composition of the selective nucleotides in the primers and the complexity of the genomic DNA determine the number and size of DNA fragments. These fragments are either group- specific or strain specific (Vandamme *et al.,* 1996; Vose *et al.,* 1995).AFLP is a reliable and powerful high resolution DNA fingerprinting technique for DNA of any origin or complexity and could be used for identification and typing purposes of

highly related bacterial strains without prior knowledge of the nucleotide sequence. It is useful as a rapid screening technique for large collection of bacterial isolates as it yield reproducible results that are comparable to DNA –DNA hybridization. The method does, however, involve high set-up costs, with a moderate cost per test. It also requires purified high molecular weight DNA. The banding patterns produced are very complex and similar sized fragment may not be homologous.

2.9.3.4 Repetitive sequence- based PCR (rep-PCR)

Enterobacteria contain families of short interspersed repetitive elements. These include the repetitive extragenic palindromic (REP) element, the enterobacterial repetitive intergenic consensus (ERIC) sequence and the Box element. The function of these elements is unknown, but it has been suggested that they are involved in stabilizing mRNA, translational coupling between genes homologous recombination and the binding of DNA polymerase. The repetitive extragenic palindromic (REP) sequences are 38-bp long, consisting of six degenerate positions and a 5- bp variable loop between each side of a conserved palindromic stem. Fingerprints generated by rep-PCR allow the recognition of strains between laboratories. REP- and ERIC- PCR analyses yield similar levels of discrimination (Laguerre *et al.*, 1997). Both procedures are extremely rapid, they do not require the generation of bacterial cultures or the extraction of genomic DNA and have the advantage that a single set of primers could be used for the analysis of both closely related and widely divergent strains.

2.9.4 Comparison of the DNA fingerprinting techniques

Although a particular typing method may have high discriminatory power and good reproducibility, the complexity of the method and interpretation of results as well as the costs involved in setting up and using the method may be beyond the capabilities of the laboratory. The choice of a molecular typing method will therefore depend upon the needs, skill level, and resources of the laboratory. Methods such as locus- specific PCR, RAPD analysis, and rep-PCR are similar in their procedures and are generally the easiest to implement RAPD assays are more discriminating than 16s RFLP or 16s- 23s RFLP, but less discriminating than rep- PCR. RAPD assays were also found to be less versatile than rep- PCR, AS RAPD PCR reactions need to be optimized and rep- PCR reaction do not. Repetitive sequence- based PCR shows better discriminatory power than plasmid profiling or restriction analysis of the 16s rRNA gene or the 16s-23s spacer region. Studies have shown rep- PCR to be superior to other typing methods such as multilocus enzyme electrophoresis and ribotyping. Methods that cover the entire genome, such as rep-PCR, RAPD and AFLP are more informative than locus specific techniques, such as PCR- based locus- specific RFLP or ARDRA. While PFGE appears to be time- consuming, it too is not difficult to implement. The level of difficulty of a technique therefore needs to be taken into consideration, along with cost and time. One other criterion is the ability of a method to allow analysis of large numbers of samples. For high throughput, simple techniques with high discriminatory power and low cost, such as rep- PCR, may be most suitable. For sub typing bacteria, the laboratory must weigh the fact that the discriminatory of a technique such as rep- PCR is sufficiently high to yield excellent sub- typing results for a fraction of the setup costs as well as lower costs per test (Olive and Bean, 1999)

Chapter Three

Materials and Methods

This investigation comprises three stages of experimental work, the first of which is farming of sample test leguminous crops in the experimental farm of Shendi University afterwards the isolated samples of rhizobia strains were prepared for subsequent chemical and biochemical tests in the botany lab of Shendi University. It should be noted here that most of the experimental findings of the test mentioned above were done at the Environment, Natural Resources and Desertification Research Institute then asserted by PCR investigations.

3.1 *Rhizobium* and *Bradyrhizobium* Samples

Five *Rhizobium* strains used throughout this study were obtained from The Biofertilization Department, Environmental, Natural Resources and Desertification Research, Institute Khartoum, Sudan. The source and classification of *Rhizobium* and *Bradyrhizobium* strains is shown in (table1) Another strain (Shendi S1) was isolated from Sesabania plant grown in Shendi .The nodules were picked from the plant, sterilized with Chlorox for 30 seconds and washed 5times with distilled water (FAO, 1984).

3.2 Strains Preservation:-

Rhizobium and *Bradiyrhizobium* strains were preserved by streaking onYeast Extract Manitol (YEM) agar, incorporating 3g of carbonate/liter, slants in screw-caps test tubes and kept in the refrigerator at 4c for further tests.

3.3 Preparation of the culture media:-

(YEM) agar, medium was used for culturing the strains from the slopes for further testing . The medium consists of the following per liter:-

K_2HPO_4	0.5g
$MgSo_4 .7H_2O$	0.2g
NaCl	0.1g
Manitol	10.0g
Yeast Extract	1.0g
Distilled water up to	1000 ml

Sources: Somasegaren and Hoben (1994)

The media was sterilized by autoclave at 121^{o}c 15lb for 15-20 min.

Fifteen ml of the sterilized media were poured in a clean sterilized Petri and left to cool.

A loop full of the selected strains was then streaked on the YEM agar medium and incubated at 27ºC for 3-5 days.

3.4 Morphological Characteristics

3.4.1 Colony shape

The morphological characteristics of the isolates were determined according to Lupwayi and Haque (1994). A loopful of rhizobia isolates from 48 hrs old broth culture was inoculated by streak plating onto YEMA and incubated at 28 ± 2^oC for 3-5 days. After an inocubation of 5 days, individual colonies were characterized based on their color, shape, borders, size and Gram stain reaction.

3.4.2 Growth rate reaction

A measurement was done by using spectrophotometer to measure growth rate of bacteria for six days.

3.4.3 Gram stain test:-

A fixed smear of the strain under test was covered with crystal violet stain for 30 seconds then washed of with tap water, then covered with iodine for 30 seconds, the iodine washed of with tap water, the smear was then decolorized rapidly (few seconds) with alcohol, washed immediately with tap water, covered with saffranine stain for two minutes and washed again with rap water .

The slides were placed in a draining rack to air dry and examined under the microscope Benson (1994).

3.5 Chemical and Biochemical tests:-

3.5.1 Bromothymol blue test:-

To determine the acid producing ability of the strains, each isolate was grown in duplicate on YEM agar plate containing 0.025%(w/v) bromothymol blue.

After the incubation of 72h at 28 ± 1 ºC, the PH change was scored on the bases of color change of the medium (Chen and Lee).

3.5.2 Phsophorus Solubilization :-

For measurement of phosphate solubilization a single colony of rhizobial strain culture grown on yeast extract manitol (YEM) Medium was streaked on pikovskaia medium containing tricalcium phosphate (Pikovskaia et

al.,1948) and incubated at 28±1º for 3-5 days. The plates were observed for clear P-zone formation around colonies.

3.5.3 Oxidase test:-

A peace of filter paper placed in clean Petri dish 2or 3 drops of freshly prepared oxidase reagent. Using a piece of stick glass rod a colony of the Test strain removed and smeared on the filter paper .within few seconds a development of a blue- purple is shown. Benson (1994).

3.5.4 Catalase test :-

Two to three ml of hydrogen peroxide solution was poured into a test tube with a sterile glass rod, several colonies of the tested strains removed , immersed in the hydrogen peroxide solution and observed for immediate bubbling. Benson (1994).

3.6 Inoculation test:-

A pot experiment was carried out at the Faculty of Science and Technology, Shendi University to study the nodulation of crop legumes (Beans, Kidny peas, Lupin and Bake) by the strains isolated from wild legumes.

Six samples of each crop were replicated four times. Five out of the six samples were injected each with one specific inoculant (strains isolated from wild legumes), while the sixth sample being control.

3.6.1 The soil:-

The soil used was a mixture of 70% silt and 30% sand. The soils were collected from a depth of 10 – 30 cm. The mixture was then wetted and sterilized in an oven at 121ºc for 2 hours.

3.6.2 The plastic bags:-

Plastic bags (25×25 cm) were used for sowing instead of the pots. Ten small halls were made per bag for drainage of excess water to avoid water logging. Each plastic bag was filled with 2 kg of the sterilized soil mixture.

3.6.3 Seeds:-

Seeds sorted by hand to remove the up normal seeds and obtain similar size seeds. The seeds were sterilized with calcium hypochlorite:-

The seeds were placed 3-4 times in a sterilized Erlenmeyer flask then poured in calcium hypochlorite solution, closed with rubber stopper, mixed from time to time by hand over a period of one hour and rinsed 4-5 times with sterile water. (FAO, 2004).

3.6.4 Sowing:-

Six seeds were sown per plastic bag. After the seedling had reached 10-15 cm in length they were thinned to3 seedlings per bag. The bags were watered daily in order to maintain the moisture at approximately 75% of the field capacity.

3.6.5 Seedling inoculation:-

Seedling inoculation, with rhizobial strains was made by making 2 holes, each of 2-5 cm depth, 0.5 cm in diameter and at a distance of about 2 cm from each seedling. Five ml of the broth media were injected by a sterile disposable syringe (with the needle being removed) to the halls around each seedling. Inoculation was done aseptically 10 days after sowing.

3.6.6 Sampling:-

At harvest, 3 bags of each treatment were cut by the edges. The soil clump was then put on a 0.5cm mesh screen and washed gently with tap water to wash the soil from the root system. The hole plant was kept in labeled paper bags and taken to the laboratory.

In the Lab. nodules number, nodules dry weight, Dry shoot and roots were measured.

3.7. Molecular Characterization of *Rhizobium* (PCR)

3.7.1 Genomic DNA Extraction

DNA extraction from isolates was done according to the boiling centrifugation method reported by Miller (1972). A single colony was grown over night at 28℃ in YEM broth. Bacterial cell were precipitated by a centrifugation at 13000 rpm for 10 minute in a micro-centrifuge (Sanyo). The supernatant was discarded and the pellets were resuspended in 500ml deionized water. The suspension was boiled for 10 minutes in a water bath and then immediately cooled on ice. Extracted DNA was then stored refrigerated until used.

3.7.2. Agarose gel electrophoresis of the extracted DNA

The extracted DNA was electrophoresed in 1.5% agarose gel [0.75g agarose dissolved in 50 ml of 1x TBE buffer (0.089 mol/L Tris-borate, and 0.002m EDTA, pH 8.00). Then 2 µl of ethidium bromide (10 mg/ml) were added Prior to casting the gel, the comb was adjusted and the gel was poured (making sure that there were no bubbles). While the gel was solidifying, DNA mixtures were prepared for electrophoresis as follows: 1 µl of each

DNA sample was transferred to a clean Eppendorf tube and 3µl of loading dye (bromo phenol blue dye) was added to the DNA sample. The content was mixed several times using a micropipette. The comb was removed with gentle back and forth motion and the gel was then immersed in 1x TBE buffer. The buffer was added until it reached a level approximately 3-5 mm above the gel surface. The sample mixtures were loaded into the wells using plastic-tipped micropipettes. 1Kb ladder (Invitrogen) was used as a molecular weight marker. The apparatus (Habaib, U.K, 9H 310083) was closed and the power was turned on, the voltage was adjusted to 75V (400mA) and the running was continued without cooling for 20 minutes after which the gel was visualized under trans illumination cabinet (Model TM-10E, Uvitec. Product) and image was captured and photographed. Extracted DNA was then stored refrigerated until used as a template for PCR amplification.

3.7.3. Polymerase chain reaction (PCR)

For genetic diversity studies four RAPD primers were used to amplify the genomic DNA. The primers were purchased from Gene link, Inc. and Operon Tech., NY 10532. These were 10 oligonucleotide OPC9 primer (CTCACCGTCC), OPY14 (GGTCGATCTG),opl 18 (ACCACCACC) and opr 10(CCAATCCA).

PCR amplification reactions were carried out in a total volume of 20 µl. Each PCR mixtures contained (Final concentration): 5X FIRE Pol PCR Master Mix (Ready to load), 5 X reaction buffer (0.4 M Tris-HCL, 0.1 M

(NH_4) SO_4, 0.1% w/v Tween 20), 12.5 Mm dNTPs, 50 ng of the primer under test, 1 U Taq polymerase and 20 ng template DNA.

The amplification program used consisted of one cycle at 94°C for 5min, followed by 35 cycles of initial denaturation at 94°C for 1min, annealing at 32°C for 3min, extension at 72°C for 2 min and a final extension step at 72°C for 10 min.

Chapter Four

Results and discussion

The plants collected from the study area are shown in table 1. No nodules were observed in roots of any Cassia (*Senna*).This genus belongs to the subfamily Caesalpinoideae. It is known that of the leguminous species examined in many parts of the world 97% of the Papilionoideae sub family, 90% of the Mimosoideae and only 23% of the Saesalpinoideae have been found to beer nodules (de Faria et al.,1989).

Other reports also noticed that the Caesalpinoideae members are the least in supporting nodulation (Sprent,1989).The Cassia (Senna) in different parts of Sudan have been reported not to beer nodules (ENRRI,1998). All studied members of the Papilionoideae subfamily were found to beer nodules on their roots (Table 2). According to the field observations all nodules proved to be active in nitrogen fixation since the interior color of the collected nodules was pink indicating the formation of leg hemoglobin (Sprent,1989). Table 2 showed the growth rate of the rhizobial isolates tasted .Three of the 5 rhizobia under test performed fast growing rate in (3 – 5) days to form separate colonies these were *Crotalaria senegalesis*, *Desmodium dichotomm* and *Clitorea ternata*. It was understood that the fast growing nodule bacteria belongs to the genus Rhizobium were the slow growing ones belong to the *Bradyrhizobium. Sesabania sesban* belongs to *Isorhizobium* although it is a slow growing bacteria.

Progress in bacteria growth rate was observed during the second day – fourth day, while deterioration observed at fifth and sixth days. It was reported that the fast growing rhizobia belongs to rhizobium and the slow growing ones were bradyrhizobium (Hiaty 1990).

Growth rate reaction (Bromothymol) *Crotalaria senegalensis*, *Desmodium dichotomum* and *Clitoria ternate* changed the growth medium to acidic one were as the other two changed the medium to alkaline one (Table 2). It was reported by many researches workers the acid producing nodule bacteria were classified as *Rhizobium* and the alkali producing one were *Bradyrhizobium.*

The acid producing *Rhizobium* changed the color of the growing medium when Bromothymole blue added to the broth culture to yellow color producing acids .The alkaline producing *Bradyrhizobium* changed the color of the medium from blue to dark blue (Jordan, 1984). From the (Table 2). it was clear that the acidic forming bacteria with yellow medium color were *Rhizobium,* the others alkali producing, blue in color were *Bradyrhizobium.*

The results of the presumptive test showed that none of the isolates could grow in Glucose Peptone Agar, they formed colonies in 3-5 days on YEMA, did not absorbed Congo Red and showed the typical morphology of *Rhizobium* colonies on YEMA (Somasegaran and Hoben, 1994) (Table2).
The result of the plant infection test showed that rhizobia isolated from *Phaseolus trilobus* plants formed nodules on *Vicia faba* and *Vigna unguiculata* plants (Table 3) which suggests that they can be grouped with *Rhizobium leguminosarum* bv. *Phaseoli and Rhizobium* spp. A previous

study of *Rhizbium* isolates indicated that one isolate had the ability to nodulate both alfalfa and common bean (Eardly et al ,. 1985).

Isolates from *Clitorea ternatea* plants formed nodules on roots of *Phaseolus vulgaris* and *Vigna unguiculata* and they can be grouped with *Rhizobium leguminosaram* bv.*phaseoli* and *Rhizobium spp* while isolates from *Clitorea ternatea* plants formed nodules on roots of *Vicia faba, Medicago sativa* and *Vigna unguiculata.* Isolates from *Crotalaria snegalesis* and accordingly they can be classified as *Rhizobium lupines* or *Brady-rhizobium japonicum* since none of them formed nodules on roots of plants representing these groups. It was also noticed that all isolates formed nodules on roots of *Vigna unguiculata* and they are belonging to the miscellanceous cow pea group which is widely abundant in soils of the tropics (Giller and Wilson, 1991).This result is in agreement with the findings of other research studies which indicated that isolates from wild legumes belong to the miscellanceous Cowpea group (Grandhi and Godbole,1990; Nimbalka,1986).

The presence of isolates from wild legumes cross-inoculating Phaseolus vulgaris is an important finding since it is known that this crop is poorly nodulated and rarely forms effective symbiosis with rhizobia (Herridge and Bergersen, 1988). Isolates from the wild legumes might serve as a solution for this problem and might prove to be effective in nitrogen fixation with *Phaseolus vulgaris* .This necessitates extensive research on the compatibility of isolates from wild legumes with leguminous crops and their ability to form effective symbiosis with their homologous crop .

The result of the experiment showed that some of the isolates belong to more than one cross-inoculation group. This finding reflects the weakness of the cross-inoculation classification of rhizobia which has been criticized by many researchers (Segovia et al; 1993). This entails that other systems of classification should be studied and adopted for the identification and classification of rhizobia.

(Table 4) showed the some biochemical tests of the collected strain from wild legumes. All the strains were found to be gram negative, catalase positive and solubilize phosphorus except strain SHS, which was isolated from *Sesbania sesban.*

As shown in (Table 5) cross inoculation of legumes by the strains isolated from wild legumes it was clear that *Vicia faba* formed nodules by most of the strains, *Phaseolus vulgares* only formed nodules by strain SHS_1 and non of the strains formed nodules in *Trifolium sp.*

The 9 isolates were amplified using 4 different Operon RAPD primers. The primers were: OPC9, OPL18, OPR10 and OPY14.

- All of the RAPD primers gave amplification products and they were all reproducible.
- A total of 141 fragments were detected for the 9 isolates representing 25 different loci with 100% polymorphism.
- 55.6% of the 9 isolates (ENRRI21, ENRRI22, USDA209, ENRRI2 and ENRRI8) didn't produce any amplification product with primer OPC9.
- Isolate 2 gave no product with primer OPL18.
- The Similarity indices were calculated using Jaccard's coefficient.

- The most relative isolates were SHS1 and ENRRI23 with 73% similarity; while the most distant were ENRRI21 and TAL380 with similarity percentage of 26%.
- According to the similarity indices, the nine samples were grouped into three clusters each containing three isolates. Cluster 1 includes ENRRI23, SHS1 AND ENRRI3; Cluster 2 contained ENRRI22, ENRRI2 and TAL380 while Cluster 3 includes ENRRI21, USDA209 and ENRRI8.

The results of PCR experiment are recorded in table 6and depicted in figure number 8 as well. In figure 8 the uppermost cluster comprises the SH1, ENRRI3 and ENRRI23 isolates which are most genetically compatible. As the similarity reaches around 0.71 which gives rise to a far extent of genetically compatibility .

The RAPD technique was used to detect the compatibility between 9 isolates of rhizobia used in this study, which analysis clear the most relative isolates were SH1 and ENRRI23, this similarity could be attributed to compatible genetically characteristics. While the most distant were TAL380 and ENRRI21 which may be underlying to fewer shared genetically characteristics.

Table (1) source and classification of rhizobia

No	Host Plant	Short name	Local name	Subfamily	Area of collection
1	*Crotalaria senegalensis*	ENRRI 3	Fartaga	*Papilionoidea*	Gezira
2	*Phaseolus trilobus*	ENRRI 21	Phillipsara	*Papilionoidea*	"
3	*Desmodium dichotomum*	ENRRI 22	Abu-Areeda	*Papilionoidea*	"
4	*Clitorea ternate*	ENRRI 23	Clitoria	*Papilionoidea*	"
5	*Sesbania sesban*	SHS 1	Sesban	*Papilionoidea*	Shendi

Table (2) Nodulation and color of nodules formed in legumes

No	Host Plant	Short name	Nodulation	Interior color of modules
1	*Crotalaria senegalensis*	ENRRI 3	+	pink
2	*Phaseolus trilobus*	ENRRI 21	+	pink
3	*Desmodium dichotomum*	ENRRI 22	+	pink
4	*Clitorea ternate*	ENRRI 23	+	pink
5	*Sesbania sesban*	SHS 1	+	pink

(+) = nodulation
(-) = no nodulation

Table (3) growth rate and growth reaction of Rhizobia

No	Host Plant	Short name	Growth rate	Growth reaction bromothymol
1	*Crotalaria senegalensis*	ENRRI 3	Fast	Yellow / Acidic
2	*Phaseolus trilobus*	ENRRI 21	Slow	Blue / Alkaline
3	*Desmodium dichotomum*	ENRRI 22	Fast	Yellow / Acidic
4	*Clitorea ternate*	ENRRI 23	Fast	"
5	*Sesbania sesban*	SHS 1	Slow	Blue / Alkaline

Table (4) biochemical characteristics of *Rhizobium.* strains

No	Host Plant	Short name	Gram Stain	Catalase test	Phosphorus sol
1	*Crotalaria senegalensis*	ENRRI 3	- Ve	+	+
2	*Phaseolus trilobus*	ENRRI 21	- Ve	+	+
3	*Desmodium dichotomum*	ENRRI 22	- Ve	+	+
4	*Clitorea ternate*	ENRRI 23	- Ve	+	+
5	*Sesbania sesban*	SHS 1	- Ve	+	-

Table (5) Nodulation and cross inoculation

No	Host Plant	Source	*Vicia faba*	*Phaseolus vulgaris*	*Trifoliam SP*
1	*Crotalaria senegalensis*	Gezira	-	-	-
2	*Phaseolus trilobus*	"	+	-	-
3	*Desmodium dichotomum*	"	-	-	-
4	*Clitorea ternate*	"	+	-	-
5	*Sesbania sesban*	Shendi	+	+	-

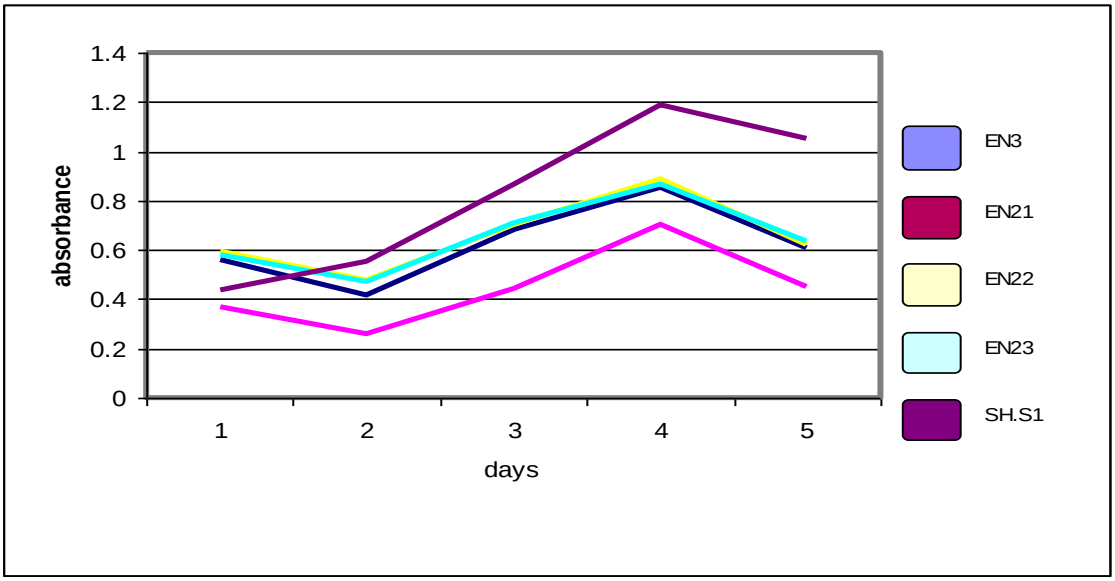


Figure. (1) Spectrophotometer absorption at wave length λ = 420 nm corresponding to different population density of bacteria

Figure (2) Pot experiment: (Bake) seedling inoculation stage.

Figure (3) kidney bean seedling inoculation stage.

Figure (4) Peas seedling inoculation stage.

Figure (5) Beans seedling inoculation stage.

Figure (6) Lupin seedling inoculation stage

Figure (7) The group of crops One week after seedling stage.

Table (6) Similarity matrix between different tested isolates of *Rhizobium* constructed from RAPD – PCR banding pattern

	SHS1	ENRRI3	ENRRI21	ENRRI23	ENRRI22	USDA209	ENRRI2	TAL380	ENRRI8
SHS1	1.00								
ENRRI3	0.71	1.00							
ENRRI21	0.41	0.42	1.00						
ENRRI23	0.73	0.70	0.53	1.00					
ENRRI22	0.52	0.35	0.30	0.38	1.00				
USDA209	0.46	0.48	0.53	0.43	0.43	1.00			
ENRRI2	0.57	0.45	0.42	0.48	0.72	0.55	1.00		
TAL380	0.65	0.48	0.26	0.50	0.57	0.38	0.62	1.00	
ENRRI8	0.52	0.56	0.53	0.43	0.42	0.59	0.47	0.30	1.00

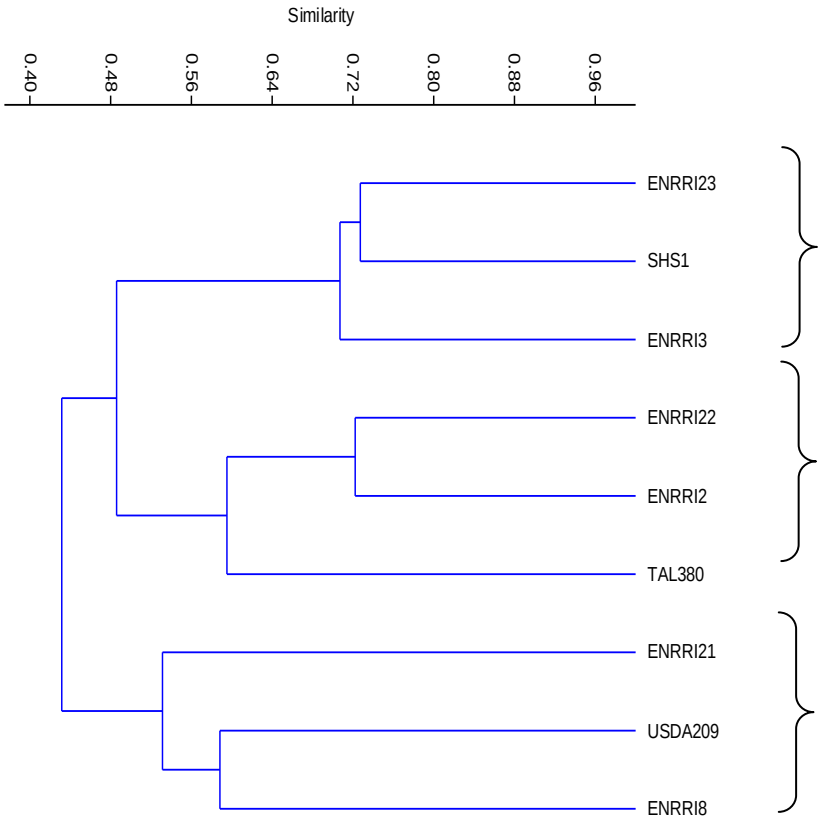


Figure (8) Dendrogram tree of Rhizobium strians isolated from wild legumes based on RAPD - PCR

Figure (9) RAPD PRIMER OPR10

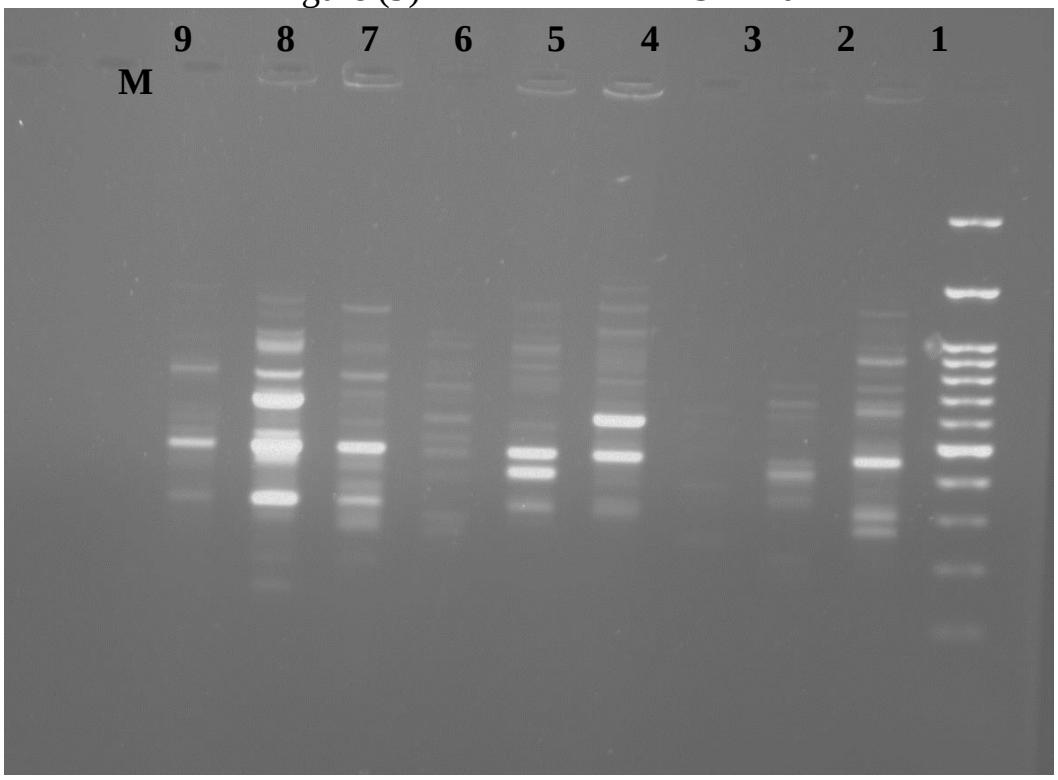


Figure (10) RAPD PRIMER OPL18

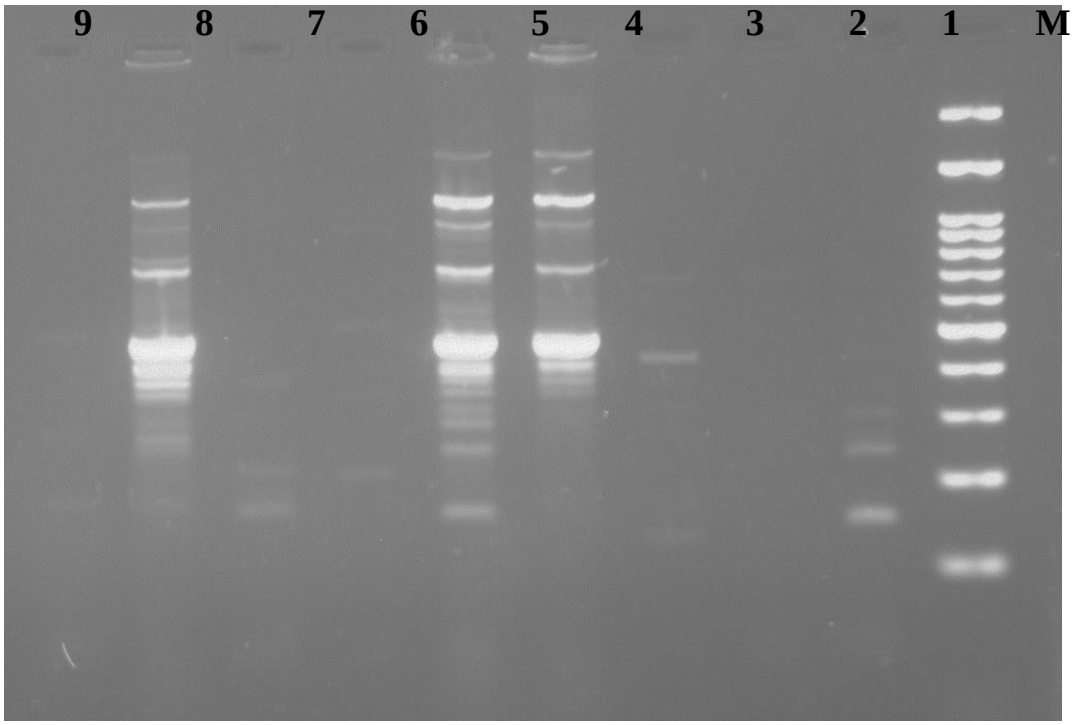


Figure (11) RAPD PRIMER OPY14

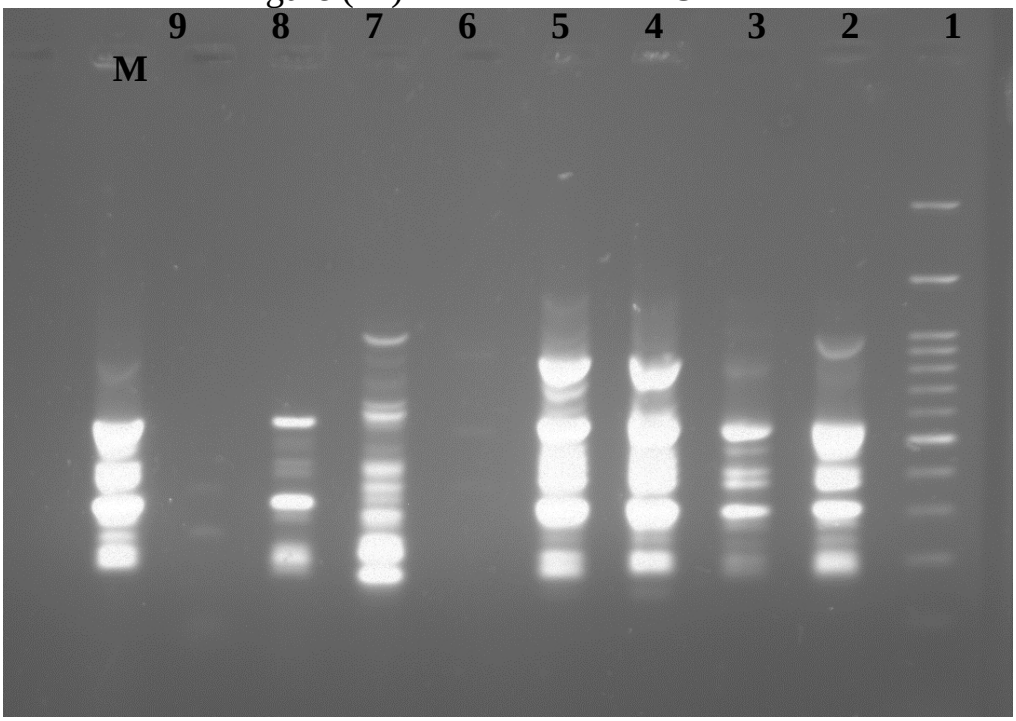
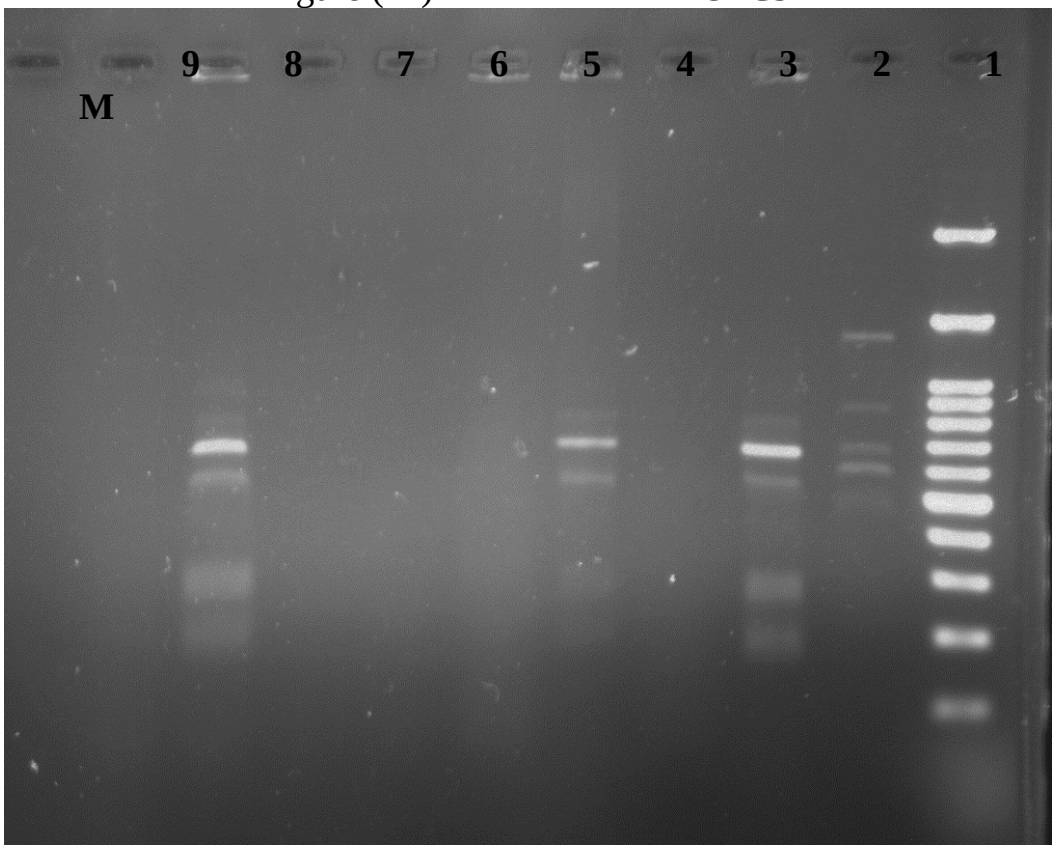


Figure (12) RAPD PRIMER OPC9



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