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# Isolation and Characterization of Nitrogen Fixing Rhizobia from Cultivated and Uncultivated Soils of Northern Tanzania

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## Abstract

**Soil bacteria[1] called rhizobia are gram-negative capable to colonize the soil immediately surrounding roots under the influence of the plant "rhizosphere" and reduce atmospheric nitrogen into the form available to plants through nitrogen fixation process. Nitrogen is the most limiting and supplied nutrient to most plants, and the determinant of plant growth. Legumes differ with most plants because they have access to nitrogen from both mineral and symbiotic sources. Small-scale farmers who are the major legume producers in Africa rarely apply fertilizers during legume production. Hence, the crop is largely dependent on fixed nitrogen from native nitrogen fixers. Isolation of rhizobia for legume production has been given a little attention in Africa due to inadequate research or negligence of researchers and unawareness of its potential in legume production as well as lack of an intention from skilled personnel to popularize the technology. Evaluation of effectiveness of isolated rhizobia is essential for inoculants preparation, host specificity recommendation and symbiotic effectiveness. The isolation, determination of their population in the soil and assessing factors affecting their population and testing the effectiveness of native nitrogen fixers with respect to right trap host crop are given a special attention in this review.**

## Keywords

**Isolation, Rhizobia, Nitrogen Fixation, Symbiosis, Nodules, Legume, Most Probable Number**

[1]Soil bacteria are called rhizobia and can be obtained from the soil by using distinct procedures. Their population in the soil depends on the land use system and the availability of host legumes among others. Trap legume crops can be used to obtain the specific rhizobia for symbiosis.

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## 1. Introduction

Nitrogen is the most limiting nutrient for growth of leguminous plants like Common beans, Soya beans, Cow peas and Garden peas because that present in the soil cannot support growth [1]. Nitrogen is essential in plant cells for synthesis of enzymes, proteins, chlorophyll, DNA and RNA, thus essential for plant growth and production of food and feed [2].

Nitrogen (N) is a constituent of proteins, enzymes, chlorophyll, and growth regulators to plants and its deficiency causes reduced growth, leaf yellowing, reduced branching and small trifoliate leaves in legumes [3]-[5]. Despite the dramatically increase in the amount of synthetic nitrogen (N) applied to crops in the last 40 years from 12 Tg/year to current 104 Tg/year [6] there has been a significant decrease in yield with considerable persistence of vicious circle of poverty to majority farmers [7] [8].

Soil microorganisms specifically bacteria called rhizobia are able to colonise the rhizosphere, infect legume roots and biologically fix nitrogen in the soil through symbiotic process [9] [10] Biological Nitrogen fixation is a process of converting elemental nitrogen into the form of ammonia ( $NH_4^+$ ) and nitrate ( $NO_3^-$ ) available to plants [11]. Rhizobia can live on plant residues (saprophytes) or entirely within plants (endophytes) or (rhizobacteria) or in close association with the plant roots [9] [12]. Based on ability to fix nitrogen, rhizobia are classified into slow (*Bradyrhizobium*) and fast growing *Rhizobia* [13].The growth of *Rhizobium* visible in Yeast Extract Mannitol Agar (YEMA) is 3 - 5 days while *Bradyrhizobium* takes 6 - 8 days [14] [15]. The process in which the rhizobia colonize the rhizosphere, infect the roots and fix nitrogen leads to plant development and grain yield improvement [2] [16]. The effectiveness of rhizobial populations in fixing nitrogen is correlated with soil fertility status where acidic soils have been reported to contain less effective rhizobia strains [17].

Legumes are one of the most diverse plants on earth widespread in tropics and temperate zones [18]. They belong to one of superfamily of angiosperms (Leguminosae/Fabaceae) the order Fabales, in eurosid clade [19]-[21]. Legumes can grow in much degraded soils because they have the ability to fix nitrogen in association with rhizobia [22] [23]. Besides its major role in the traditional diets throughout the world, legumes provide a multiple benefits to both soil and other crops through intercropping [24]-[27]. Despite vast and potential uses of grain legumes like soybean, cowpea, common bean and peas as human food, animal feed and soil fertility enhancer, they can be grown in different agro-ecological zones [28]. Small-scale farmers who are the major legume producers in Africa rarely apply fertilizers during legume production due to their low income; hence the crop is largely dependent on fixed nitrogen from native nitrogen fixers [29]. In most cases, native nitrogen fixers are competitive to inoculants but not efficient strain and possibly incompatible to the host plant [30]. Therefore, relying on native nitrogen fixers without prior information on its efficiency and compatibility with host legume leads to crop production failure. The interaction between plants and soil microorganisms occurs much in the rhizosphere [31].

The legume-rhizobial symbiosis has a large impact on success of legumes hence the atmospheric nitrogen the organisms fix can be more than the fertilizer nitrogen an average farmer can afford to buy and apply [32]. Therefore, legume-rhizobia symbiosis can provide easy and inexpensive way to enhance soil fertility and improve crop production [33]. The root nodule rhizobia approximately reduce 20 million tons of atmospheric nitrogen to ammonia which is 50% - 70% of the world biological nitrogen fixation [34]. The higher fixed nitrogen in hosts determines the success of symbiotic relationship between legumes and rhizobia [35]. However, host range expansion may be limited by the symbiont distribution while hosts can potentially acquire different rhizobia when invading new habitats [36].

Tanzania is among the countries endowed with mega-biodiversity of several biosphere reserves, world heritage sites and protected areas which constitute 35.6 percent of the territory [37] [38] Isolation and testing effectiveness of nitrogen fixing rhizobia from mega-biodiversity of Tanzania creates a platform for rhizobia inoculant production for improving crop production through legume-cereal intercropping.

## 2. Isolation of Nitrogen Fixing Rhizobium/Bradyrhizobium from Soils

Rhizobia are very important for crop production because they form symbiotic relationship with legume the process that converts atmospheric elemental Nitrogen ($N_2$) into ammonia ($NH_3$) accounting for 65% of the nitrogen currently utilized in agriculture [2]. The isolation and characterization of rhizobia is a valuable biological resource for finding bacterial strains with effective rhizobium-legume association to maximize the agricultural production [39] [40]. Rhizobia are phenotypically and numerically diverse of which many remain unidentified [41]

In a study for characterization of indigenous rhizobia nodulating chickpea in India, 53 rhizobial strains were isolated and 28 selected for diversity analysis [42]. Zahran *et al*. [43] isolated 68 rhizobia species in Egypt which were reported to be good candidates for establishing symbiosis in various Egyptian environments. During the study for isolation and characterization of *Rhizobium* specie and determination of their potency for growth factor production, 260 bacteria were isolated on plate count Agar up on which 53 were nitrogen fixing rhizobia and 43 types of morphologies were found with 5 effective rhizobia strains producing plant growth factors [44]. The study for isolation and biochemical characterization of *rhizobium meliloti* from root nodules of alfalfa (*medico sativa*) revealed twenty five 25/50 samples of root nodule with presence of *Sinorhizobia meliloti* [45]. The continuous isolation and characterization of new strains from diverse environment is of paramount importance.

There are about 19,700 legume species currently known to grow around the world with few respective microsymbionts studied [39] [40]. However, the wide forest reserves in Tanzania with varying characteristics is the noble platform for isolation of effective rhizobia strains for nitrogen fixation. Farmers in northern Tanzania mostly intercrop cereal with common beans (*Phaseolus vulgaris*), Cow pea (*Vigna unguiculata*) and Garden pea (*Pisum sativum*) [46]. Most farmers in Tanzania are not aware about the use of native rhizobia inoculants as biofertilizers because the knowledge is not the first priority in agricultural production and even the rhizobia inoculants are not readily available. Therefore, isolation of efficient native nitrogen fixing rhizobia for common beans (*Phaseolus vulgaris*), Soybean (*Glycine max*), Cow pea (*Vigna unguiculata*) and Garden pea (*Pisum sativum*) and produce them as inoculants to improve legume production is important.

## 2.1. Legume-Rhizobia Symbiosis

Legumes are believed to have emerged since 59 - 60 Million years ago (Ma) early in the Tertiary period and its subfamilies to have evolved between 56 - 50 Ma [18] [47] and can survive even in poor soils where there is not enough nitrogen to support other types of plants [23]. However, rhizobia are thought to be present before the legumes for nodulation because Sino rhizobium-Bradyrhizobium split was reported about 500 Ma [48] [49]. Bacteria of the genus *Bradyrhizobium*, *Rhizobium Sinorhizobium* and others induce nitrogen-fixing nodules on the roots of legumes such as peas, beans, cow peas and soybeans [50].

The process of symbiosis is such that rhizobia supply a constant source of reduced nitrogen to the host plant in exchange of nutrients and energy for its activities [51] because plants cannot directly utilize nitrogen stored in the soil as organic matter except in $NH_4^+$-N and $NO_3^-$-N inorganic forms [52]-[54]. The interaction between root nodules and their symbiotic bacteria has been studied through proteins (proteomics) produced by both partners during their signal exchange and growth of symbiosome. The study has identified hundreds of proteins with development and functioning of rhizobial symbiosis [55]-[57]. The pathogen-responsive proteins induced by a variety of biotic and abiotic stimuli protect plants against pathogenic fungi, bacteria, viruses and adverse environmental conditions [58].

When nitrogen in the soil is inadequate, legumes release flavonoids which signal to rhizobia that the plant is seeking symbiotic bacteria [59] [60]. In response, the rhizobia releases nodulation factor which stimulates the plant to create deformed root hairs [61]. Rhizobia then form an infection thread for allowing them to enter the root cells through root hairs [62].

When the rhizobia are inside the root cells, the cells divide rapidly to form nodule [63]. Then the rhizobia convert atmospheric nitrogen into ammonia, a form that is directly used by the plant for synthesis of amino acids and nucleotides, the plant provides the bacteria with sugars, hence the symbiosis is established [64]. Biological nitrogen fixation has gained attention in recent years because it substitutes inorganic fertilizer and is environmental friendly farm inputs essential for poor resource farmers [65]. It also limits groundwater's pollution by nitrates [40]. There are two major groups of bacteria fixing nitrogen within nodules of vascular plants; rhizobia (Alpha-proteobacteria) associating with legumes and Frankia (in actinobacteria). These are filamentous slow growth rate bacterium forming hyphae colonies without an aerial mycelium and have vesicles and spores, the unique differentiated developmental structures critical to its survival [66].

Grain legume like soybean, cowpea, common bean and peas are used in a wide range of products and are important crops for sustainable agriculture [67]. More studies on interaction between rhizobia and legume are essential in unexploited area. Therefore isolation of native nitrogen fixing rhizobia from the soils obtained from farmers' fields and from virgin land through the use of legume trap host crop provides a perfect match between legume and rhizobia for efficient nitrogen fixation leading to improve legume production.

## 2.2. Host Specificity and Symbiotic Effectiveness

Host specificity refers to the ability of particular rhizobia species to form nodules on specific legumes [68]. The approach of using effective or superior exotic rhizobia strains as inoculants has failed in various environments due to various reasons including the use of ineffective and non-competitive rhizobia strains as inoculants [69] [70]. The host specificity leads to a perfect match between legume and rhizobia resulting into effective nodules (deep red inside) formation and nitrogen fixation [71]. If cross inoculation with no perfect match has occurred, ineffective nodules (green or white inside) or no nodules may be formed and nitrogen fixation does not occur [72].

The host specificity and symbiotic interaction is conferred by the particular root exudates (flavonoids) and the Nod factor structure composition [65]. It is enhanced by bacterial recognition of the flavonoids produced by a host species providing opportunity for plant choice as the only correct flavonoid for induction of symbiotic gene expression in a particular rhizobium strain [73]. The particular flavonoid signal perceived by bacteria is mediated in part by the transcriptional regulator NodD, varying functionally among rhizobial strains [74]. The lipo-chitin oligosaccharide (LCO) ‘‘nod factors,’’ is then produced to influence the regulation of plant genes leading to recognition of specific legume host [73]. Bacterial nod factors are composed of four to five beta 1 - 4 linked N-acetyl glucosamine units (a chitin backbone) and a fatty acid [58]. The nod factors differ in their fatty acids, lengths of their sugar backbones, saturation of the acyl unit and decorations (glycosylation, sulfation, and methylation) of the reducing and non-reducing ends of the backbone [75]-[77]. The nod factors produced by rhizobia are diverse, and plant discrimination of the same contributes the second level of specificity to the interaction and create an opportunity for plant partner choice [75]. The *rhizobium* cell walls are composed of varying polysaccharide which provides another opportunity for plant partners choice using the ‘‘lock and key’’ cascade that determines the degree of plant and bacterial specificity [75]. Some researchers have reported that rhizobia of different genera can infect the same plant species but some plant species can strictly be infected by rhizobia from only one specific genera [78] [79].

Cowpea has been reported to be nodulated by rhizobia isolated from soybean, ground nuts and Bambara ground nuts but these legumes cannot be nodulated by rhizobia isolated from cow pea [80]. This is an essential phenomenon when providing recommendation on efficient inoculants for legume production [81] [82] and developing efficient nitrogen fixing rhizobia inoculants because inoculants are introduced into new environments previously not been cultivated and which may lack compatible rhizobia strain [83]. Strain 042B for soybeans in China was reported to have high symbiotic efficiency than *Bradyrhizobium japonicum* and displayed early appearance of nodules with higher nitrogenase activity [84]. Therefore, identification of rhizobia strains in African farmers' fields using specific hosts and testing its efficiency in nitrogen fixation and effectiveness in cross inoculation is essential for improvement of legume production.

## 2.3. Nitrogen Fixation and Crop Productivity

Legumes growth and productivity is normally limited by fixed nitrogen in all environments with suitable climate and available water to support life [85]. Soil fertility can be enhanced through the concept of using, improving and restoring the symbiotic rhizobia [86]. The fact that rhizobia inoculants are inexpensive source of biofertilizers, is a means for sustainable legume supply to the growing population hence is environmental friendly [87]. Legume is considered the major nitrogen fixers [88]. When legumes are ploughed or cut back to the ground, the root nodules release all the valuable biological fixed nitrogen to the soil for the following crop [89].

The estimated contribution of prokaryotes to the nitrogen input of soil ranges from 0 to 60 Kg/ha/year [90] or estimated to be about 175 million metric tons annually and about 70% of all annual fixed nitrogen on the earth; the rest is by micro-organisms, autotrophs or heterotrophs called “free fixers” [91]. Biological nitrogen fixation contributes about 100 million tons of nitrogen for terrestrial ecosystems, 30 to 300 million tons for marine ecosystems and 20 million tons from chemical fixation due to atmospheric phenomena [92]. There has been an increase in the use of nitrogenous chemical fertilizers for cereal and other agricultural crops production since the Second World War [93]. The increase has led to various consequences such as water pollution and eutrophication due to leaching of nitrogen in the soil by rain and irrigation [94]. In order to minimize the consequences, the use of nitrogen-fixing rhizobia can play an important role in the life of host plant because it ensures their nitrogen supply, defense against pathogens and pests as well as adaptation to various environmental stress [91].

Botha *et al.* [88] in South Africa reported that CB 1809 strain for soybeans was 60% superior of the isolates

tested in efficient nitrogen fixation from Bergville and Morgenzon and was similar to 73% of isolates from Koedoeskop. Sadowsky *et al.* [95] in USA reported that three phaseolus plant introduction out of 684 genotypes examined were found to form effective symbiotic nitrogen-fixation with the *Rhizobium fredii* strains and 9 nitrogen fixing efficient strains of *Phaseolus vulgaris* was observed to have between 5 to 10 nodules per plant. In Zimbabwe, smallholder farmers consider nitrogen fixing rhizobia inoculants as having magic properties due to high growth rate and dark green colour of the inoculated legume plants compared to uninoculated [96]. Farmers in Africa are legume producers despite the challenge of low essential macro and micronutrients in the soil. There are no adequate reports on relationship between nitrogen fixation and crop productivity records for different farming systems used in Africa.

The identification of native strains capable of fixing nitrogen will provide an inexpensive solution for enhancing legume production if properly utilized by the majority of small scale farmers in Africa.

## 3. The Factors Affecting Rhizobia Population, Legume and Nitrogen Fixation

Nitrogen fixing rhizobia cannot express their full potential in fixing nitrogen if the environment and the plant is in poor state [97] [98]. The process of nitrogen fixation depends much on the functional state of the legume plant [99] and the optimum environmental conditions supporting the macro and microsymbionts. The nitrogen fixing rhizobia vary in their tolerance to major environmental factors [100]. Environmental stresses can affect the host plant and symbiotic rhizobia [101]. The most threatening environments for rhizobia functions are marginal lands with low rainfall, acidic soils with poor water holding capacity, nutrient stress and temperature extremes [51]. The proposed critical levels of mineral requirement for effective legume growth for soils in Tanzania are as shown in **Table 1**.

### 3.1. Temperature Extremes

High temperatures have effects on the root nodule structure, function and root hair infection [100]. The best temperature for nodule functioning in common beans (*Phaseolus vulgaris*) is between 25˚C - 30˚C [102] [103]. The optimum temperature range for rhizobia has been reported to be 2˚C 5 to 31˚C in culture media but rhizobia isolated from hot and dry Sahel Savannah environment were reported to grow well at 40˚C [104]. The favorable recommended temperature for root hair development and large number of infection is between 15 and 20˚C. The limits of low temperature for crops native to temperate region is 2$^{o}$C while for tropical species is 10˚C [105]. Legume species can fix nitrogen at different critical levels of temperature such as 35˚C - 40˚C for soybeans and cowpeas, and 30˚C for peas [106].

The 68 rhizobia species isolated in Egypt grew at temperature ranging from 20˚C - 35˚C though some species grew at 35˚C - 40˚C and at maximum of 50˚C [43] Successful isolation of high temperature tolerant rhizobia for common bean has been reported in various regions (107). The rhizobia population in relation to temperature for across different zones in Africa still needs more research. The low temperature experienced at the highlands and high temperature with low moisture content in lowlands has resulted into varied crop yield from one season to another. Therefore, isolation of native rhizobia species from extreme temperature range as those found in Africa is essential for obtaining temperature tolerant rhizobia species for improvements of legume yield.

### 3.2. Drought (Soil Moisture Deficiency)

Both rhizobia and legume can exist in soils with low moisture levels with the lowest population densities reported in most desiccated environments [51]. Drought reduces the rhizobia population in soils, inhibits nodulation and $N_2$ fixation [107]. Nitrogen fixation process is highly sensitive to the deficiency of soil moisture [107]. Rhizobia population in relation to drought has received little attention in Tanzania and Africa at large. Most dry lowlands in Africa are characterized with low moisture content and high annual temperature range. Therefore, successful isolation of rhizobia from such environment will definitely result in obtaining good rhizobia candidates for establishing successful symbioses in drought environments useful for production of common bean and other legumes.

### 3.3. Soil Acidity and Related Stress

Soil acidity and related problems of manganese and aluminum toxicity as well as calcium deficiency seriously

**Table 1.** Proposed critical levels of mineral requirement for soils in Tanzania.

| Mineral | Proposed critical levels | Reference |
|---|---|---|
| Nitrogen (N) | 2 g/Kg soil | [113] |
| Phosphorus (P) | 10 - 15 mg/Kg of soil | [114] |
| | **Exchangeable cations** | |
| Potassium (K) | 0.20 cmol/ Kg of soil | [115] |
| Magnesium (Mg) | 2 cmol/Kg of soil | [116] |
| Calcium (Ca) | 5.0 cmol/ Kg of soil | [117] |
| Aluminum (Al) | 1.0 cmol/ Kg of soil | [116] |
| Cation exchange capacity (CEC) | 6.0 - 12.0 cmol/Kg of soil (for poor soils) | [113] |

affect nodulation, $N_2$ fixation and plant growth [108]. Nitrogen fixation by rhizobia to most leguminous plants is effective at neutral or slightly acidic soils [109] [110]. Researchers have reported that most legume species fail to nodulate at pH less than 5.0 because cannot withstand acidic condition [111]. A study conducted in Kenya revealed that common beans (*Phaseolus vulgaris*) can be nodulated by both rhizobia from low pH (acidic soils) and high pH soils [100] [110]. Rhizobia isolated in Egypt grew at pH ranging from 6 - 8 with some being able to tolerate acidic pH ranging from 3.5.to 4.0 and alkalinity at pH ranging from 9 - 10 [43]. *Rhizobium tropici* is a species adapted to acidic soils than any other species of *Rhizobium* reported to nodulate *Phaseolus vulgaris* [112]. Bother *et al.*, [79] in South Africa reported that the Bradyrhizobia population serotype 135 for soybeans was observed to be adapted to alkaline soils at Koedoeskop while serotype 122 was adapted to soils of neutral or acid pH thus could not survive well in Koedoeskop soils. In China it was reported that strain 042B was adapted to alkaline soils with pH 10.7 and a wider range of temperature from 10˚C - 42˚C and can grow faster in unfavourable environments and utilized a wide range of carbohydrates than other strains nodulating soybeans providing an essential commercial value for industrial inoculum production [84].The relationship between rhizobia population and soil pH for Tanzania and Africa at large still needs more research due to soil characteristics varied with altitude and rhizobia diversity. Therefore; isolation of rhizobia from varied locations with wide range of soil pH is a pavement to acquire effective native rhizobia tolerant to low and high soil pH.

### 3.4. Salt and Osmotic Stresses

Researchers have reported the detrimental effect of salt on growth and survival of rhizobia [118] [119]. The response of legume to salinity varies much depending on soil properties, climatic conditions and plant growth stage [120] [121]. Salt and osmotic stress can affect the initial stage of legume-rhizobial interaction and nodule formation than it does to rhizobia [122]-[124]. The root hair formation on plants is more sensitive to salt than rhizobia cells hence rhizobia can tolerate salinity from 4.5 to 5.2 $dsm^{-1}$ [125] [126]. The *Rhizobium leguminosarum* for common beans (*Phaseolus vulgaris*) can tolerate up to 350 mM NaCl concentration in broth culture while those for *Vigna Unguiculata* can tolerate up to 450 mM NaCl concentration [104]. Some rhizobia species can tolerate moderate salinity soils and fix nitrogen effectively [127].

In order to establish a successful rhizobia-legume symbiosis for saline environments, efficient salt tolerant native rhizobia strains should be isolated from saline soils [118] [128]. The study conducted in Meru and Hai Districts northern Tanzania reported that Bradyrhizobia and rhizobia inoculants are efficient supplier of nitrogen to common beans and soy beans respectively and that inoculation is a better option to poor resource farmers who cannot afford to buy expensive inorganic fertilizers [119]. This process is environmental friendly source of nitrogen which is renewable and can replace or complement mineral fertilizer inputs [120] [121].

Therefore, isolation and estimation of the population density in relation to salt and osmotic stress for native rhizobia nodulating common beans, cowpeas, soybeans and peas across favorable to unfavorable rhizobia growth conditions across Africa is a means for improvement of legume production.

## 4. Rhizobia Population Estimates Using the Most Probable Number (MPN)

The most probable number (MPN) plant-infection is a technique for the enumeration of rhizobia in soils and in inoculants for legume inoculant testing program. The MPN technique relies upon the pattern of positive or nega-

tive nodulation responses of host plants inoculated with consecutive series of dilutions of sample containing rhizobia [83]. The technique is applied under the following major assumptions: 1) a single viable rhizobium cell inoculated onto its specific host in a Nitrogen-free medium will cause nodule formation; 2) nodulation is the proof of infective Rhizobia; 3) the validity of the test is demonstrated by the absence of nodules on uninoculated plants; and 4) absence of nodules on inoculated plants is proof of the absence of infective rhizobia. The MPN of rhizobia in hot and dry region and in the region receiving heavy rainfall has been reported to be low while in the region receiving moderate rainfall, the population is high [126] [128]. Cultivation of legume in an area has been reported to influence the population of rhizobia.

The study conducted in Iowa fields grown with Soybean within the previous 13 years reported the MPN of *Bradyrhizobium japonicum* to be correlated with whether soybeans had or not been grown at the site within the previous 13 years [124] [125].

The study conducted in India at the field grown legume for at least 15 years ago showed no yield response to inoculation because native nitrogen fixing rhizobia for nodulating cowpeas and common beans were still present in the soil with the population ranging from $6 \times 10^0$ to $1 \times 10^4$ per gram of soil but *Rhizobium japonicum* for soybeans were not found in any of the soil samples [51]. The study conducted in Tunisia using MPN reported the increased number of nodules and shoot dry yield to more than twofold when *R. gallicum* strain 8a3 were inoculated to common beans and the nodule count was observed to be higher even from the soil with population of $10^6$ native rhizobia $g^{-1}$ soil [129]. In South-West Spain, the study conducted using MPN count reported that rhizobia population ranged between $3.6 \pm 42.4$ rhizobia per gram of soil in uncropped soil and about $4 \times 10^3$ rhizobia per gram of soil in cropped soil.

However, most isolated strains were more efficient in nodulating common bean than the control and there were no association between soil properties and presence of common bean nodulating rhizobia [130]. In Brazil using MPN technique, [131] reported that rhizobia population nodulating common bean ranged between $7.6 \times 10^4$ and $1.57 \times 10^3$ cells per gram of soil in the plot with 4% aluminum saturation and unlimed plot respectively. In Nigeria using MPN, Aliyu *et al.* [132] found that native rhizobia population estimates for soybeans were very low in slightly acidic soil and were not found in moderately acidic soils. However, the rhizobia for nodulating cowpea were relatively high in slightly acidic soils and significantly high in moderately acidic soils. The rhizobia estimates using MPN in soils from Embu district in Kenya varied with the system of land use; the population range for land use under coffee, tea, maize-beans intercrop and fallow was between 1.1 - $2.3 \times 10^2$ cells per gram of soil while $6.1 \times 10$ and 0 cells per gram of soil were recorded for land use under Napier and natural forests respectively [133] [134]. In Poland using MPN technique, *R. leguminosarum* bv. *Phaseoli* population for common bean was observed in 21/32 soils ranged between log 2.23 - 5.34 [135]. The study conducted in Japan by [136] revealed that the native rhizobia population isolated from cultivated soil nodulated to Yezin-3 (Rj4) and Yezin-6 (non-Rj) were $1.16 \times 10^4$ and $1.16 \times 10^5$ cells per gram of soil respectively. The population was associated with the content of nitrogen and silt-clay fractions in the soil indicating that it was affected by the absence of plant host [136] [137].

Dudeja and Ghurana [138] in India reported that inoculated Bradyrhizobia population varied with time in different soil layers from $10^1$ - $10^2$ in July to $10^2$ - $10^3$ in August to September and 47 rhizobia per gram of soil in October. However, he increase in soil moisture content led to increase in rhizobia population to $10^3$ per gram of soil constantly soon after the onset of rainfall. The study conducted in Isinya District Kenya using MPN reported that rhizobia collections were found in varying densities in all the sampled plots and the natural grasslands had low rhizobia concentration (575 cells/g soil), the population declined with repeated cultivation of non-legume crops but increased sharply (30,000 cells/g soil) with the introduction of grain legumes [139]. Maingi [137] in Kiboko at Makueni District, Southeast Kenya (semi-arid to arid conditions) using MPN count reported that the total Bradyrhizobia population was between $2.59 \times 10^4$ and $1.89 \times 10^5$ per gram of soil. The population size of taxonomically defined slow growing Bradyrhizobia in Kiboko was between $2.59 \times 10^2$ and $1.89 \times 10^3$ cells per gram of soil sample while the approximate Bradyrhizobia population specific to TGx genotype was between $7.81 \times 10^2$ and $5.67 \times 10^3$ cells per gram of soil [140].

In Kaguru at Meru District, East Kenya (semi-humid climate) the approximate total Bradyrhizobia population was between $1.04 \times 10^2$ and $7.56 \times 10^3$ cells per gram of soil. The population size of taxonomically defined slow-growing Bradyrhizobia was between $1.33 \times 10^2$ and $9.72 \times 10^2$ cells per gram of soil while the approximate Bradyrhizobia population specific to TGx genotype was between $2.37 \times 10^2$ and $1.73 \times 10^3$ per gram of soil [140]. The rhizobia abundance and nitrogen depletion was reported to be led by vegetation clearing and cul-

tivation of cereal crops without intercropping with legume [139]. Regular crop rotation with grain legumes can replenish soil fertility through biological nitrogen fixation and significantly improve crop yields and food security. The repeated cultivation of symbiotic legumes can lead to rapid increase of rhizobia population and significant improvement of soil Nitrogen fixation [139].

Due to the fact that inoculant price is low compared to the potential benefits they provide, farmers should be encouraged to inoculate each season of the production year [141]. Rhizobia population has been reported to be high in legume cultivated areas and in wild vegetation whether leguminous or not [51]. It is therefore essential to conduct the research for MPN of native rhizobia in both uncultivated (wild) and cultivated regions in Africa to establish the critical levels and create the platform for rhizobia inoculation in soils with varied characteristic.

## 5. Estimation and Characterization of Rhizobia Population Using Serological Techniques

Serological techniques has been used in the study of rhizobia for strain identification, ecological investigation of serological relatedness of strains and their antigenic composition [142] The techniques has also been used to identify some organisms depending on their cells immune response to foreign organism that enters their body [143]. The technique applies agglutination, immuno-diffusion and immuno-fluorescence techniques in investigation of rhizobia cells [144]. Most studies report indicates that rhizobia are serologically heterogeneous group of organisms [145] Different strains isolated from the same host could serologically be unrelated [146]. The strains isolated from different nodules on the same plant could serologically be unrelated [147]

Agglutination reaction has been used to assess the serological relatedness of strains and species of rhizobia [148]. The studies conducted in Hawaii to examine serological relatedness of 25 strains of slow-growing *Rhizobia japonicum* by agglutination identified 6 somatic serogroups [143] [149] Raposeiras, and Marriel [150] in Brazil reported that SLA 2.2 native rhizobia strain and CIAT 899 commercial strains are competitive strain for bean inoculation in soils with low fertility and reduced rhizobia population. Gao and Yang [48] in China reported that strain 042B could form nodules and fix nitrogen to both alfalfa and soybeans with nodule occupancy ranging from 82% - 90% while that of strain USDA110 ranged from 78% - 46%. Bizarro and Giongo [151] in Brazil reported that 27/75 isolates from soybeans were similar to original strain with strong correlation obtained in their genetic variability.

Immuno-diffusion is another technique used extensively to investigate the serological relationships between various strains and species of *Rhizobium* [152]. It has the resolving power in distinguishing between antigenically identical and closely related but not identical strains [153]. The studies for immunodiffusion of 62 fast growing strains of lotus rhizobia indicated that while fast and slow growers shared no common somatic antigen, internal antigen were shared by fast growing strains [143].

Fluorescent Antibody (FA) is among the most used technique for direct examination and identification of rhizobia strains in the culture media, nodules and direct enumeration of specific strains from the soil [154] .The technique is essential because needs only small amount of antigen and antibody and is the only technique capable for the study of rhizobia insitu [155]. Enzyme-linked Immunosorbent Assay (ELISA) is another technique mostly used for identification of bacteria in the soil or in plants. It uses antibodies and colour change to identify a substance. Moawad *et al.*, [156] in Egypt assessed the competition for nodulation using FA technique and reported that Phaseolus 163 inoculant strain occupied 30% - 40% in both soils and 38% - 50% of nodules on Bronco cultivar and at least 50% of the nodules on the Bronco were occupied by native rhizobia. Serological techniques in characterization of native rhizobia effectiveness in fixing nitrogen from the mega-biodiversity in Africa are essential for legume production.

## 6. Evaluation of the Nitrogen Fixing Capability of Isolated Strains and Their Effects on Legume Production

The symbiotic Rhizobia-legume system is the major contributor of biologically fixed nitrogen as compared with non-symbiotic nitrogen-fixing bacteria [157]. The system is estimated to contribute $1.44 \times 10^8$ metric tons of nitrogen per year globally [158]. The application of nitrogen fertilizers in Kenya was reported to increase the dry matter and grain yield in common beans (*Phaseolus vulgaris*) but led to decreased number of nodules per plant.

Rhizobial inoculants application increased both the nodule number and dry weight although the effect was not realized on plant growth and grain yield and the application of farm yard manure only increased the number of

nodules during short rain season [159]. The study conducted in Egypt for assessing the performance of phaseolus bean rhizobia in the Nile Delta soils reported a positive response to inoculation of Giza 6 in terms of nodule numbers, dry matter and plants biomass accumulation and CE3 strain enhanced plant growth and high nitrogen uptake compared with Phaseolus 163 strain [160]. Mehrpouyan [161] reported that in Zanjan province the nodule number and weight was increased in inoculated treatments and the nitrogen fixation was higher while the average yield increased about 43% compared with the non-inoculated (control).

In Khuzestan province the yield of inoculated common beans was 35% to 69 % higher than controls while the grain yield for inoculated soybeans increased twice compared with the maximum fertilizer treatments and 10 folds relative to the control. The percentage of grain protein content was reported to be higher in inoculated treatments than the control. In Iowa, *Bradyrhizobium japonicum* was reported to have different competitive ability on soybeans nodule formation. The isolated native rhizobia strains were effective in nitrogen fixation on common beans because they were able to compete with the exotic rhizobia species. Gicharu and Gitonga [162] reported that Greenhouse inoculation of multi-strain on climbing common bean (*Phaseolus vulgaris*) in Kenya produced the highest number of nodules and yield than the control but in the field, the highest dry weight was produced by USDA 2676 inoculant. This means that the native rhizobia in the control were competitive and effective in fixing nitrogen to meet the host nitrogen requirements level thus there were no need to use new inoculants at such field [163]. The study conducted by Maingi [164] in Kenya revealed that most isolates were rhizobia with different morphology, the isolated strain 446 was more effective in fixing nitrogen compared with *Rhizobium leguminosarum* biovar *phaseoli* and the nodulated legume plants had the highest shoot dry weight than the non nodulated plants. All rhizobia strains isolated from Amazon soils during the study for evaluating plant growth-promoting traits of *Rhizobium* strains for their co-inoculation in common beans (*Phaseolus vulgaris*) were found to fix nitrogen efficiently as free living bacteria [165].Weaver and Frederick [166] reported that there were no increase in yield even when there were less than $1.1 \times 10^1$ native rhizobia per g of soil regardless of majority nodules being formed by inoculum strain. Also inoculation to common beans formed no nodules when the number of native rhizobia was $1 \times 10^2$ per g of soil but the nodule number was increased when the number of rhizobia was below $6 \times 10^0$ per g of soil and the yield of soybeans increased by 62%.

The number of native rhizobia was inversely related to the inoculant rhizobia in terms of inoculation and the successful competition. One of the reason for the failure to achieve successful inoculation with efficient rhizobia is the stiff competition for nodule sites posed by native rhizobia [167]. Abaidoo and Van Kassel [168] reported a higher accumulated shoot dry matter in inoculated soybeans intercropped treatments than in monocroping systems with inoculated and non-inoculated treatments. Furthermore, common beans dry matter accumulation was higher in uninoculated than inoculated treatments; The results suggests that there were high competition for nutrients in common beans than in soybeans intercropping systems from native rhizobia species. However, higher nodulation and nitrogen fixation was found in common beans intercropped with maize planted in the same hole than otherwise, possibly due to nitrogen stress caused by maize utilizing the fixed nitrogen. Conversely, Allen and Obura [169] reported the increase in dry matter and nitrogen when maize was intercropped with cowpea and decrease from 23% - 26% when maize was intercropped with inoculated soybean. These results suggest that a benefit to non-legume crop on fixed nitrogen will depend on efficiency of legume in fixing nitrogen. Inoculated plants have been reported to develop vigorously with high shoot dry matter contents from effective symbiosis of about 84% [170]. Most researchers have reported the increased number of pods, yield, and seed number per pod, dry weight of pods per square meter, biomass, Leaf Area Index and 100 seed weight in inoculated than non-inoculated treatments with no significant difference for grain harvest index [171] [172]. Nitrogen fixation is reported to be affected by the nature of the rhizobia population such that low rhizobia population may not nodulate the host adequately and the ineffective population may fail to nodulate the host to its nitrogen requirements [173].

Nitrogen fixing rhizobia have been reported to vary in their ability to compete, infect and fix nitrogen in their hosts due various factors such as host symbiont compatibility, soil salinity, soil pH, mineral and heavy metal toxicity, temperature extremes, inadequate or extreme soil moisture and nutrient deficit [174]. The only alternative to acquire successful inoculation is by using effective native rhizobia strains. Therefore evaluation of the capability of isolated native nitrogen fixing rhizobia for legume provides a way forward for yield improvement.

## 7. Conclusion

Isolation and testing effectiveness of nitrogen fixing rhizobia from mega-biodiversity ecosystem of Africa as

well as monitoring the factors affecting the rhizobia, legume and symbiosis providing effective rhizobia is essential as it may result into the identification of supper inoculants for improving legume growth and yield and later providing economic benefit to legume producers. Therefore, more research and emphasis is required to popularize this cheap and eco-friendly technology for majority of smallholder farmers in Africa.

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