Plant Growth Promoting Bacteria Play a Major Role in Nutrient Uptake of Cowpea Plants

Ajayi Olaoluwa O.[1*], Dianda Mohammed[2], Fagade Obasola E.[1], Nwadike Blessing[1]

[1]*Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Nigeria*
[2]*International Institute for Tropical Agriculture, Ibadan, Nigeria*
**Corresponding author*

Abstract: **Plant growth promoting bacteria including *Agrobacterium spp., Azotobacter spp., Pseudomonas sp.* and *Paenibacillus sp*., which are known to promote the growth of plants were co- inoculated co-inoculated with Rhizobia (which are known Nitrogen fixers) in cowpea to study and determine their effects on the uptake of nutrients such as Nitrogen, Phosphorus, Calcium, Sodium, Magnesium and Potassium which all play specific roles in the development of plants and are important for their growth. The experimental cowpea plants were growen for eight weeks under screen house conditions during which Nitrogen- free nutrient was applied to them weekly. Upon harvesting, the percentage quantity of these nutrients in the dried shoot was determined. It was observed that the consortium of *Agrobacterium* spp., *Azotobacter spp*. and *Paenibacillus sp.* improved the uptake of nitrogen and phosphorus, a corsortium of *Pseudomonas sp*. and *Paenibacillus sp*. improved the uptake of nitrogen, magnesium and calcium while the consortium of *Azotobacter* spp improved the uptake of calcium. There was no specific pattern in the uptake of sodium and potassium. There was significant correlation between the percentage Na and the percentage Mg, Ca, K, P and N in the shoot of the plants. Plant growth promoting bacteria have significant roles to play in nutrient uptake and can also contribute to nitrogen fixation in cowpea plants.**

Keywords: **Nutrient uptake, Plant growth promoting bacteria, Cowpea**

I. INTRODUCTION

Nitrogen fixing bacteria especially members of the family Rhizobiacea have been known to be able to fix nitrogen when they are found to be in friendly symbiotic relationships with leguminous plants. This phenomenon is described as nitrogen fixation and is known as nature's nitrogen factory (14). Although much work has been done on legume – *rhizobium* symbiosis, it has been found that other microorganisms are also important and play useful roles in nitrogen fixation. These other endophytic bacteria which can be found in legume nodules are able to penetrate the nodules alongside *rhizobia* but have been ignored for a long time (1,14) there is therefore little information on the roles they play in enhancing plant growth particularly with respect to increased absorption of macronutrients such as phosphorus, calcium, magnesium etc. and their importance as plant growth promoting rhizobacteria (PGPR). Nitrogen (N_2) is an essential nutrient required for plant growth as it is a prominent component of amino acids, nucleic acids and chlorophyll. Deficiency of nitrogen results in chlorosis which is characterized by yellowing of leaves, stunted growth, slow growth, etc. Despite its abundance in nature, N_2 it is unavailable to plants because of its inert nature. Phosphorus is a fundamental component of the substances that are the building blocks of genes and chromosomes (16,21). It is an essential part of the process of carrying the genetic code from one generation to the next, giving the blueprint for all characteristic of plant growth and development (18,19). Sufficient phosphorus is also required to enhance different plant organs growth, promote nodulation and early maturity in legumes (7). Potassium is required for every major step of protein synthesis. The process of the "reading" the genetic code in cells of plants to produce proteins and enzymes that regulate all growth processes would be impossible without adequate K. When plants are deficient in K, there is no synthesis of proteins inspite of an abundance of available nitrogen (N). Instead, there is accumulation of protein "raw materials" (precursors) such as amino acids, amides and nitrate. The enzyme nitrate reductase catalyzes the formation of proteins, and K is likely responsible for its activation and synthesis (8).

Calcium (Ca) is taken up by plants as the calcium ion (Ca^{++}). A structural nutrient, it is an essential part in cell walls and membranes and is required for the formation of new cells. For this reason, early season availability of supplemental Calcium has a distinct effect on fruit set. Once deposited in plant tissues, Calcium is not remobilized. Therefore, young tissue is affected first under conditions of deficiency. Since Calcium is not mobile, the requirements of a crop for Ca^{++} after early fruit set are commonly supplied in the form of nutritional sprays (16). Plant uptake of magnesium is in the form of the magnesium ion (Mg^{++}). The chlorophyll molecule, which is essential for photosynthesis, contains magnesium (21). Sodium is not an essential requirement for plants but is used in small quanties by plant where it affects photosysnthesis by altering the balance of water, aids metabolism and synthesis of chlorophyll. It deficiency does not show any symptom in plants (15,17). Cowpea is a

II. MATERIALS AND METHODS

2.1 Study area and sample collection: Samples were collected from three sites in Nassarawa State which are Shamage Local government (N 080 37| 47.7| E 007^0 46| 48.4| Elevation 244

m), Ogba/ Ubbe Egon local government (N 080 51| 55.4| E 080 25| 34.5| Elevation 399 m), Mandara Kokona local government (N $08^0$50| 29.8| E 008 12| 37.1| Elevation 364 m). Nassarawa State was selected because at the outset of this research the nodule samples were readily available and it is also an area where large cultivation of cowpea is a common practice. A hand trowel was used to mark a circle with a radius of approximately 15 cm around the plant base, and section was cut out to a depth of about 20 cm. A spade was then used to slowly lift out the clump. The soil particles were carefully removed from the root material mechanically. Detaching of the secondary roots from the plant was avoided as nodules may be found on the lateral roots as well as the tap root. A sieve of 0.5 mm size and mesh was then placed under each root sample to catch nodules that may become detached from the root. The root was then carefully washed under a gentle stream of water from a tap in a seive (13). The samples were then wrapped in Aluminum foil paper and transported by road to the laboratory and stored in a fridge.

2.3 Isolation of Rhizobia from Root Nodules: Microorganisms were isolated from nodules on Congo red agar (22) using spread plate method. Five undamaged nodules samples were selected at random from each site. They were place in sterile water for about 15 to 20 mins to rehydrate them after which they were surface sterilized using 3 % sodium hypochlorite for 3 minutes. They were then rinsed in sterile water; then further sterilized with 95 % ethanol and rinsed with six changes of sterile water. The nodules were transferred into sterilize petri-dishes and crushed with flamed glass rod. A few drops of sterile water were added to the crushed sample. A loop full of crushed nodule was streaked on congo red agar and then incubated at 28^0C for 5 - 7 days (20, 22). Selected isolates present were picked, *rhizobial* isolates were selected based on their cultural appearances i.e their ability to absorb congo red dye thus appearing white on the congo red media (20, 22). Isolates were labelled, purified and stored on yeast mannitol agar slants and nutrient agar slants. The major media used for isolating and culturing of *rhizobia* were Congo red, Yeast Mannitol Broth, and Nutrient agar. Identification of Plant growth promoting bacteria was done using and identified using biochemical methods and the bergy's manual (6,14).

2.4 Prepation of broth for inoculation: Pure cultures of the *rhizobia* isolates were obtained and introduced into 100 ml Erlenmeyer flasks containing 50 ml of yeast-mannitol broth in duplicates. The pure cultures of the Non *rhizobial* microorganism which were to be co–innoculate with the rhizobial isolates were also obtained and introduced into one of the duplicates *rhizobial* broths. For *Rizobial* isolates (R1, R2, R3) obtained from Shamage, the following plant growth promoting rhizobacteria (PGPR) were added *Agrobacterium spp*, *Azotobactar sp*, and *Paenibacillus wynnii* (consortium A) while the *Rhizobial* isolates obtained from Mandara (R4,R5) were co-inoculated with *Paenibacillus wynnii* and *Pseudomonas aeroginosa* (Consortim B) and the *rhizobia*l isolates from Ogba (R6, R7, R8, R9) were co- inoculated with two *Azotobacter spp*. (Consortium C). The broth inoculation was inoculated with bacterial cfu/ ml of about $10^{\wedge 6}$ (for PGPRs) and $10^{\wedge 9}$(for *rhizobia*). The inoculated broth were incubated at room temperature (28°C) on a Rotary shaker for 7 days and then used to inoculate plants at 1 week of growth (22).

2.5 Pot Experiment: Sea sand was collected from Lagos bar beach and washed repeatedly with water to remove debris and reduce pH to about 6.8 which is suitable for *rhizobia* growth. The crushed gravel and medium sized gravel were also washed till the water was clean. The sea sand, crushed gravel and peat were mixed in a ratio 6:6:1 and mixed until it was evenly distributed. The mixture was then sterilized at 121^oC and 1.05 kg cm^{-2} for 15 minutes. The medium sized gravel was also sterilized (22). 500 ml pots sterilized using 5 % of JIK (3 % w/v sodium hypochlorite) after which it was thoroughly rinsed with sterile water. Seeds were planted in 500 ml pots filled with sterile sand and allowed to germinate. One week after planting (WAP), the cowpea plants were thinned to one viable plant per pot.1 ml of the inoculums which were already prepared as described above, was introduced into the cowpea plants using sterile pipette. A completely randomized block experimental design was used and the 20 treatments (including the Nitrogen control (N+ treatment to which nitrogen was applied)) were replicated in each block

2.6 Application of nutrient to Plants: Cowpea plants were allowed to grow for 8 week during which they were given 20 ml of nutrient solution consisting of both micro and macro nutrient. To prepare nutrient solution given to plant, the stock solutions were mixed using 100 ml of macro- stock solution and 10 ml micro- stock solution made up to 10 liters using distilled water. The nutrient solution was sterilized at 121^oC and1.05 $kgcm^{-2}$ for 15 minutes and was aseptically given to the plants weekly.

The solution for the N+ treatment (control containing nitrogen) was prepared using 5% of N in KNO_3 this was sterilized at121^o c and1.05 kg cm^{-2} for 15 mins after which 50 ml of the solution was added to the plant weekly.

2.7 Harvesting: The plants were harvested 8 weeks after planting (WAP) by cutting at the base with a secateur. The shoots and roots were collected and placed in labeled paper bags and placed in an oven were they were dried at 68^0C for 72 hours until constant weight was obtained.

2.8 Determination of nutrients in plant shoots: The total Nitrogen in plant shoots was determined using the micro Kjeldahl method (9) phosphorus was determined by the molybdenum blue method as described by (11). The concentrations of Ca, K, Na and Mg in shoot were determined following the method described by (6). Unnodulated plants of the cowpea N+ treatment was used as a reference plant. Dried plant samples were digested after which the readings for sodium and calcium were taken using a flame photometer while the readings for potassium and magnesium were taken

using an atomic spectrometer from the diluted digested samples.

2.9 Statistical analysis: The collected data were analyzed for correlation (Pearson's) using SPSS version 20 and Fisher's least significance was used to compare means at $p \leq 0.05$ and $p \leq 0.01$.

III. RESULTS

3.1 Nitrogen: Plant samples were processed and analyzed for total-N. The total yield from the percent total-N in the plant material and the dry matter yield were calculated, and the difference in total-N yield between the cowpea and the reference crop obtain, to give the estimate of N_2 fixation. Total Nitrogen for at R1, R2, R3, R4, R5 when co-inoculated with consortium A and B respectively were higher than their counterparts which were inoculated with only the *rhizobia* , while R6, R7, R8, R9 to which *Azotobacter spp.* had lower Nitrogen value than their counterparts to which only rhizobia strains were added. All treatments gave a higher N value than that of the N^+ treatment with a percentage increase ranging between 7.8 – 50.9 % See Fig 1.

3.2 Phosphorous: The rhizobia strains that were co-inoculated with consortium A (R1, R2, R3) had a higher percentage shoot P than their counterparts inoculated with only rhizobia while R5 and R6 which was co-inoculated with consortium B and consortium C respectively also had higher percentage shoot P values with only *rhizobia* inoculants although others in their group showed a negative response. About 60 % of the treatments had a higher P value than N^+ treatment (See Fig 2).

3.3 Calcium: All plants inoculated with only *rhizobia* and those co-inoculated with PGRB had a higher percentage shoot Ca compared to the N^+ control plant. Consortium C i.e R6,R7,R8,R9 to which *Azotobacter spp*. was added showed consistency in improving the uptake of calcium when compared with their counterparts to which only rhizobia was added. While R2 and R5 also had higher values of calcium uptake the other in their group did not

3.4 Potassium: All inoculated plants had a lower percentage shoot K value than N^+ control. Fig 3.

3.5 Sodium: All treatments had a lower percentage shoot Na value than the N^+ (See fig 6) including those to which the three groups of PGRBs were added. There was also no consistent pattern in the absorption of sodium suggesting that this group of PGRBs including *Agrobacterium spp*., *Paenibacillus wynnii Pseudomonas aeroginosa* and *Azotobacter spp.* did not have any effect on the uptake of sodium but that other factors or PGRBs may be responsible for its uptake. See fig 6.

3.6 Magnesium: Consortium B i.e treatments R4, R5 to which they were added were able to improve the uptake of magnesium while the consortium of A and C showed no consistency in increasing the uptake of magnesium. 83.3% of the treatments had a higher mg value than the N^+ treatment, While R2, R7 and R9 also showed increased the mg content on addition of PGRB compared to the others in their group obtained are shown in fig 5.

3.7 Correlation between Macronutrients: There was significant correlation between Na, P and N at P 0.05, K and Na at P 0.01, Mg and Can at P 0.05, Can and Na at $P \leq 0.01$, Mg and Na at $P \leq 0.01$(Table 1).

IV. DISCUSSION

This research was carried out to study the effect of Plant growth promoting bacteria isolated from the nodules of cowpea can increase the absorption of some macro nutrients including Nitrogen, Phosphorous, Magnesium Calcium, Potassium and Sodium. Total Nitrogen for R1, R2, R3, R4, R5 on addition of PGPBs was higher than those that were inoculated with only the *rhizobia*, and all other treatments had a higher N value than that of the N^+ treatment giving similar results to the work of Eutropia *et al*., (2013) (where there was significant uptake of nutrient was observed in experiments using soybean), the work of Ahmad *et al*., (2008) and Young *et al*., (2001). All rhizobial had a higher P value on addition of PGPRs except R9 and R8. About 60% of the treatments had a higher P value than N^+ this was similar to the result obtained by Eutropia *et al*., (2014) where significant uptake of P was observed.

All inoculated plants had a lower percentage shoot K than the N^+ this could be as result of KNO_3 was applied as a source of nitrogen for the N^+ treatment thus a higher amount of Potassium was available to the N^+ plants. Absorption of Na showed no particular pattern and all inoculated plants had a lower percentage shoot Na value than the N^+ control treatment. This suggests that these group of PGRBs including *Agrobacterium spp., Paenibacillus wynnii, Pseudomonas aeroginosa* and *Azotobacter spp*. did not have any effect on the uptake of sodium but that other factors or PGRBs may be responsible for its uptake a similar report was given by Mehrpouyah *et al*., (2012) and Eutropia *et al*., (2013). All treatments had a higher percentage shoot Ca value compared to the N+ control treatment. Consortium i.e R6, R7, R8, R9 to which *Azotobacter spp.* were consistently able to improve the uptake of calcium when compared with their counterparts to which only rhizobia was added as was also reported in work of Makoi *et al*., (2013) and Yahya-Abadi; (2008).For magnesium, all treatments had a higher percentage of magnesium than the N^+ treatment except R5, R7, R8 co-inoculated with *Azotobacter spp*. while consortium B increased the uptake of magnesium consistently. Rhizobia strain 5 showed ability to work with all the PGRBs used by increasing the uptake of all the nutrients after it was co-inoculated with them. Sodium showed significant correlation with all the Macronutrients having negative correlation with N, Ca, and Mg but positive correlation with P and K.

V. CONCLUSION

Consortium A (*Agrobacterium spp*, *Azotobactar sp.*, and *Paenibacillus wynnii*) when co-inoculated along-side rhizobia

strains were able to increase the uptake of nitrogen and phophorous, while Consortium B (*Paenibacillus wynnii* and *Pseudomonas aeroginosa*) increased the uptake of nitrogen, magnesium and calcium and Conortium C (Azotobacter spp.) increased the uptake of calcium. Plant growth promoting bacteria therefore play significant roles in enhancing nutrient uptake in plants although their mechanism of action is yet to be understood.

FIGURES

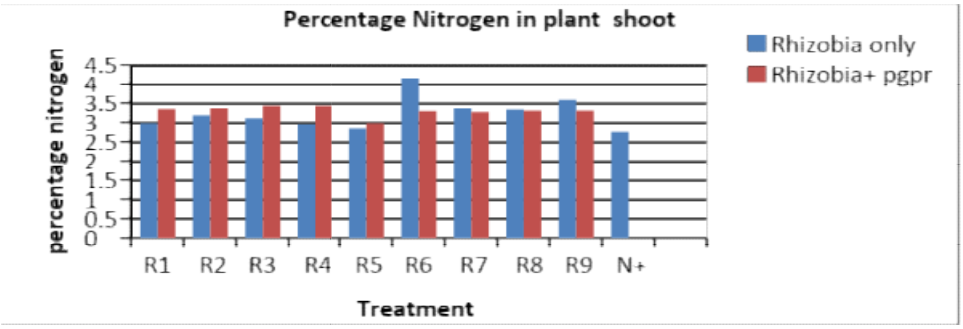

Fig1: Percentage Nitrogen in the dried shoot of plant

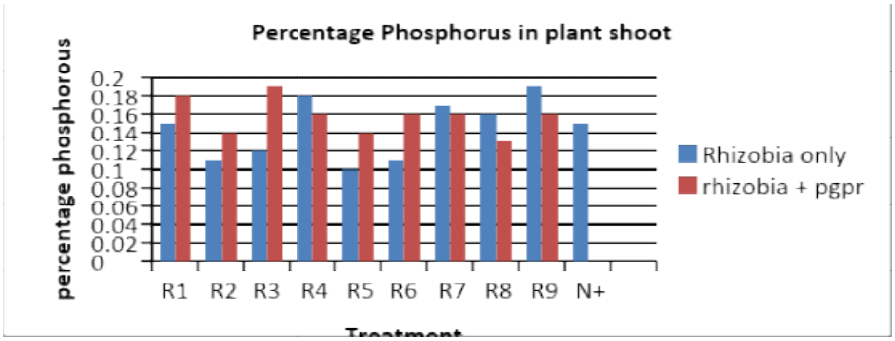

Fig 2: Percentage Phosphorus in the dried shoot of plant.

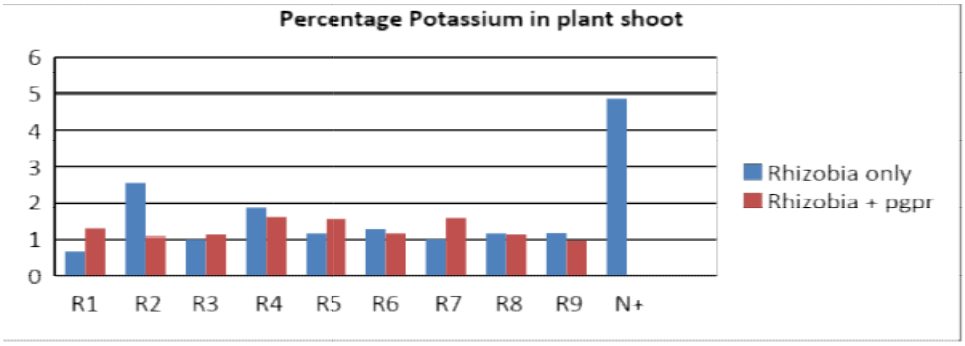

Fig 3: Percentage Potassium in the dried shoot of plant.

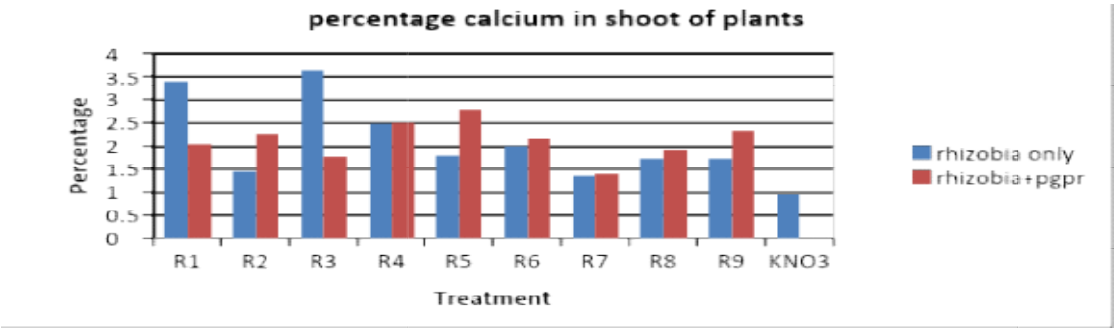

Fig 4: Percentage Calcium in the dried shoot of plant

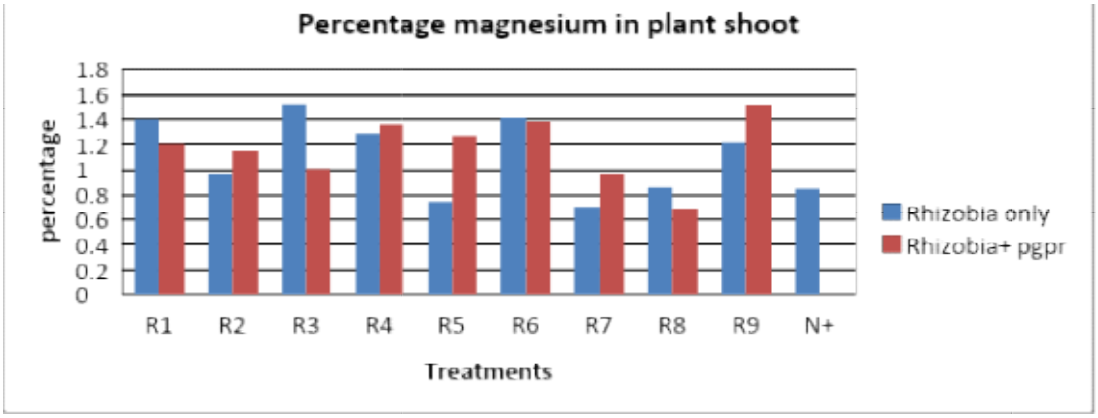

Fig 5: Percentage Magnesium in the dried shoot of plant

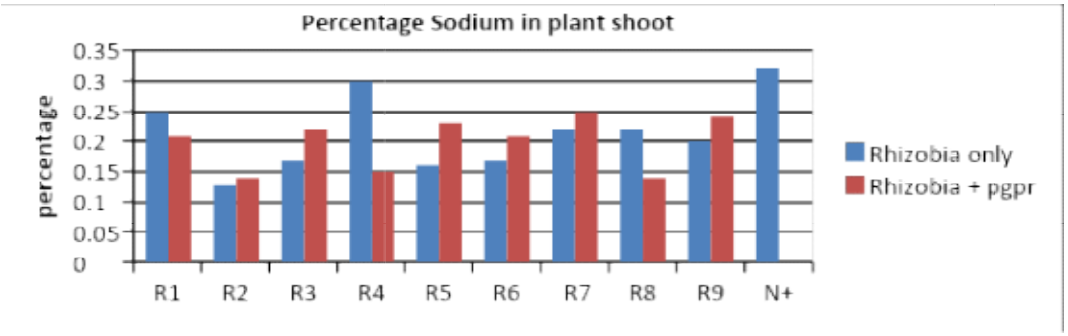

Fig 6: Percentage Sodium in the dried shoot of plant

Table 1: Correlation between percentage macronutrient in shoot of experimental plants

	N	P	K	Ca	Mg	Na
N	1	.496**	-.111	.055	.146	-.339**
P	.496**	1	.079	-.216	-.101	.429**
K	-.111	.079	1	-.060	.063	.334**
Ca	.055	-.216	-.060	1	.788**	-.267*
Mg	.146	-.101	.063	.788**	1	-.253*
Na	-.339**	.429**	.334**	-.267*	-.253*	1

**. Correlation is significant at $P \leq 0.01$ level (2-tailed).

*. Correlation is significant at $P \leq 0.05$ level (2-tailed).

ACKNOWLEDGEMENT

This work was supported by the Soil Microbiology Unit, International Institute of Tropical Agriculture Ibadan, Oyo State Nigeria.

REFERENCE

[1] Alvaro Peix., Martha .H., Ramirez- Bahena., Encarna Velazquez., Eulogio.J. Bemor. (2015). Bacteria Associations with Legumes. *Critical reviews in plant science.* 34: 17- 42.

[2] Eutropia V. T and Patrick A. N. 2013. Bradhyrhizobium japonicum inoculation and phosphorous supplementation on growth and chlorophyll Accumulation in soybean (*Glycine max L.*). *American Journal of Plant Sciences*. 4: 2281- 2289.

[3] Frans.J.M., and Maathius (2014). Sodium in plants.: Perception, Signalling and regulation of sodium refluxes. Journal of experimental botany. 65:849-858.

[4] Gangasuresh P., Muthuselvi V., Muthulakshmi E., Muthumari S. and Maniammal G., (2010) "Synergistic Efficiency of Phosphate Solubilizer Associated with Nitrogen Fixer on the Growth of Soybean (*Glycine max*),". *International Journal of Biological Technology*, 1: 124-130.

[5] Harrigan , W.F and McCance, M.E. (1966). Laboratory Methods in Microbiology . department of Bacteriology, the west of Scotland. Agricultural college Auchicurive,

[6] Hesse P.R. (1971). A textbook of the soil chemistry analysis. John Murray ltd. London. Pp120-307.

[7] Kamara A. Y., Kwari J. , Ekeleme F. , Omoigui L. and Abaidoo R.(2010). "Effect of Phosphorus Application and Soybean Cultivar on Grain and Dry Matter Yield of Subsequent Maize in the Tropical Savannas of North-Eastern Nigeria,"*African Journal of Biotechnology*, 7: 2593-2599.

[8] Kalavati P and Modi H.A. (2012). Importance of potassium in growth – A Review. *Indian Journal of Plant Science*. 1: 2319-3824.

[9] Kjeldahl, J. (1883). New method for the determination of nitrogen in organic substances, *Zeitschrift fur analytische chemie. 22*: 366-383.

[10] Makoi J.H.J.R, Bambara S., Ndakidemi P.A (2013). " Rhizobium Inoculation and the supply of Molybdenum and lime Affects the uptake of macronutrient in common bean(P.vulgaris L.) plants".AAustralian Journal of crop science 7: 784-793.

[11] Murphy J. and Rilley J. P.(1962). A modified single solution method for the determination of phosphate in natural water. Analytical chemistry Acta. 27:31-36.

[12] Olutiola, P.O., Famurewa,O, Sonntag, H.G. (2000). An introduction to general microbiology (A Practical approach), reprinted. Nigeria: Bolabay Press Ltd. 157-175.

[13] Onyango B.O, Koech P.K, Anyango B, Nyunja R.A, Skilton R.A, Stomeo F.(2015) Morphological, genetic and symbiotic characterization of root nodule bacteria isolated from Bambara groundnuts (Vigna subterranea L. Verdc) from soils of Lake Victoria basin, western Kenya. *J App Biol Biotech*. 3 (01): 001-010.

[14] Pandya M., Naresh Kumar, G., and Rajkumar, S. 2013. Invasion of rhizobial in-fection thread by non rhizobia for colonization of Vigna radiate root nodules.FEMS Microbiol. Lett.348: 58–65.

[15] Peoples, M.B., Herridge, D.F., Ladha, J .K. (1995). Biological nitrogen fixation: Investments. Expectations and actual contribution to agriculture. *Plant and Soil* 174: 3-28.

[16] Philip J. White and Martin R. Broadly (2003). Calcium in Plants. *Annals of Botany* 92:487-511.

[17] Subbaran G.V, Ito.O., Berry W.L., Wheeler R. M., (1941). Sodium –A functional plant nutrient. *Critical reviews in plant science*. 52: 237-244.

[18] Samavat S., Samavat S. , Mafakheri S. and Shakouri M. J. (2012) "Promoting Common Bean Growth and Nitrogen Fixation by the Co-Inoculation of *Rhizobium* and *Pseudomonas fluorescens* Isolates," *Bulgarian Journal of Agricultural Sciences*,18: 387-395.

[19] Shahid M. Q. , Saleem M. F., Khan H.Z. and Anjum S. A.(2009) "Performance of Soybean (Glycine maxL.) under Different Phosphorus Levels and Inoculation," *Pakistan Journal of Agricultural Sciences*, 46: 237-241.

[20] Somasegaram P and Hoben H.J.(1985). Methods in legume-rhizobium technology NifTAL project and MIRCEN. University of Hawaii Maui.

[21] Uchida R. (2000). "Essential Nutrients for Plant Growth: Nutrient Functions and Deficiency Symptoms. Plant Nutrient Management in Hawaii's Soils," College of Tropical Agriculture and Human Resources, University of Hawaii Manoa, 31-55.

[22] Woomer, P. L., Karanja. N., Stanley M. (2011). A revised manual for rhizobium methods and standard protocols (www.n2africa.org).

[23] Yahya-Abadi M.(2008). Evaluation of Nitrogen fixation potential and nutrients uptaking in some common bean sysmbiosis bacteria. *Preccedings of 10th Iranian congress of crop production and plant breeding 19-25 august karaj, Iran.*75

[24] Young, J. M., Kuykendall, L.D., Martinez-Romero, E., Kerr, A. and Sawada, H. (2001). A revision of rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all spiecies of Agrobacterium Conn 1942 and Allorhizobium undicola de Lajudie *et al*., 1998 as new combinations: *Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola and R. vitis*. *International Journal of systemic and evolutionary Microbiology* 51: 89-103.