Effect of co-inoculation of Non- Rhizobial Microorganisms (NRMs) with rhizobia on Nodulation and Leaf chlorophyll content on cowpea *Vigna ungigulata* [(L.) Walp.] (Tvx3236)

AJAYI . Olaoluwa .O.[1,2*], DIANDA. Mohammed.[2], FAGADE Obasola E.[1], NWADIKE Blessing[1]

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

Abstract: **- Screen house experiment was carried out to study the effect of other microorganisms besides rhizobial living in the root nodules of cowpea plants, (Non-Rhizobial Microorganisms (NRMs). The NRMs used include *Agrobacterium* spp, *Pseudomonas* sp. *Paenibacillus* sp and *Azotobacter spp*. Treatments included 9 strains of rhizobial applied singly and also co-inoculated with NRMs applied to cowpea (Tvx 3236) in four replicates using block design to observe the effects of NRMs on leaf chlorophyll content and also on the nodulation of cowpea. It was observed that nodulation was significantly affected by the presence of a consortium of *Agrobacterium* spp, *Pseudomonas* sp. and *Paenibacillus* sp but it was not affected by the presence of *Azotobacter sp*. On the other hand chlorophyll was significantly affected by the presence of a consortium of *Azotobacter spp*. and a consortium of *Agrobacterium* spp, *Pseudomonas* sp. while *Paenibacillus* sp did not show significant effect on the leaf chlorophyll.**

Key words: **Nodulation, Chlorophyll, Rhizobia, Non-Rhizobial Microorganisms (NRMs).**

I. INTRODUCTION

Nitrogen (N_2) constitutes about 78 % of the atmospheric gas; it 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. Legumes with the help of nitrogen fixing bacteria especially members of the family Rhizobiacea (1, 2), fix nitrogen within their nodules in friendly symbiotic relationships termed Biological Nitrogen Fixation (BNF) (3, 4).

Nodules are the site of nitrogen fixation and their colour is used to classify their effectiveness. Nodules harboring efficient rhizobia are usually large and they contain leghemoglobin and are colored pink, brown or red and a green and a black coloration (may be observed when they are effective but have begun to degenerate). Nodules formed by inefficient rhizobia are small and white (2). Several authors have reported the presence of other bacteria in the nodules of legumes such as *Agrobacterium*, (5, 6, 7), *Burkholdera* and *Cupriavidus* (8), *Azotobacter* (7) C*lostridium* spp and *Azospirillum* sp (9) *Paenibacillus pabuli*, *P. amyliticus* and *Methylobacterium mesophilium* (10) which we have called Non- Rhizobial Microorganisms (NRMs). Numerous studies have indicated that co-inoculation of *Bradyrhizobium* and certain Plant Growth Promoting Rhizobacteria (PGPR), can positively affect symbiotic nitrogen fixation by enhancing both root nodule number or mass (11, 12) and increasing nitrogenase activity (13, 14).

Chlorophyll is a major requirement for photosynthesis and biological nitrogen fixation which are two key metabolic processes for legume growth, development and yield. It has been reported that there is a close relationship between nitrogen supply, net leaf photosynthesis and measurement of chlorophyll content in leaf thus the supply of Nitrogen through nitrogen fixation can be a rate limiting process for photosynthesis as well as yield formation (15). The determination of leaf chlorophyll concentration with hand-held chlorophyll meters has been widely used in recent years (16), as it is a rapid and non-destructive technique and can be easily applied in field experiments. This is because of the close relationship between chlorophyll content and nitrogen concentration which can be interpreted as reflecting the plant's nutritional status. The chlorophyll values obtained are highly correlated and can both be used for measuring chlorophyll content (17).

Cowpea is an annual herbaceous plant (18, 19) and about 7.56 million tonnes of cowpea are produced annually on about 12.75 million hectares of land (20). More than 5.4 million tons of dried cowpeas are produced worldwide, with Africa producing nearly 5.2 million. Nigeria, accounts for 61 % of production in Africa and 58 % worldwide. This research was carried out to study the effect of co-innoculation of rhizobial with NRMs on nodulation and chlorophyll in cowpea.

II. MATERIAL AND METHODS

2.1 Sample site: Nodule 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].

2.2 Sample collection: A hand trowel was used to mark a circle with a radius of approximately 15 cm around the plant. This section was cut out to a depth of about 20 cm. A spade was then used to slowly lift out the clump. A sieve of 0.5 mm size and mesh was placed under the root sample to collect nodules that may become detached from the root of the plant (21). The samples were then wrapped in Aluminum foil paper and transported by road to Ibadan.

2.3 Sample preparation: The roots were carefully washed under a gentle stream of water from a tap in a bowl (22, 23). The nodule samples were transported to the laboratory and stored in at 8°C in a fridge until they were to be used. Microorganisms were isolated from nodules on Congo red agar (24, 25, 26) using spread plate method.

2.4 Isolation of microorganisms from root nodules and their characteristics: Five (undamaged) nodules samples were picked randomly from each site. They were placed 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. The nodules were rinsed with sterile water after which they were further sterilized with 95 % ethanol for 30 seconds and rinsed with six changes of sterile water. The nodules were then 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° C for 5 - 7 days (23). After the incubation period identified isolates were selected, Rhizobial isolates were differentiated by their ability to absorb congo red dye (white colouration) media (23). Isolates were labelled, purified and stored on yeast mannitol agar slants. The pure isolates were inoculated on Yeast Mannitol Agar slants (26), slants were placed in an incubator and allowed to grow for 7 days before they were stored in the fridge to restrain growth of the rhizobium. Others suspected not to be rhizobial were stored on nutrient agar slants. The isolates were identified using biochemical test (27, 28).

2.5 Determination of Chlorophyll: Leaf chlorophyll content was determined using a chlorophyll meter from the fourth to the seventh week (16). The measurments were recorded in spad units. This was done by placing he leaf in-between the teeth of the meter which has a sensor the measures the chlorophyll based on the colour and transpiration rate.

2.6 Experimental design/ Set-up: The Complete Randomized Block Design (CRBD) was used in carrying out this research experiment. 20 treatments were used each having four replicates. Peat mixed (evenly) with sea sand and crushed gravel in a ratio 6:6:1 was sterilized at 121° C and 1.05 kg cm^{-2} for 15 mins. This mixture used in place of soil was filled into 500 ml Pots (sterilized using 3 % w/v sodium hypochlorite) after which it was thoroughly rinsed six times with sterile water to ensure complete removal of all traces of sterilizing agent). Four cowpea seeds were planted in each pot; kept in a screen house and allowed to germinate.

Cowpea Tvx 3236 which is a fast maturing variety was used for this experiment. Pure cultures of the rhizobia isolates were introduced into 100 ml Erlenmeyer flasks containing 50 ml of yeast-mannitol broth, also a second set of pure rhizobia cultures co–inoculated with Non- rhizobial microorganism were applied as treatments see table 2. Inoculated broth solutions were incubated at room temperature (25-30° C) on a Rotary shaker for 7 days. One week after planting (WAP), the cowpea plants were thinned leaving one viable plant per pot. Inoculum (of 1ml containing about 1 x 10^9 cells ml^{-1}), was introduced into the cowpea plants using sterile pipette. Control treatments were also used the KNO_3 (N^+) and those planted in unsterilized normal soil (NST) to which no treatment was applied. Plants were allowed to grow for 8 weeks during which two replicates were given nutrient solution weekly. The plants were watered daily using sterile distilled water. The nutrient solution was made up of micro and macro nutrient (21, 29). Leaf chlorophyll content was measured from the fourth to the seventh week (16).

2.7 Harvesting and preparation of sample: Cowpea plants were harvested 8 weeks after planting (WAP) using a secateur to cut the stem close to the base. The shoots and root were collected and wet weight was taken. The nodules were detached from root and placed in labelled envelops. The shoot and roots were dried in an oven at 68 °C for 3 days and dry weight was taken. The nodules were allowed to dry naturally and then placed in labelled bottles containing dehydrated silica gel and stored away (22).

Table 1: Peat properties

Properties	Units	Values
Water holding capacity		33.38
Moisture content	%	4.3
pH	-	6.14
Organic Carbon	%	88.8

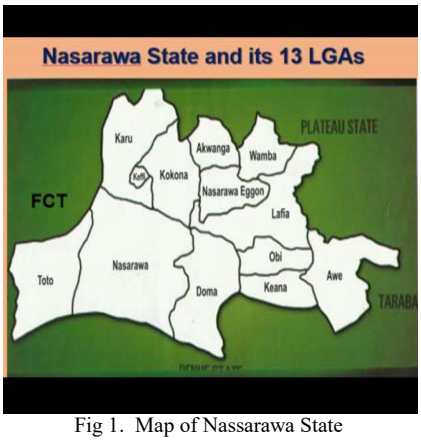

Fig 1. Map of Nassarawa State

III. RESULTS

3.1 Isolates obtained: A total of nine rhizobia isolates were isolated from the samples, three rhizobia isolates were obtained from nodules obtained from Shamage local government, four from Ogba/ Ubbe Egon local government and two from Mandara Kokona (Table 2). The other microorganisms (NRMs) isolated included *Azotobacter* spp, , *Panibacillus* sp, *Agrobacterium* spp, *Pseudomonas* sp .

3.2 Effect of co-inoculation of rhizobial with NRMs on nodules: The nodule mass of the treatments with NRMs were higher than their counterpart with only rhizobia except for *Rhizobial sp 8* which was lower, and *Rhizobial sp 1* and *Rhizobial sp 5* which had almost similar value with each other. Those with the N^+ treatment had no nodules. Nodule color were mostly pinkish indicating that they were actively involved in fixing nitrogen. They also had higher nodule numbers than those from NST except *Rhizobial sp* 3, *Rhizobial sp* 3+ consortium A, *Rhizobial sp* 2 + consortium A, *Rhizobial sp 9* although it was observed that their nodules were large and had a higher average mass per nodule compared to those from the NST experimental plants which were small. The percentage increase in total nodule mass ranged from 69.39 % to 44.26 % while the percentage increases for the mass per nodule ranged from 10.31 % to 329.59 % (Table 2)

3.3 Effect of co-inoculation of rhizobial with NRMs on chlorophyll: The chlorophyll value for all the treatments were higher than that of the N^+ treatment, while some were higher than that of those in potted plants. *Rhizobial sp 1*, *Rhizobial sp 4*, and *Rhizobial sp 5* had higher chlorophyll reading than their counter parts containing NRMs (non rhizobial microorganisms). It was observed that the chlorophyll content increased steadily from 4 WAP to 7 WAP indicating that over time more nitrogen was being fixed in plant nodules.

IV. DISCUSSION

The nodule mass of all experimental plants which were subjected to treatments was higher than those of the plants in potted non sterile soil. This indicated that the rhizobia strains were more effective in fixing nitrogen than the ones in the unsterilized soil. Their mostly pink colour indicated they were actively involved in fixing nitrogen. The treatments with NRMs had higher nodule mass and average mass per nodule than their counterpart with only rhizobia except for treatment with consortium c (*Azotobacter* spp.). This correlates with the work of Polonenko *et al*., 1987(11) and Yahalom *et al*., 1987(12) which concluded that co-inoculation of Bradyrhizobium and certain PGPR, can positively affect symbiotic nitrogen fixation by enhancing nodule number, nodule mass and increasing nitrogenase activity.

The chlorophyll reading for all the treatment were higher than that of the N^+ treatment, while some were higher than that of those in potted plants. *Rhizobial sp* 1, *Rhizobial sp 4*, and *Rhizobial sp 5* had higher chlorophyll reading than their counter parts containing NRMs (non rhizobial microorganisms), this showed that the consortium of *Paenibacillus* and *Azotobacter sp* did not significantly improve the chlorophyll content but were able to affect the nodulation of the plant, while rhizobial strains 6, 7 8 and 9 when co-inoculated with *Azotobacter* showed higher chlorophyll this showed that *Azotobacter* spp. contributed significantly to the increase in chlorophyll content although they did not improve the nodulation of the plant. Nitrogen was being fixed by rhizobia sp. in plant nodules as was observed by the chlorophyll content which increased steadily from week four to week seven. This is similar to the findings of Eutropia *et al*., 2013 (30) where rhizobial inoculation significantly increased leaf chlorophyll content and the work of Zerpa *et al*., 2013 (31) and Lambers *et al*., 2006 (32). The consortium of *Agrobacterium* spp, *Pseudomonas* sp. and *Paenibacillus* sp. were able to significantly affect both the chlorophyll nodulation and nodule mass.

V. CONCLUSION

Application of consortium C which contained only *Azotobacter* spp. alongside rhizobia did not enhance total nodule mass or the average mass per nodule of experimental plants although chlorophyll values were significantly affected by their presence. Application of consortium A (*Agrobacterium* spp, *Pseudomonas* sp. and *Paenibacillus* sp) and B (*Paenibacillus sp* and *Azotobacter sp*) on the other hand contributed significantly to the increase in nodule mass and average mass per nodule of experimental plants and chlorophyll values were also significantly affected showing they positively contributed to nitrogen fixation. They should therefore be considered for use along-side rhizobia in commercial inoculant.

Table 2: Average total nodule mass, average nodule number and the percentage increase

Treatment	Ave. total Nodule mass (mg)	% Increase in total nodule mass	Ave. mass per nodule (mg)	% Increase in mass per nodule	Ave Nodule Number	Site source
Rhizobial sp 1	102.25 ± 8.70	122.95	4.88	118.83	21 ± 9.06	Shamage
Rhizobial sp + (consortium A)	249.00 ± 33.20	444.26	9.58	329.56	26 ± 11.39	shamage
Rhizobial sp 2	78.50 ± 21.50	71.585	4.08	82.96	19 ± 14.41	shamage
Rhizobial sp 2.+ (consortium A)	94.75 ± 14.95	107.10	7.9	254.26	12 ± 4.24	shamage
Rhizobial sp 3	95.25 ± 16.79	108.19	4.65	108.52	21 ± 6.14	shamage
Rhizobial sp 3+(consortium A)	103.00 ± 19.90	125.14	9.2	312.56	11 ± 2.36	shamage
Rhizobial sp 4	82.5 ± 6.80	80.33	3.15	43.49	26 ± 9.07	mandara
Rhizobial sp 4+ Consortium B	93.25 ± 16.20	103.83	3.11	38.12	30 ± 3.20	mandara
Rhizobial sp 5	106.00 ± 26.50	131.69	4.08	82.96	26 ± 15.73	mandara
Rhizobial sp 5 + Consortium B	100.30 ± 18.90	119.23	4.36	10.31	23 ± 1.57	mandara
Rhizobial sp 6	82.25 ± 14.50	79.78	2.46	95.52	34 ±9.47	ogba
Rhizobial sp 6 + Consortium C	93.25 ± 12.00	103.83	3.45	54.708	27 ± 13.22	ogba
Rhizobial sp 7	75.75 ± 24.74	65.57	2.46	10.31	31 ± 28.46	ogba
Rhizobial sp7 + Consortium C	83.50 ± 8.50	82.51	3.09	38.57	27 ± 4.51	ogba
Rhizobial sp 8	101.50 ± 21.67	121.86	5.41	142.60	19 ± 9.07	ogba
Rhizobial sp 8 + Consortium C	77.50 ± 6.40	69.39	2.74	22.87	28 ± 11.68	ogba
Rhizobial sp 9	112.70 ± 18.00	146.64	5.73	156.95	20 ± 12.97	ogba
Rhizobial sp 9, + Consortium C	89.50 ± 19.60	95.63	3.38	115	27 ± 4.58	ogba
Potassium Nitrate	-	-	-	-	-	N+
Non sterile soil (NST)	45.75 ± 3.80		2.23		21 ± 3.11	CONTROL

KEY - N+ : Nitrogen treatment, Wap: week after planting,

Consortium A: *Agrobacterium* spp, *Pseudomonas* sp , *Paenibacillus* sp.

Consortium B: *Paenibacillus* sp, *Azotobacter* sp

Consortium C: *Azotobacter* spp.

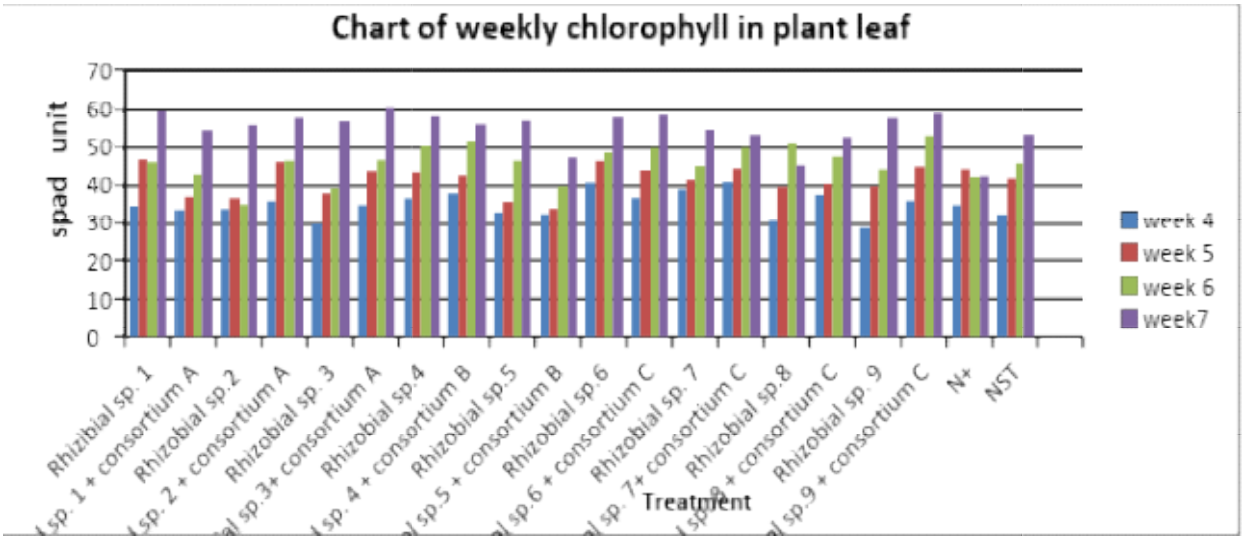

Fig 2. Chart showing weekly average chlorophyll readings of plants.

ACKNOWLEDGEMENT

This study was supported by the International Institute of Tropical Agriculture IITA. (Presented at the Isteam Conference Ghana, 2018).

REFERENCES

[1] 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.

[2] Valverde, Angel, Igual, Jose M., Peix, Alvaro, Cervantes, Emilio, Velazquez, Encarna. (2006) *Rhizobium lusitanum* sp. nov. a bacterium that nodulates *Phaseolus vulgaris*. *Int J Syst Evol Microbiol* 2006 56: 2631-2637.

[3] Angelini, J., Castro, S., Fabra, A. (2003). Alterations in root colonization and nodC gene induction and inpeanut-rhizobia interaction under acidic conditions. *Plant Physiol. Biochemistry* 41: 289-294.

[4] Hardarson, G. (1993). Methods for enhancing symbiotic nitrogen fixation. *Plant and Soil* 152: 1-17.

[5] Sprent J.I (2001) nodulation in legumes. Royal botanica gardens kew uk.

[6] Murugesan,S. Manoharan, C. Vijayakumar R. and Panneerselva A.(2010) Isolation and Characterization of Agrobacterium rhizogenes from the Root Nodules of Some Leguminous Plants. *International Journal of Microbiological Research* 1: 92-96.

[7] Young , J.M., Kuykendall, L.D., Martinez- Romero, E., Kerr, A., Sawada, H.(2001). A revision of Rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all species 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 Evolutionary Microbiology* 51: 89-103

[8] Moulin, L., Munive, A., Dreyfus, B., Biovin-Masson, C. (2001).Nodulation of legumes by members of the bold beta-subclass of Proteobacteria. *Nature.* 411: 948-950

[9] Li, C.Y., Hung, L.L., (1987) Nitrogen fixing (acetylene reducing) bacteria associated with ectomychorizae of Douglas-fir. *Plant and Soil*; 98: 4254-428

[10] Paul, L. P., Vanconver, B,C. Nitrogen fixation associated with tuberculate ectomychorriza on lodgepole pine (Pinus contorta). Ph.D thesis. University of British Colombia, Canada.

[11] Polonenko, D.R., Scher, F.M., Kloepper, J.W., Singleton, C.A., Laliberte, M., Zaleska, I. (1987). Effect of root colonizing bacterium on nodulation of soybean roots of Bradhyrhizobium japonicum. *Canadian Journal of Microbiology* 33: 478-503

[12] Yaholom, E., Okon, Y., Dovrat, A.(1987). Azospirillium effects on susceptibility to Rhizobium nodulaton and nitrogen fixation of several forage legumes. *Canadian Journal of Microbiology*. 33: 510-514

[13] Iruthayathas E. E, Gunasekaran S, Vlassak K. 1983. Effect of combined inoculation of Azospirillum and Rhizobium on nodulation and nitrogen fixation of winged bean and soybean. Scientia Horticulturae 20 : 231- 240

[14] lagawadi AR, Gaur AC. 1988. Associative effect of Rhizobium and phosphate-solubilising bacteria on the yield and nutrient uptake of chickpea. Plant and Soil 105 : 241-246

[15] Sinclair T.R. (2004): Improved carbon and nitrogen assimilation for increased yield. In: Boerma H.R. and Specht J.E. (eds.) Soybeans: Improvement, production, and uses. Third edition, Series Agronomy, No 16, American Society of Agronomy, Madison, WI. 537-568.

[16] Uddling J., Gelang-Alfredsson J., Piiki K., Pleijel H. (2007). Evaluating the relationship between leaf chlorophyll concentration and SPAD-502chlorophyll meter readings. *Photosynth. Res*. 91: 37-46

[17] Vollmann J., Walter H., Sato, T., Schweiger, P. (2011): Digital image analysis and chlorophyll metering for phenotyping the effects of nodulation in soybean. *Computers and Electronics in Agriculture* 75: 190-195.

[18] Awonaike K. O, Kumarasinghe K.S and Danso S. K. A. 1990. Nitrogen fixation and yield of Cowpea (Vigna unguiculata) as influenced by cultivar and Bradyrhizobium strain. *Field Crops Research*. **24**: 163- 171.

[19] Peksen A, Peksen E and Bozoglu H. 2004. Relationship among some seed traits, laboratory germination and field emergence in cowpea [*V. unguiculata* (L) Walp] genotypes. *Pakistan Journal of Botany*. **36** : 311 –320.

[20] IITA. 2007. Cowpea. IITA annual report. http://www.iita.org/cms/details/cowpea_ project_details. aspx? Zoneid =63&articleid=269

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

[22] 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.

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

[24] Bryan, C. S. (1938). Identification of Phytomonas, Azotobacter and Rhizobium or Achromobacter upon initial isolation. *Soil Science* 45:185-187. Cited by Barbara and Thomas (1983)

[25] Dye, M. (1980). Functions and maintenance of a Rhizobium collection,. In N. S. Subba Rao (ed.), Recent advances in biological nitrogen fixation. Holmes and Meier Publishers, Inc., New York. 435. Cited by Barbara and Thomas (1983).

[26] Vincent, J.M. (1970). A Manual for the Practical Study of Root Nodule Bacteria, Blackwell Scientific, Oxford. Cited by Barbara and Thomas (1983)

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

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

[29] Somers E, Vanderleyden J, Srinivasan M. 2004. Rhizosphere bacterial signalling: a love parade beneath our feet.Crit. Rev. Microbiol.30:205–235

[30] 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.

[31] Zerpa, m., Mayz, J., Mendez, J. (2013). Effects of Bradhyrhizobium japonicum Innoculant on Soybean (*Glycine max* (L)) Growth and Nodulation. *Annals of Biological Research*. 4: 193-199

[32] Lambers H. , Shane M. W., Cramer M. D., Pearse S. J., Veneklaas E. J. (2006). "Root Structure and Functioning for Efficient Acquisition of Phosphorus: Matching Morphological and Physiological Traits," Annual Botanical, Vol. 98: 693-713.