Nitrogen Fixing Bacterial Interaction in Groundnut (Arachis hypogea L.) Growth and Yield Under Nursery Experiment

Karuppusamy. S[1], Franklin C. Jose[2]
[1,2]*Department of Botany, Government Arts College, Udhagamandalam – 643 002, Tamil Nadu, India*

Abstract **- Groundnut (Arachis hypogea L.) is a highly demandable leguminous crop, which is rich nutritious and regular consumed on various processing ways and it could be enhance the soil fertility in tropical and subtropical regions. However, this crop production was low due to ecological and commercial factors. In ecological aspects, several alternatives were suggested by the researchers to improve the groundnut productions, among the alternative methods bioinoculants are suitable to ecological and social feasible. In the present study, two diazotrophic bacteria of Rhizobium and Azospirillum were treated with groundnut plants under nursery condition on individual and combined applications. The treated plants were harvested in different time intervals like 30, 60 and 90 days after inoculations. The harvested plants morphometric, nodule number and dry weights, leaf area and chlorophyll contents, soil and plant tissue nitrogen content, leghaemoglobin content and formation of nuts numbers were recorded. The present study results were suggested to possibility by better chances of survival and yield in the field conditions.**

Index Terms **- Groundnut; Arachis hypogea; Bioinoculants; Diazotrophic bacteria; Rhizobium; Azospirillum.**

1.INTRODUCTION

The groundnut is a major oil leguminous crop in India, it was occupies an area of nearly 9 million ha and with an annual production of 7.5 million tons of nuts and shells. However, the productivity in India is very low (847 Kg ha^{-1}), which is much less than the world average (1148 Kg ha^{-1}) (Bhownik, 1996). While, the groundnut plays a very vital role in the human food chain, providing high protein grain and ecosystem particularly increasing soil fertility as well as its one of the crops that alternate with rice in the crop rotation system in subtropical and tropical regions.

Soil microbial activity is considered to be a main parameter in ecosystem functioning. The diazotrophic bacteria such as the associative and symbiotic are beneficial microorganisms in the root zone of leguminous plants, being reported as very essential for plant establishment and growth, especially under nutrient unbalanced conditions. Seeds or soil inoculation of microbes is a common practice for enhancing the growth and development of some agricultural crops (Champawad, 1990) and can be very advantageous in sustainable agriculture. The effect of any microbial inoculation is the result of interactions between the plants and the rhizosphere in habitants. It was well documented that combined diazotrophs results in better performance (SubbaRao, 1985; Paula *et al*., 1992; Biro *et al*., 1993; Garbaye, 1994). Several similarly studies are documented on the interactions of bioinoculants in the rhizosphere of leguminous plants. However, the associative and symbiotic diazotrophic bacterial interaction inoculation studies in the *A. hypogea* plants under a particular suit of environmental conditions are lacking. The present is focused on the analysis of nitrogen fixing bacterial interaction in *A. hypogea* under nursery experiment.

2. MATERIALS AND METHODS

2.1. Maintenance of the Experimental Plants

Experiments were carried out in the private nursery near Maruthamalai Hills, Coimbatore, Tamil Nadu, India. Groundnut plants inoculated with N_2 fixing bacteria of *Rhizobium* and *Azospirillum* individually and combined and the control (uninoculated) plants were raised in polybags and arranged in a complete randomized block design. Plants were watered as and when necessary throughout the duration of the experiments. The positions of the polybags were

altered once in every 15 days to expose seedlings to uniform conditions.

2.2. Substrate and Showing

Arachis hypogea L. (Co-2) seeds were obtained from Seed Bank, Tamil Nadu Agricultural University, Coimbatore. The seeds were directly sown in polythene bags (23 x 13 cm size) after N_2 fixers inoculations according To treatments, each polybags filled with *ca* 3 kg of sand: red soil: cow dung (1:2:1). The soil had a pH of 7.8 and electric conductivity of 42.35 mScm-1, 0.14 mg Kg^{-1}total nitrogen, 0.016 mg Kg^{-1} of total phosphorus, 0.12 mg Kg^{-1} exchangeable potassium and 3.68% organic carbon prior to cow dung amendment. The indigenous *Rhizobium* and *Azospirillum* populations were respectively 2.37 and 2.16 propagules g^{-1} soil.

2.3. *Rhizobium* and *Azospirillum* inoculums

Inocula of *Rhizobium* and *Azospirillum* were obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. Five g of charcoal based Bacterial Inocula (10^9 CFU g^{-1}) was placed as a thin layer about 2 cm below the soil surface in each bag ofspecific treatment before sown seeds.

2.4. Harvest and Measurements

Arachis hypogea plants were harvested at 30, 60 and 90 days after microbial inoculations with their entire root systems. Growth parameters such shoot height, root length, shoot and root dry weight, nodule number and dry weight and leaf number were measured. Populations of *Rhizobium* and *Azospirillum* were estimated by dilution plate method (Wollum, 1982).

2.5. Quantitative Estimation of the Microorganisms

Rhizosphere soil samples were collected and placed in a polyethylene bags sealed, brought to the laboratory, shade dried and stored at 4°C until further analysis. Dilution plate counting method was employed for the enumeration of microbial population in the soil samples. Appropriate dilution viz., 10^{-5} was chosen for *Rhizobium* [Yeast extract agar (SubbaRao, 1986)] and *Azospirillum* [N-free semi solid malate medium (Dobereiner*et al*., 1976)].

2.6. Leaf Area

The leaf area was recorded using the LI-3000 portable leaf area meter (Li-Cov, USA).

2.7. Estimation of Chlorophyll

One gram of fresh leaves were homogenised with glass mortar and pestle in 80% acetone. The homogenate was filtered through a cheese-cloth. The residue was re-extracted with 80% acetone and filtered. The filtrates were poled and centrifuged at 7000g for 10 min. The clear supernatant was made up to 20 ml with 80%acetone and its optical density (OD) was measured at 645 and 663 nm. Total chlorophyll was calculated by using the following formula and the results were expressed in mg g-1 fresh weight.

$$\text{Total chlorophyll (mg g-1)} = \frac{20.2\ A_{645} + 8.02\ A_{663}}{A \times 1000 \times W} XV$$

Where,

A: Distance travelled by light in the cell (1 cm)

V: Volume of the extract in ml

W: Fresh weight of the sample in g

2.8. Analysis of Leghaemoglobin

Fresh nodules weremacerated with double volumes of phosphate buffer and filtered through two layers of cheese-cloth. The nodule debris was discarded. The turbid reddish brown filtrate was centrifuged at 10000 g for 15 min. and diluted suitable. Three ml of the extract was added to an equal volume of alkaline pyridine reagent and mixed thoroughly. The solution turns greenish-yellow due to the formation of ferric hemochrome. The hemochrome was divided equally into two tubes. To one portion few crystals of sodium dithionite was added to reduce the hemochrome, stirred without aeration and read at 556 nm. To the second portion a few crystals of potassium hexacyanoferrate was added to oxidize the hemochrome and read at 539 nm. Leghaemoglobin was calculated by using the formula mentioned below and the results were expressed in mM (Appleby and Bergersen, 1980).

$$\text{Lb concentration (mM)} = \frac{A\ 556 - A\ 539 \times 2D}{23.4}$$

Where, D: The initial dilution.

2.9. Analysis of Soil Nutrient Content

The total soil nitrogen (N) and available phosphorus (P) were determined respectively by micro-Kjeldahl

and molybdenum blue methods of Jackson (1973). Exchangeable K was extracted from the soil in ammonium acetate solution (pH 7) and measured with a digital flame photometer (Jackson, 1973). Soil organic carbon was determined according to Piper (1966).

2.10. Analysis of Plant Nutrient Content

The plant samples were oven dried and ground to a fine powder in Willy Ball mill. These samples were used for the estimation of N,P and K. One hundred mg of plant samples were wet digested in 2 ml of concentrated H_2SO_4 containing catalyst ($CuSeO_3$). The digested samples were made up to 50 ml, and N content in the extract was estimated by micro-Kjeldahl method. Two hundred mg of tried plant samples were wet digested in 10ml of a triple acid mixture (Nitric acid, Sulphuric acid and Perchloric acid in the ratio of 9:2:1). The digested samples were made up to 100 ml for P estimation. Phosphorus was estimated colorimetrically following the vanadomolybdate method (Jackson, 1973). Potassium content in the aliquot of the triple acid extract was estimated by the emission spectrophotometry using EEL flame photometer (Jackson, 1973).

2.11. Statistical Analysis

The data were statistically analysed by Analysis of Variance (ANOVA) and treatment means were separated using Duncan's Multiple Range Test (DMRT).

3. RESULTS

Morphometric Analysis

Arachis hypogea plant shoot height was significantly higher in the *Rhizobium* and *Azospirillum* combined inoculation soils at entire period of studies such as 30, 60 and 90 DAI; however the plat root length was higher on *Azospirillum* inoculated soils at 30 days after inoculation (DAI), at the same time *Rhizobium* and *Azospirillum* combined inoculation soils had showed maximum at 60 and 90 DAI (Table 1). The plant leaf numbers were showed higher in combined inoculation soils at entire study periods, whilethe plant leaves total chlorophyll levels had showed maximum on *Azospirillum* inoculated soils at 30 DAI, but in the study period of 60 and 90 DAI has shown the *Rhizobium* and *Azospirillum* combined inoculation soils (Table 2).

Arachis hypogea root nodules formation was occurred on all soils at 30 DAI and *Rhizobium* inoculated soils had showed maximum numbers at 30 and 60 DAI, however at 90 DAI had the shown maximum numbers, which is compared with control soils it has shown more than 4 folds higher. In the case of plant root nodules dry weight measures, *Rhizobium* inoculated soils was higher at 30 DAI and *Rhizobium* and *Azospirillum* combined inoculation soils was shows higher at 60 and 90 DAI (Table 3).

In the nursery condition, *A. hypogea* plants were grown in polybags and recommended dosage of *Rhizobium* and *Azospirillum* inocula was inoculated on individual as well as combined into one week aged healthy plants grown polybags, the *Rhizobium* and *Azospirillum* combined inoculation soils has shown maximum bacterial population at entire period of studies such as 30, 60 and 90 DAI (Fig.1). The nitrogen fixing diazotrophs were inoculated the plants grown polybags, accordingly chosen to analysis of soil as well as tissue nitrogen only at 90 DAI. *Rhizobium* and *Azospirillum* combined inoculation soils has shown maximum levels of nitrogen accumulation in plant rhizosphere soils and plant tissues (Fig. 2).

The diazotrophic bacteria are fix the nitrogen in leghaemoglobin in leguminous plants. Thus leghaemoglobin analysis is obligatory to confirm the root nodule viability and functional activity in nitrogen fixing progression. In the present has clearly expressed the *Rhizobium* individually inoculated plants nodules shows maximum level of leghaemoglobin content at entire study period, however this maximum level was marginally higher than the *Rhizobium* and *Azospirillum* combined inoculation plant nodules (Fig. 3). In the case nuts produced by ground nut plants, there was no nuts formation at 30 DAI and subsequently the *Rhizobium* individually inoculated plants had showed maximum number of nuts at 60 and 90 DAI (Fig. 4).

4. DISCUSSION

In general plant growth and biomass were significantly increased in combined inoculation treatment. These findings were generally agreed with early studies, where simultaneous inoculation of different microorganisms has been reported to increase plant growth (Wong and Stenberg, 1979; Reddell *et al*., 1988; Muthukumar *et al*., 2001). Plant growth promotion by rhizobacteria is well known and

influence to N_2-fixation in leguminous plants (Russo, 1989; Isopi *et al*., 1994; Osundina, 1998). The *Rhizobium* and *Azospirillum* combined inoculation was increased plant shoot height, root length, leaf numbers on significant levels, since the increased cytokinin activity in the shots in response to diazotrophic population which can promote leaf growth through increased cell division and cell expansion (Bass and Kupier, 1989).

Similar results have been reported for dual inoculated *Trifolium alexandrinum*, *T. subtermeum*, *Medicago sativa* and *Glycine max* (Smith and Daft, 1977; Smith *et al*., 1979; Asimi *et al*., 1980; Petterson *et al*., 1990). However, Petterson *et al*. (1990) indicated that the nodule development in the lower parts of the roots in *Medicago sativa* and *Trifolium alexandrinum* are affected by other microbes due to indirect response of the host plants.In ground nut, the biomass and nutrient contents were significantly increased when bioinoculants were added to unsterilized soil. The increase in nitrogen of *A. hypogea* plants in *Rhizobium* + *Azospirillum* combination indicates that the increased nitrogen in the soil due to additional diazotrophic bacterial activity is taken up by the roots. However, inoculation with asymbiotic dinitrogen fixers like *Azospirillum* may improve plant growth and yield due to the supplementing the growing plants with fixed nitrogen and growth promoting substances (Pacovsky *et al*., 1985; Pacovsky, 1989; Sumner, 1990; Muthukumar *et al*., 2001).

The bacterial population was positively indicated with combined inoculations, most of the evidence indicates that the establishment and function many microorganisms in the rhizosphere, may not only influence plants but also the co-occurring microbial members in the soil community (Kloepper *et al*., 1991; Lynch, 1990). The specialized activities such as production of vitamins, amino acids, hormones etc., may be operating in bacteria-bacteria interaction and other soil microorganisms. In addition to, the present inoculum of *Rhizobium* + *Azospirillum* were able to improve other rhizosphere microorganisms (Azcon-Aguilar and Barea, 1992; Barea *et al*., 1998).

In conclusion, *A. hypogea* plants inoculated with diazotrophic (*Rhizobium* + *Azospirillum*) microorganisms mixture on substantial increase in plant growth, nutrient content and yields, these responses were either marginal or reached up to a several fold when inoculated plants were compared with the uninoculated control plants. Inoculation of *A. hypogea* plants with a mixture of bioinoculants caused higher vigour to plants in the nursery, which raises the possibility by better chances of survival and yield in the field conditions.

REFERENCES

[1] Appleby, C.A. and Bergersen, F.J., 1980. Methods for evaluating biological nitrogen fixation in: Biochemical methods. S.Sadasivam and A.Manickam. (eds.). New Age International (P) Limited, Publishers. New Delhi.

[2] Asimi, S., Gianinazzi-Pearson, V. and Gianinazzi, S., 1980. Influence of increasing soil phosphorus levels on interactions between vesicular–arbuscular mycorrhizae and *Rhizobium* in soybeans. *Can. J.l of Bot*. *58*, pp.220-225.

[3] Azcón-Aguilar, C., and Barea, J.M., Azcón, R. 1992. Interactions between mycorrhizal fungi and other rhizosphere microorganisms, In: *Mycorrhizal functioning; an integrative plant-fungal process.* M.J.Allen (ed.) Chapman and Hall, New York, N.Y.pp 163-198.

[4] Barea, J.M., Andrade, G., Bianciotto, V., Dowling, D., Lohrke, S., Bonfante, P., O'gara, F. and Azcon-Aguilar, C., 1998. Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for biocontrol of soil-borne fungal plant pathogens. *App. Environ. Microbio. 64*, pp.2304-2307.

[5] Bass, R., and Kupier, D.1989. Effect of vesicular-arbuscular mycorrhizal infection and phosphate on *Plantago major* sp. *pleospema*in relation to internal cytokinin concentration.*Physiol. Plant* 76: 211-215.

[6] Bhowmik, T.P. 1996. Collar rot of groundnut in India and its management.Disease scenario in crop plants.Vol. II- cereals, pulses, oilseeds and cash crops.V.P.Agnihstri, Om Prakash, Ram Kishum and A.K.Misra (eds.) International Books and Periodicals supply service. Pitampura. New Delhi.

[7] Biro, B., Voros, I., Koves-pechy, K. and Szegi, J. 1993.Symbionteffect of *Rhizobium* bacteria and AM fungi on *Pisumsativum*in recultivate mine spoils. *Giomicrobiol*.*J*. 11: 275-284.

[8] Champawat, R.S., 1990. Response of chickpea (Cicerarietinum) to Rhizobium and vesicular

arbuscular mycorrhiza dual inoculation. *ActaMicrobiologicaPolonica*, *39*(3/4), pp.163-169.

[9] Dobereiner, J., Marriel, I.E. and Nery, M., 1976.Ecological distribution of *Spirillumlipoferum*Beijerinck. *Can.J. of Microbiol*. *22*: pp.1464-1473.

[10] Garbaye, J., 1994. Helper bacteria: a new dimension to the mycorrhizal symbiosis. *New phytol*. *128*: pp.197-210.

[11] Isopi, R., Lumini, E., Frattegiani, M., Puppi, G., Bosco, M., Favilli, F. and Buresti, E., 1994. Inoculation of *Alnuscordata* with selected microsymbionts: Effects of *Frankia* and *Glomus* spp. on seedling growth and development. *Symbiosis*17: 237-245.

[12] Jackson, N.L. 1973. Soil chemical Analysis.Pretince Hall. New Delhi.

[13] Kloepper, J.W., Schroth, M.N. and Miller, J.D. 1980. Effects of rhizosphere colonization by plant growth promoting rhizobacteria on potato plant development and yield. *Phtopathol.*70: 1078-1082.

[14] Lynch, J.M., 1990. Microbial metabolites. In: *The rhizosphere.*, (Lynch, J.M. (eds.). pp.177-206.

[15] Muthukumar, T., Udaiyan, K. and Rajeshkannan, V., 2001.Response of neem (*Azadirachtaindica A.Juss*) to indigenous arbuscular mycorrhizal fungi, phosphate-solubilizing and asymbiotic nitrogen-fixing bacteria under tropical nursery conditions. *Biology and Fertility of Soils*, *34*, pp.417-426.

[16] Osundina, M.A., 1998. Nodulation and growth of mycorrhizal *Casuarinaequisetifolia*. J.R. and G.Firstin response to flooding.*BiolFert Soils*, *26*, pp.95-99.

[17] Pacovsky, R.S., 1989. Carbohydrate, protein and amino acid status of *Glycine-Glomus-Bradyrhizobium* symbioses. *Physiol Plant*, *75*, pp.346-354.

[18] Pacovsky, R.S., Fuller, G. and Paul, E.A., 1985. Influence of soil on the interactions between endomycorrhizae and *Azospirillum* in sorghum. *Soil Bio.andBiochem*. *17*, pp.525-531.

[19] Patterson, N.A., Chet, I. and Kapulnik, Y., 1990. Effect of mycorrhizal inoculation on nodule initiation, activity and contribution to legume productivity. *Symbiosis.*8: 9-20.

[20] Paula, M.A., Urquiaga, S., Siqueira, J.O. and Döbereiner, J. 1992.Synergistic effects of vesicular-arbuscular mycorrhizal fungi and diazotrophic bacteria on nutrition and growth of sweet potato (*Ipomoea batatas*). *Biol. Fertil. Soils*, 14:61-66.

[21] Piper, C.S.1996.Soil and plant analysis.Hans publications. Bombay.

[22] Reddell, P., Rosbrook, P.A., Bowen, G.D. and Gwaze, D., 1988. Growth responses in *Casuarinacunninghamiana* plantings to inoculation with *Frankia*. *Plant and soil*, *108*, pp.79-86.

[23] Russo, R.O. 1989. Evaluating alder-endophyte (*Alnus acuminate – Frankia – mycorrhizae)* interactions. I. Acetylene reduction in seedlings inoculated with *Frankia*Ar13 and *Glomusintraradices*under three phosphorus levels. *Plant Soil*118: 151-155.

[24] Smith, S.E. and Daft, M.J., 1977. Interactions between growth, phosphate content and nitrogen fixation in mycorrhizal and non-mycorrhizal *Medicagosativa*. *Aust. J*. Plant. Physiol ***4,*** pp.403-413.

[25] Smith, S.E., Nichola, D.J.D. and Smith, F.A., 1979. Effect of early mycorrhizal infection on nodulation and nitrogen fixation in *Trifoliumsubterraneum L. Aust. J. Plant.Physiol*, *6*, pp.305-316.

[26] SubbaRao, N.S. 1985. Effect of combined inoculation of *Azospirillum brasilence*and vesicular arbuscular mycorrhiza on pearl millet (*Pennisetumamericanum*). Plant Soil 81: 283-286.

[27] SubbaRao, N.S., 1986. Cereal nitrogen fixation research under the BNF coordinated project of the ICAR. In Cereal nitrogen fixation: proceedings of the Working GroupMeeting. *ICRISAT*.23-30 pp.

[28] Sumner, M.E., 1990. Crop responses to *Azospirillum* inoculation. In *Advances in Soil Science 12* pp. 53-123.

[29] Wollum, A.G.II. 1982. Cultural methods for soil microorganisms. In: Page, A.L., Miller, R.H., Keeney, D.R. (eds.). Methods of soil Analysis, Part-2.Chemical and microbiological properties. American Society of Agronomy, Medison, Wis. Pp. 781-802.

[30] Wong, P.P. and Stenberg, N.E., 1979. Characterization of *Azospirillum* isolated from

nitrogen-fixing roots of harvested sorghum plants. *App. Environ microbial.*, ***38***, pp.1189-1191.

Figure 1. Bioinoculants treated groundnut plants soil bacterial population under nursery condition

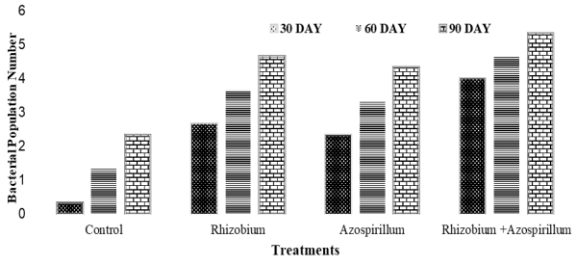

Figure 2. Bioinoculants treated groundnut plants soil and tissue nitrogen content under nursery conditions at 90th Day

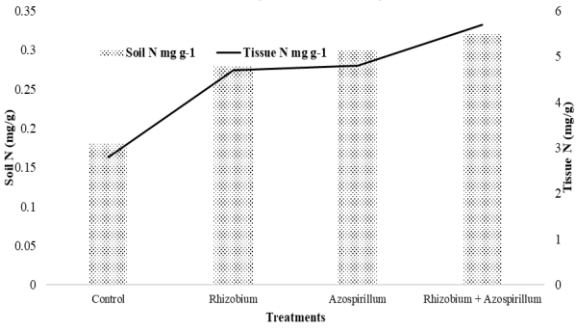

Figure 3. Bioinoculants treated groundnut plants nodule leghaemoglobin content under nursery condition

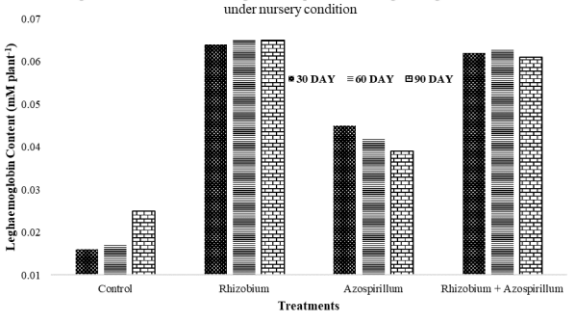

Figure 4. Bioinoculants treated groundnut plants number of nuts formation under nursery condition

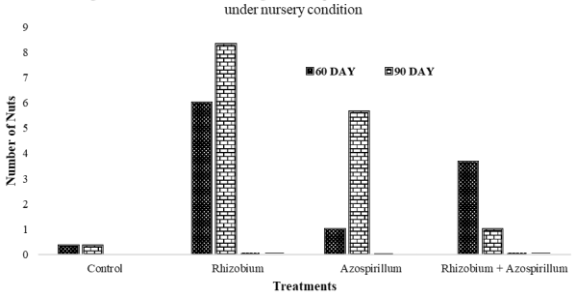

Table 1. Bioinoculants treated groundnut plants growths under nursery condition

Treatment	Shoot height (cm plant^{-1})			Root length (cm plant^{-1})		
	30 DAI	60 DAI	90 DAI	30 DAI	60 DAI	90 DAI
Control	7.93 a	21.63 a	26.03 a	17.26 a	18.63 a	29.57 a
Rhizobium	14.50 b	45.33 c	46.33 b	26.26 b	40.43 c	46.23 c
Azospirillum	18.36 c	26.33 b	47.43 c	31.10 c	33.60 b	42.73 b
Rhizobium + *Azospirillum*	25.60 d	46.50 d	54.47 d	29.60 d	42.50 d	48.63 d

Table 2. Bioinoculants treated groundnut plants leaf numbers and total chlorophyll content under nursery condition

Treatment	Leaf Number			Total chlorophyll (mg plant^{-1})		
	30 DAI	60 DAI	90 DAI	30 DAI	60 DAI	90 DAI
Control	12.33 a	45.00 a	84.54 a	19.70 a	19.83 a	18.36 a
Rhizobium	32.33 c	144.67 c	163.37 c	35.72 c	35.92 b	34.68 d
Azospirillum	26.00 b	113.00 b	153.84 b	31.67 b	38.34 c	29.46 b
Rhizobium + *Azospirillum*	35.67 d	147.67 d	239.15 d	35.97 d	36.77 d	33.90 c

Table 3. Bioinoculants treated groundnut plants nodule number and dry weight under nursery condition

Treatment	Nodule No.			Nodule Dry Wt.		
	30 DAI	60 DAI	90 DAI	30 DAI	60 DAI	90 DAI
Control	0.33 a	1.33 a	19.33 a	0.0005 a	0.0012 a	0.0377 a
Rhizobium	16.67 d	49.33 d	34.00 b	0.0075 d	0.033 b	0.0518 c
Azospirillum	0.66 c	38.33 c	61.00 c	0.0007 b	0.0362 c	0.0455 b
Rhizobium + *Azospirillum*	16.33 b	18.33 b	84.33 d	0.0049 c	0.0424 d	0.1094 d