African Journal of Basic & Applied Sciences 3 (6): 278-284, 2011
ISSN 2079-2034

Stress Tolerant *Rhizobium* Enhances the Growth of *Samanea saman* (JECQ) Merr.

[1]*K. Karuppasamy,* [2]*S. Nagaraj and* [3]*K. Kathiresan*

[1]School of Genetic Engineering, Madurai Kamaraj University, Madurai, India
[2]Centre for Advanced Studies in Botany, University of Madras, Guindy, Chennai, India
[3]Department of Botany, Saraswathi Narayanan College, Madurai, India

Abstract: Tree legumes are nodulated by a diverse group of rhizobia (*Rhizobium* and *Bradyrhizobium*). The potential of these root nodule bacteria as symbionts with tree legumes are usually exposed to fluctuated environmental conditions. The present study aims at using stress tolerant rhizobia isolated from nodules of *Sesbania grandiflora* and *Erythrina variegata* grown on different stressed soils such as dry land soil (DLS), dye industry effluent soil (DES), saline soil (SS) and calcareous soil (CS). Using these rhizobia as inoculants it is shown that the growth of tree legumes *Samanea saman* could be improved. The growth characteristics were analyzed after 45 and 90 days and biochemical characteristics at the end of 90th day of plant growth. At the end of 90th day of plant growth the highest increase 86.49% in shoot length and 33.43 % in root length was recorded using *Rhizobium* strain isolated from plant grown in CS-strain and DLS-strain. As for the biomass accumulation, the highest increase 210.11% in shoot dry weight and 135.89% in root dry weight was recorded. While there was 267% increase in the number of nodule, the fresh mass and dry mass also showed a marked increase of 231.4% and 220.1%, respectively under CS-strain treatment.

Key words: *Rhizobium* · Nitrogen fixation · *Samanea saman* · *Sesbania grandiflora* · *Erythrina variegate* · Stressed soil

INTRODUCTION

Forests play a vital role in providing rainfall, improving economic wealth, maintaining ecological balance and increasing the productivity. The Increasing population reflects on the environment, resulting in the reduction of biological productivity and erosion of biodiversity. Waste land statistics indicate that about 63.85 million hectares, which amounts for 20.17 per cent of the total geographical area, exist as waste lands in India (Community Forestry Projects and NGOs, 2004). For agricultural purposes man began to destroy forests which created an ecological imbalance. To increase the crop yield he began to use various chemical fertilizers indiscriminately, as a result of which degradation of soil in the tropics has occurred leading to a reduction in biological productivity and diversity. Reduction of vegetation cover causes decrease in soil fertility and soil organic matter and causes erosion. Natural periodic droughts also enhance this adverse development [1]. Land degradation not only disturbs natural plant communities but also affects plant-microbe symbioses, which is a critical factor in helping further plant growth in degraded ecosystems [2]. India's total geographical area is 369 m ha. Of this, 8.53 m ha is water logged, 3.58 m ha is occupied by alkali soils, 4.5 m ha is occupied by acid soils and 5.50 m ha is occupied by saline and coastal sandy areas [3]. Tolerance of salinity and alkalinity are important attributes sought in tree legumes. Salt-tolerant strains isolated from *Acacia redolens* grown on saline area was found to produce effective nodules on both *A. redolens* and *A. cyclops* grown in sand at salinity levels up to 80 mM NaCl [4]. The growth, nodulation and N_2 fixation of *Acacia ampliceps* inoculated with salt tolerant *Rhizobium* strains in sand culture were resistant to salt levels up to 200 mM NaCl [5]. Various strains of *Sinorhizobium meliloti* and *R. leguminosarum* were able to grow at more than 300 mM NaCl. Rhizobia isolated from woody legumes like *Hadysarum, Acacia, Prosopis* and *Leucaena* can tolerate up to 500 to 800 mM NaCl [6]. Adaptation to acidity varies quite markedly among the tree legumes. *Sesbania grandiflora* and *Sesbania sesban* are extremely well adapted to acidic soils. Leena Rasanan [7] reported that drought tolerant *Sinorhizobium* nodulated species of

Corresponding Author: K. Karuppasamy, School of Genetic Engineering, Madurai Kamaraj University, Madurai, India, Mobile: +91 9994201522, E-mail:kksamysnc@gmail.com

Acacia, Prosopis and *Sesbania* in dry land areas and increased the N_2 fixation rhizobia were isolated from the nodules of *Calliandra calothyrsus, Gliricidia sepium, Leucaena leucocephala* and *Sesbania sesban*. Specificity for nodulation and N_2 fixation varied greatly among these legumes [8]. Therefore, the improvement of soil fertility and afforestation in wastelands are necessary to protect the vegetation cover, forest cover and future life. We can use these waste lands in afforestation programme by planting leguminous trees. Kadiata, [9] reported the N2 fixed (measured as a percentage of the total nitrogen) in the tree legumes *Leucaena*, *Albizia* and *Cliricidia* ranged from about 20 to 74, 28 to 72 and 44 to 84% respectively. In one of the studies [10] determined the contribution of some tree legumes to soil fertility. Tree legumes fix about 43 to 581 kg of N ha^{-1}, compared to about 15 to 210 kg of N ha^{-1} for grain legumes. These trees are helpful to improve the fertility of alkaline, acidic and saline wasteland soils. Nitrogen fixation in legumes depends on the formation of nodules of *Rhizobium*. Without sufficient nodule mass filled with an efficient *Rhizobium*, nitrogen fixation will be inadequate. Inoculation usually corrects these problems. Those soils that lack adequate numbers of rhizobia need rhizobial inoculation to establish a successful symbiosis [11]. Observation of the development of nodules in the stressed soils, leads one to believe that these strains could be highly efficient, as they produce root nodules in the legumes under difficult condition. The present paper describes the isolation of rhizobial strains from tree legumes grown in different stressed soils and using them as inoculants to improve the growth performance of tree legumes and reclamation of soil fertility.

MATERIALS AND METHODS

Collection and Analysis of Soil: Dye industry effluent soil (DES), Saline soil (SS), Calcareous soil (CS), Dry land soil (DLS) and Red soil were collected from different places. These soils were tested for parameters such as pH, Electrical conductivity (according to standard procedures), organic carbon and organic matter [12].

Isolation of *Rhizobium*: Stress tolerant strains of rhizobia were selected based on the growth performance of host plants, nodule number and dry weights of nodules (Table 1). Rhizobia isolated from *Sesbania grandiflora* grown on saline soil, Calcareous soil and Dye industry effluent soil were taken and rhizobia isolated from *Erythrina variegata* grown on dry land soil and garden soil was taken for testing their potential on *Samanea saman*. The rhizobial strains were isolated using healthy and pink nodules only and cultured at 28°C on YEM agar with Congo red [13]. Distinct rhizobial colonies were picked out, purified and maintained.

Growth Conditions and *Rhizobium* Inoculation: The healthy *S. saman* seeds were surface sterilized with 0.05% mercury chloride and treated with *Rhizobium* inoculums and seed were sown in polythene bags filled with mixture of red soils and sand (1:1) ratio. Saplings are grown under natural condition at Research Center in Botany, Saraswathi Narayanan College Campus, Madurai, India. Different rhizobial treatments viz., seed treatment and soil treatment were given

Estimation of Growth Parameters, Pigments and Metabolites: Various growth parameters were analyzed at end of 45^{th} and 90^{th} day of plant growth using data obtained from 10 replicates for each treatment (Table 2). At the end of 90^{th} day of plant growth fresh tissues from fully expanded leaves of same age were analyzed, in triplicate, for the content of chlorophyll [14], starch [15], soluble sugar [16] soluble protein [17] and nitrate reductase activity [18].

Table 1: Nodule characteristics of *Sesbania grandiflora* and *Erythrina variegata* saplings grown in different stressed and garden soils used for *Rhizobium* isolation

	Sesbania grandiflora			*Erythrina variegata*		
Soil types	Nodule numbers	Nodule fresh weigh(g)	Nodule dry weight(g)	Nodule numbers	Nodule fresh weigh(g)	Nodule dry weight(g)
Saline soil	74.55	3.21	1.06	33.1	1.41	0.46
Calcareous soil	35.8	0.99	0.30	15.45	0.86	0.20
Dye industry effluent soil	94.95	4.67	1.57	40.8	1.12	0.37
Dry land soil	65.95	2.52	0.72	80.9	2.74	0.81
Garden soil	105.2	6.8	2.1	120.6	7.1	2.8

Values are means of 10 replicates from each sample

Table 2: Influence of rhizobial isolates from stressed soils on the growth characteristics and fresh and dry weight of *Samanea saman*

Growth parameter	No of days	CON-UT	CON-T	DLS-T	DES-T	SS-T	CS-T
shoot length (cm)	45	8.65	10.20(25.00)	11.55(41.54)	11.62 (42.4)	10.3 (26.23)	12.25(50.12)
	90	23.7	30.65(29.32)	35.45(49.59)	38.35 (61.8)	38.5 (62.46)	44.2 (86.49)
Root length (cm)	54	17.9	24.65(37.71)	31.65(76.85)	27.6 (54.14)	25.34(41.56)	25.65 (43.3)
	90	35.3	35.50 (0.57)	47.10(33.43)	42.45(20.25)	35.50 (0.57)	35.60 (0.85)
Shoo Fresh weight (g)	45	1.004	1.36 (35.85)	1.41 (39.94)	1.72 (71.13)	1.77 (75.59)	11.99 (99.0)
	90	7.3	10.10(38.42)	10.36(38.87)	11.9 (63.04)	11.95(63.70)	20.69(183.5)
Root Fresh weight (g)	45	0.23	0.32 (42.47)	0.41 (80.97)	0.59(160.17)	0.50 (120.4)	0.62 (175.7)
	90	3.06	4.46 (45.73)	5.07 (65.98)	4.56 (49.13)	5.68(85.70)	7.15 (133.6)
Shoot Dry weight (g)	45	0.405	0.45 (12.09)	0.50 (23.20)	0.58 (43.20)	0.59 (44.93)	0.67 (64.94)
	90	2.205	2.89 (30.88)	3.38 (53.21)	3.41 (54.59)	3.41 (54.82)	6.84(210.11)
Root Dry weight (g)	45	0.087	0.11 (22.98)	0.16 (125.3)	0.20 (125.3)	0.18 (102.3)	0.21 (136.9)
	90	1.117	1.32 (17.96)	1.78 (58.90)	1.78 (58.90)	1.84 (64.54)	2.36(135.89)
Nodule numbers	45	14.3	46.5(225.17)	43.1 (201.3)	25.1 (75.52)	62.7(338.46)	42.9 (200.0)
	90	72.7	158.3(117.7)	147.6(103.1)	152.4(109.6)	169.0(130.0)	266.8(267.0)
Nodule Fresh weight(g)	45	0.021	0.05 (142.9)	0.06 (195.2)	0.064(204.8)	0.07 (214.3)	0.07(228.6)
	90	0.996	1.069 (7.32)	1.36 (36.55)	1.72 (73.09)	1.77 (80.32)	3.30 (231.4)
Nodule Dry weight (g)	45	0.004	0.008(125.0)	0.01 (125.0)	0.01 (150.0)	0.01(175.0)	0.01 (200.0)
	90	0.154	0.165 (7.14)	0.20 (31.17)	0.23 (49.26)	0.26 (71.43)	0.49 (220.1)

Values are means of 10 replicates and figures in parenthesis are percentage increase over the control. (Con-UT: Control Untreated, Con-T: Control with garden soil strain, DLS-T: Dry Land Soil Strain, DES-T: Dye Effluent Soil Strain, SS-T: Saline Soil Strain and CS-T: Calcareous soil strain)

RESULTS

Physico-Chemical Characteristics of Different Problem Soils: The physico-chemical characters of the different soils used for the present study to grow *Sesbania grandiflora* and *Erythrina variegata* are presented; the saline soil was light gray in colour, with a pH of 8.13 in the alkaline range. The electrical conductivity was 4.0dsm^{-1}, the organic carbon was 0.03 per cent and organic matter was 0.05 per cent. The calcareous soil was gray in colour, with a pH of 8.27 in the alkaline range. The electrical conductivity was 1.4dsm^{-1}, the organic carbon was 0.09 per cent and organic matter was 0.16 per cent. The dye industry effluent soil was light brown in colour, with a pH of 7.91, which was in the slightly alkaline range. The Electrical conductivity was 2.5dsm^{-1}, the organic carbon was 0.45 per cent and organic matter was 0.78 per cent. The dry land soil was red in colour with a pH of 5.42 in the acidic range. The Electrical conductivity was 0.12 dsm^{-1}, the organic carbon was 0.12 per cent and organic matter was 0.21 per cent. The garden soil, mixed with sand 1:1 ratio, used to grow *Samanea saman,* was red in colour with a pH of 6.51 in the slightly acidic range and an electrical conductivity of 0.93dsm^{-1}. Organic carbon was 4.24 per cent and the organic matter was 7.21 per cent. *Erythrina variegata* and *Sesbania grandiflora* were grown in the above mentioned problem soils and after three months of growth, the characteristics of nodules were analyzed. Plants in which the nodule characteristics were better, was used for isolating the rhizobial strain. The characteristics of nodules of both the plants grown in all the four stressed soils are given in Table 1. The characters indicate that *Sesbania grandiflora* produced better nodules in saline soil, calcareous soil and dye industry effluent soil and *Erythrina variegata* produced better nodules in dry land soil.

Growth Systems and Experimental Design

Effect of Inoculation and Growth Characteristics of *Samanea saman:* The effect of rhizobial isolates from the various stressed soils on the growth characteristics of *Samanea saman* is presented in Fig. 1. Among the different strains used an increase in the shoot length, root length and total length was observed in all the treatments at the end of 45 day as well as 90 day of growth. The calcareous soil strain produced the maximum increase in the shoot length 50.12% in 45 day and 86.49% in 90 day, followed by the saline soil strain 26.23% in 45 day and 62.46% in 90 day. The increase in percentage of root length (76.86%) in 45 day and (33.43%) in 90 day was influenced by soil strain followed by dry land soil strain followed by others soil strain, when compared to the respective control. Like the shoot and root length, all the rhizobial isolates used caused an increase in the total length also (Table 2). The difference between the performances of different rhizobial strain indicates a variation in infection and effective nodulation.

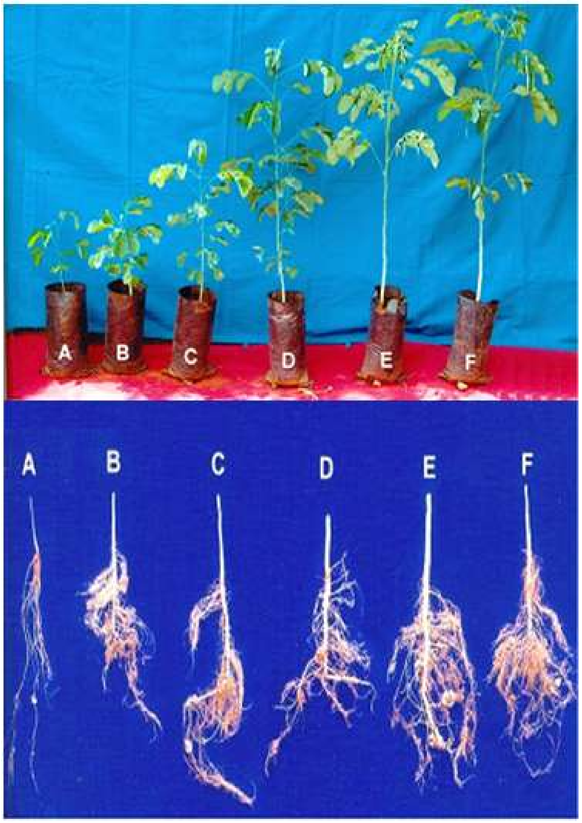

Fig. 1: The Sapling of *Samanea saman* inoculated by stress tolerant rhizobial strains isolated from different stressed soils. (A-Untreated control, B-Control treated (garden soil strain), C-Dry land soil strain, D-Dye industry effluent soil strain, E-Saline Soil Strain, F-Calcareous soil strain)

Biomass Production: Like the length of plant, the fresh weight of the plants also increased under all the treatments. While the increase in shoot fresh weight was significant in all the treatments, maximum increase of 99.0% in 45 day, 183.5% in 90 day and root fresh weight of 99.0% in 45 day,133.5% in 90 day was caused by the calcareous soil strain followed by other soil strains. Similar to the fresh weight, a significant increase in the dry weight also was noticed (Table 2).

Nodule Formation and Nitrogen Fixation: A significant increase in number, fresh weight and dry weight of nodules resulted because of the influence of the rhizobial strains used. Observations made at the end of 45 and 90 day showed a significant increase in number of nodules in the calcareous soil 267.0%, 130.0% in the saline soil strain followed by others soil strain, compared to respective control. The maximum nodule fresh weight

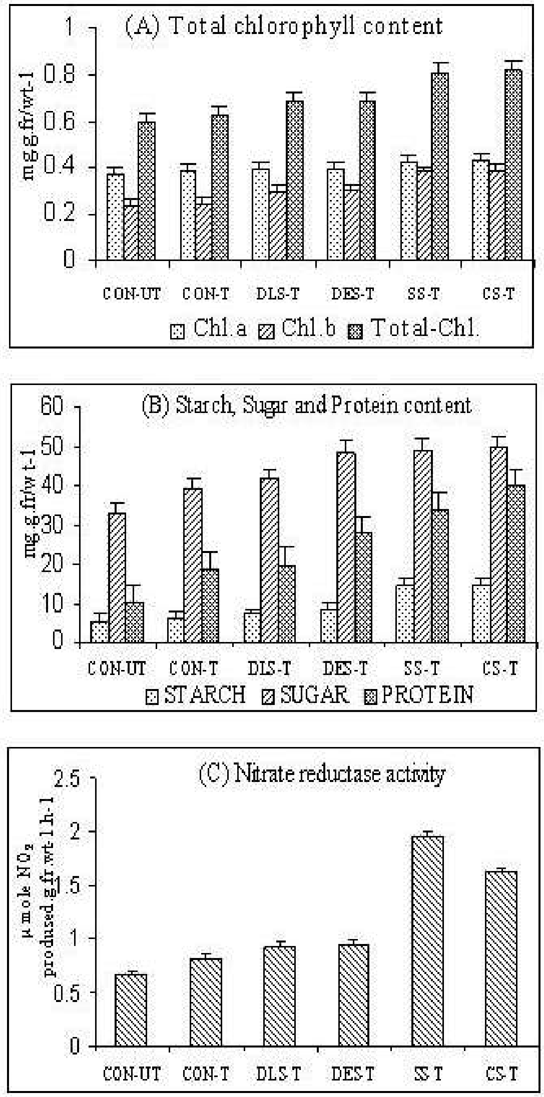

Fig. 2: Effect of Rhizobial isolates from stressed soils on the biochemical characteristics (A) Chlorophyll content, (B) Starch, Soluble Sugar and Protein content, (C) Nitrate reductase activity) of *Samanea saman.*

231.4% was caused by the calcareous soil strain followed by the saline, dye industry effluent and dry land soil strains at the end of 90 day of plant growth when compared to the respective control. Similar to the fresh weight a significant increase in dry weight was also recorded.

Biochemical Characteristics of *Samanea saman*

Estimation of Total Chlorophyll: The chlorophyll content increased in all the treatments. Increase in chl *b* was found to be more than the increase in chl *a* in all the treatments including treated control. Chl *a* content two fold increased using in calcareous soil and saline soil isolates followed by other isolates. Chl *b* increased significantly in all the treatments, significant increased in the calcareous soil strain closely followed by the saline soil strain. The increase in total chlorophyll was average when compared to chl *a* and chl *b*. The maximum increase was noticed in the calcareous soil treatment followed by the saline soil treatment and others when compared to the treated control (Fig. 2A). An increase in the chlorophyll content is a direct indication of the increased rate of photosynthesis which could have occurred in these plants.

Estimation of Starch, Sugar and Protein Content: Starch content was found to increase significantly in all the treatments. Maximum increase occurred in the calcareous soil strain treatment followed by other soil strains treatments (Fig 2B). The effect of rhizobial isolates on the soluble sugar content in leaf was relatively lesser when compared to the starch content. Unlike sugar content, the increase in the soluble protein due to the action of the rhizobial isolates was highly significant. The increase was maximum in the calcareous soil strain treatment followed by others when compared to the untreated control.

Total Nitrate Reductase Activity: Nitrate reductase activity will always increase with better nitrogen nutrition. The maximum activity occurred in the saline soil isolates followed by calcareous soil isolates. In the other two treatments, the values were almost equal (Fig. 2C).

DISCUSSION

A successful host-symbionts relationship is essential for the nitrogen fixing ability. Many of the earlier workers have also indicated that there is host specificity for the various rhizobial strains and species. Sivakumar and Kumutha [19] in their results found that an increase in shoot length of 17.5, 24.5 and 8.8 per cent occurred with the use of TNAU 14, PGR 1 and BGR 1 rhizobial isolates and root length also with a maximum increase recorded using PGR 1 (8.4 per cent) followed by TNAU 14 (8.0 per cent). Moussa *et al.* [20] found that the effect of inoculation on plant growth was significantly effective on shoot hight,stem diameter of all the species (*Albizia adianthiflora, A. attissima, A. furuginea, A. zygia, Erythrophleum guineensis, E. ivorensis, Millettia rhodantha, M. zechiana*) grown in nursery condition. Rachel *et al.* [23] reported that *Rhizobium* inoculation significantly enhanced the root length (13-48 per cent) and shoot length (6-133 percent) in *Vigna mungo* and *Vigna radiata* after 40 days of growth. Our results are in agreement with the results of these studies.

The dry biomass accumulation indicates a better influence on the root growth than on the shoot growth. Biomass accumulation also indicates the better influence of the rhizobial strains of the calcareous soil followed by other strains. Singh *et al.* [21] reported that more dry matter accumulation with the use of SML 357 strain was due to more leaf area at maturity, while in case of SML 134 strain, it was due to more growth in terms of plant height. Kantar *et al.* [22] reported that inoculation with bacterial strains isolated from perennial wild chick peas at high altitudes significantly increased the fresh and dry weights of the plants and also increased yield and yield components of chick pea in the field compared with the uninoculated control. Sivakumar and Kumutha [19] also found an increase in the fresh and dry weights of the plants. The increase in fresh weight was 6.8, 7.5 and 3.4 per cent when compared with uninoculated seeds of ground nut. These results are in agreement with the results of the present study.

An increase in the rate of photosynthesis could make more carbon skeletons available for the synthesis of amino acids. Better nitrogen availability could also help this. When there is an increase in the synthesis of amino acids, there would be an increase in the protein synthesis also. This could influence all the other related synthetic metabolic pathways which would cause an increase in overall growth. Rachel *et al.* [23] observed an increase in the total chlorophyll from 3.75 mg g^{-1} fr.wt. in control to 4.22 mg g^{-1} f.w. in *Rhizobium* inoculated *Vigna mungo* while in *Vigna radiata,* the total chlorophyll in control was 4.01 mg g^{-1} f.w and in *Rhizobium* inoculated plants, it was 4.46 mg g^{-1} f.w. Kantar *et al.* [22] in their studies in the field trials found that inoculations with HF 274 and HF 177 increased the chlorophyll content.

Better accumulation of starch and sugar could be a direct consequence of increased photosynthesis caused by more chlorophylls and better growth influenced by an increased metabolic rate due to the availability of more nitrogen. An increase in proteins could be related directly to the availability of more nitrogen. Rachel *et al*. [23] showed an increase in soluble sugars by 96 % in *Vigna mungo* and 231 per cent in *Vigna radiata* due to rhizobial inoculation. Khanna and Gupta [24] showed an increase in proteins up to 526 % in *Pisum sativum* and increase in starch up to 120 % by applying *Rhizobium.* Khanna and

Gupta [24] showed an increase in proteins up to 526 % in *Pisum sativum* and increase in starch up to 120 per cent by applying *Rhizobium*. Similar trend is also observed in our study.

Nitrate reductase activity is a direct indication not only for more fixation of nitrogen but also for the increased protein synthesis. Sri Ramachandrasekharan and Muthukkaruppan [25] revealed that *Rhizobium* inoculation in soybean increased nitrogenase activity up to 8.5 % over the control. Rachel *et al.* [23] also showed that rhizobial inoculation on *Vigna radiata* increased nitrogenase activity up to 125 % over the control. These results are in agreement with the results of the present study. The *Rhizobium*-legume symbiosis is superior then other N_2 fixing system with respect to N_2 fixing potential and adaptation to various environments such as salinity, alkalinity, acidity and drought conditions were identified. These associations might have sufficient straight necessary to establish successful plant growth and N_2 fixation under various environmental conditions. In this study it is suggested that the stress tolerant rhizobia isolated from the nodules of *Sesbania grandiflora* grown in calcareous soil and saline soil give better nodulation and nitrogen fixation in various environmental condition and improve the soil fertility.

ACKNOWLEDGEMENT

The authors express their gratitude to the Principal, Saraswathi Narayanan College and special thanks to Dr. C. Sankaranarayanan, Head, PG study and Research Center in Botany, Saraswathi Narayanan College, Madurai, India, for providing laboratory facilities.

REFERENCES

1. Kirmse, R.D. and B.E. Norton, 1984. The potential of *Acacia albica* for desertification control and increased productivity in Chad. Biolo. Conserv., 29: 121-141.
2. Zahran, H.H., 1997. Diversity, adaptation and activity of the bacterial flora in saline environments. Boil. Fertile Soils, 25: 211-223.
3. George, P.S., 1994. Management of renewable natural resources / sustainability of agriculture-Rapporteur's report. Indian J. Agric. Economics, 49: 41-45.
4. Craig, C.F., C.A. Atkins and D.T. Bell, 1991. Effect of salinity on growth of four strains of *Rhizobium* and their infectivity and effectiveness on two species of *Acacia.* Plant and Soil, 133: 253-262.
5. Zou, N., P.J. Dart and N.E. Marcar, 1995. Interaction of salinity and rhizobial strains on growth and N_2 fixation by Acacia ampliceps. Soil Biol. Biochem., 27: 409-413.
6. Zahran, H. Hussein, 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol. Mol. Boil., 63: 968-989.
7. Leena Rasanen, A., 2002. Biotic and a biotic factors influencing the development of N2-fixing symbiosis between rhizobia and the woody legumes *Acacia* and *Prosopis.* Academic Dissertation in Microbiology. University of Helsinki, Finland.
8. Abdullahi, B. And E.G. Ken, 2001. Symbiotic specificity of tropical tree rhizobia for host legumes. New Phytol., 149: 495-507.
9. Kadiata, B.D., K. Mulongoy and N.O. Isirimah, 1996. Time course of biological nitrogen fixation, nitrogen absorption and biomass accumulation in three woody legumes. Biol. Agric. Hort., 13: 253-266.
10. Dakora, F.D. and S.O. Keya, 1997. Contribution of legume nitrogen fixation to sustainable agriculture in Sub-Saharan Africa. Soil Biol. Biochem., 29: 809-817.
11. Mor, S., R.C. Dogra and S.S. Dudeja, 1995. Effect of adhesives on rhizobial survival, distribution, nodulation and nitrogen fixation in summer and winter legumes. Indian Journal of Microbiology, 35(2): 115.
12. Walkey, Block, 1934. An examination of Degtjareff method for determining soil organic mater and a proposed modification of the chromic acid titration method. Soil Sci., 34: 29-38.
13. Vincent, J.M., 1970. Annual for the Practical Study of the Root Nodule Bacteria IBP Vol. 15. Oxford, Blackwell Scientific Publications.
14. Arnon, D.E., 1949. Copper enzymes in isolated chloroplast. Poly phenol oxidase in *Beta vulgaris*. Plant Physiol., 24: 1-15.
15. Mc Cready, F.M., J. Guggolz, V. Silvera and H.S. Owen, 1950. Determination of starch and amylase in vegetables. Anal. Chem., 52: 156.
16. Dubois, G.K.A., J.K. Hamilton, P.A. Hobars and T. Smith, 1956. Colorimetric determination of sugar and related substances. Anal. Chem., 28: 351-356.
17. Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall, 1951. Protein measurements with folin phenol reagent, J. Biol. Chem., 13: 263-5.

18. Muthuchelian, K., K. Paliwal and A. Ganam, 1989. Influence of shading on net photosynthetic and transpiration rates, stomatal diffusive resistance and nitrate reductase and biomass productivity of wood legume tree species (*Erythrina variegata* Lam.) Proc. Ind. Aca. Sci. Plant Sci., 99: 539-596.
19. Sivakumar, V. And K. Kumutha, 2003. Effects of *Rhizobium* and molybdenum on nodulation, yield and yield contributing characters of groundnut. J. Ecobiol., 15: 451-455.
20. Moussa, D., M. Antonic, M. Sergio, B. Amadou, Bernard and G. Antoine, 2004. Occurrence of nodulation in unexplored leguminous trees native to the West African tropical rainforest and inoculation response of native species useful in reforestation. New Phytologist, 166: 231-239.
21. Singh, M., H.S. Sekhon and J. Singh, 2005. Growth and nodulation characteristics of Mungbeen (Vigna radiate (L) wilczek) genotype in response to phosphorus application. Crop Res., 29: 101-105.
22. Kantar, F., E. Elkoca, H. Oguteu and O.F. Algur, 2003. Chick pea yields in relation to *Rhizobium* inoculation from wild chick pea at high attitudes. J. Agronomy and Crop Science, 189: 291-297.
23. Rachel, R.D., K. Santhagru and P. Gunasekaran, 2001. Effect of supplementary UV-B Radiation on growth, Nodulation and Nitrogen fixation in *Vigna mungo* L. and *Vigna radiate*(L.) Wilzek. Indian. J. Microbiol., 4: 157-161.
24. Khanna, P.K. and A.K. Gupta, 2005. Changes in Growth, Yield and Some Biochemical Attributes in Pea (*Pisum sativum*) with Rhizobium and Sulphur Applications. Society for Plant Physiology and Biochem., 32/1: 25-28.
25. Sri Ramachandrasekharan, M.V. and S.M. Muthukkaruppan, 2004. Effect of sulphur and *Rhizobium* on biological nitrogen fixation, nutrient uptake and seed yield in soybean. J. Ecobiol., 16: 235-236.