J. Mycopathol, Res, 48(2) : 321—328 (2010)

Symbiotic interactions between *Rhizobium* and *Neptunia prostrata* (Lam.) Baill.

ROMESH SAGOLSHEMCHA; M. BABITA DEVI; A.I. SINGH; A.H. SINGH* AND N. IRABANTA SINGH
Aerobiology, Microbiology, Biotechnology and Plant Pathology Laboratory Department of Life Sciences, Manipur University, Canchipur, Imphal 795 003, Manipur
**College of Agriculture, Central Agricultural University, Iroishemba, Imphal, Manipur*

Nodulation process between *Rhizobium* and *Neptunia prostrata* were examined under microbiologicallly controlled condition as well as under its natural habitat. The plant did not developed root hair in its natural habitat and laboratory conditions, which was the primary site of rhizobial entry for most leguminous plants. Entry of rhizobia into *N. prostrata* occurred through ruptured tissue at the site for adventitious and lateral root emergence. Microscopic examination revealed that the nudule of *N. prostrata* were always with the root but not with the stem. The indeterminate nodule appeared macroscopically, 1-11 mm in length and 1-3 mm in diameter. After colonizing the infection sites, Rhizobia initiated early development of nodules at the base of the root. Rhizobia then migrated from the site of infection to deeper in the nodule through intercellular spaces and eventually formed multibranched infection thread. The infection thread invaded the host cells intracellularly. Rhizobia were then released from the infection thread and accumulated into the host cells forming into enlarged bacteriods within symbiosomes. Cross inoculation test of *Rhizobium* sp. AN-01 of *N. prostrata* nodule isolate revealed the rhizobium readily nodulated in certain economic leguminous crops and also its inability to nodulate several other crop legume species.

Key words : *Rhizobium, Neptunia prostrata,* infection thread, symbiosis, root nodules

INTRODUCTION

Neptunia prostrata (Lam.) Baill, known as water mimosa, is a miniature tropical, perennial herb with long floating stem. It is an aquatic legume, native to several continents of the humid tropics and is used for both human consumption and as green manures for rice cultivation in India and some Asiatic countries (Subha Rao *et al.,* 1995). Water mimosa is one of the most important native vegetables or aquatic plants in Manipur. It grows actively during rainy season with distinct nodes and internodes floating stem containing white spongy internodal tissue and nodes with bright red nodule and adventitious roots (Schaede, 1940; Allen and Allen, 1981).

Neptunia prostrata contains 88% moisture, 5.4 mg/ 100 mg vitamin C; 1.2% crude fat; 1.6% crude fiber; 13% crude protein and 5.4% total ash (Arora, 1981). Moreover, it is medically a very valuable plant. The plant is refrigerant and astringent (Chopra *et al.,* 1956). The leaves of *N. oleracea* have pheophorbide 'a' and its related compounds which can be used as a possible anti-tumour promoters (Nakamura *et al.,* 1996). Further, *N. prostrata* is administered in various ailing treatment among the tribal of N.E. India such as for nose bleeding, sore tongue, diarrhea with blood, white discharged, epilepsy etc. (D'Souza, 1993).

Neptunia prostrata is unusual into that it normally develops buoyant floating stems that grows profusely on the surface of freshwater ponds and slow moving water bodies, and in this aquatic environment it develops many stem associated nitrogen fixing root nodules of various sizes and shapes. The genera *Devosia* sp. (Rivas *et al.,* 2002) and *Allorhizobium* (de Lajudic *et al.,* 1998) are also reported in the nodules of *N. prostrata,* but

Subha Rao *et al.*, (1995) have reported from India that nodules on this legume are formed by *Rhizobium* sp.

Schaede (1940) has described that nodules are being formed on adventitious roots arising from floating stems and the rhizobium enters through the epidermal cells as the sole means of infection and formed nodules. James *et al.* (1992) have agreed with his first interpretation. They have found that the nodules have vascular connection to the adventitious root and not with the stem itself. They have argued against *Neptunia* bearing true stem nodules.

James *et al.* (1992) have studied *Neptunia plena – Rhizobium* symbiosis and found out nodule invasion that as an intercellular spread of rhizobium followed by penetration of host cells by means of infection thread with subsequent nodule development. Similar observations are also made by Subha Rao *et al.* (1995) with his study on *N. natans-Rhizobium* symbiosis. James *et al.* (1992) have described that *Neptunia plena* develops root hairs when grown in Leonard jars filled with vermiculite, but infection thread are not formed in early stage of nodulation, either in the root hairs or the root cortex when inoculated with *Rhizobium.*

Subha Rao *et al.* (1995) have studied *Neptunia-Rhizobium* symbiosis in greater details. They have observed the rhizobium enters the plants through natural ruptured wounds caused by emergence of young lateral and adventitious roots. After having entered the large cavity of the wound at the junction of lateral roots, the bacterium colonizes open pockets or fissure between dead host cells at the periphery of the young nodules. The bacterium then enteres the host intracellularly with formation of infection thread. The vegetative bacterium then divides by binary fission forming intracellular symbiosomes with endosymbiotic bacterium. They have observed that symbiosomes containing mature bacteriods are embedded in an unusual and elaborate array on electron dense, fibrillar material occupying the entire matrix between the bacterial and peribacteriod membrane.

The present paper deals with the studies of the following objectives :- (i) structure and development of nodule of *Neptunia prostrata* in its natural habitat in Manipur, (ii) process of root nodule formation in the host plant in a microbiologically controlled environment, and (iii) host range of the *Rhizobium* isolated from *Neptunia prostrata* to certain important economic leguminous plants of Manipur.

MATERIALS AND METHODS

The aquatic legume was found growing in all the four valley districts of Manipur which include Imphal east, Imphal west, Thoubal and Bishnupur districts. The plant samples were brought to the laboratory to examine their features and nodule structure. Seeds were harvested from the survey site and were sundried and kept for further investigation. A group of 5 plants of pencil size of 30 cm in length from tip were selected randomly from each survey site. The plants were carefully washed in continuously running tap water to remove debris. Number, size and fresh weight of nodules were recorded. Firm and intact nodules of similar size were excised using sharp razor blade. Thin sections of nodule were stained with methylene blue and observe under bright field microscope.

Bacterial isolates were isolated from fresh nodules of plant samples by the standard method on YMA medium (Vincent, 1970). Single isolated colonies was picked and checked for purity by repeated streaking and by microscopic examination.

Neptunia prostrata seed were scarified and surface sterilized with 0.1 $HgCl_2$ for 5-10 min. followed by washing in sterile distilled water. The seeds were then left for 30 seconds in absolute alcohol followed by quick washing with sterile distilled water until all traces of alcohol were removed. The seeds were germinated on sterile Petridish containing 2 ml of sterile water with double layered paper towels for 24 hrs. Seedlings were transferred to sterile culture tube containing $Ca_3(PO_4)_2$ 0.2%; K_2HPO_4 0.05%; $MgSO_4$ $7H_2O$ 0.02%; NaCl 0.02%; $FeSO_4$ 10 ppm; $MnSO_4$. $4H_2O$ 1 ppm; Na_2MoO_4 1 ppm for growth supplement of the plant. Each culture tube contains 10 ml of the broth and supported the growth of 3 seedlings for up to 8 weeks. 0.1 ml of 48 hrs old cultures were inoculated for root nodulation trials. Inoculated plants were compared with control uninoculated plants.

Plant microscopy by SEM method

Surface features of stem, roots and nodules of *Neptunia prostrata* were examined by scanning electron microscope. The tissues were fixed overnights in Karnovsky's fixative at 4°C. They were then washed with 0.1 Sodium Cacodylate buffer with three changes of 15 minutes each at 4°C, then dehydrated progressively in an acetone series, dried by Tetra methyl Silane method (TMS) for 5-10 min and brought to room temperature to dry. Finally mounted on Brass/Aluminum stubs and Sputter coated with 35 mm of gold.

Plant microscopy by TEM method

Stem, root and nodules were fixed overnight in Karnovsky's fixative at 4°C, they were then washed with 0.1 M Sodium Cacodylate buffer, post fixed with 1% Osmium tetraxide in 0.1M Sodium Cacodylate buffer at 4°C, then washed with the same buffer, dehydrated progressively in an acetone series at 4°C. The tissues were then dehydrated in 100% acetone for 30 min at room temperature, and then cleaned with Propylene oxide, infiltrated with Propylene oxide, and again infiltrated with Propylene oxide and embedding medium. Finally the tissues were then embedded and polymerized. Ultra-thin sections of the tissues were then cut off and observed under TEM.

Cross inoculation test

Nodulation ability of *Rhizobium* isolate from *N. prostrata* was examined on certain economically important leguminous plants found in Manipur. The leguminous plants were grown on plastic pots filled with sterilized vermiculite. Nodulation on the test legumes were checked as described by Vincent (1970). The plants were irrigated regularly with sterile distilled water and incubated at mixed of 14 hrs (25°C) light and 10 hrs (20°C) dark.

RESULTS AND DISCUSSION

Morphology of root nodules of Neptunia prostrata

Neptunia prostrata produced abundant nodules. The nodules were of various shapes and sizes (Figs.1a, 1b). They appeared macroscopically 1-11 mm in length and 1-3 mm in diameter. The nodules were formed very close to the stem and appeared to be connected to them. Root nodules were also formed at other part of the root system but they were concentrated at the base of the root (Fig.1a). the main advantages of having nodules on roots arising from floating stem is that air pathways are kept to a minimum (James *et al.*, 1992) and hence simple diffusion of 0_2 through the interconnected pathway of stem/root/nodule/aerenchyma/lenticels is sufficient to supply the respiratory needs of the nodule (Loureiro *et al.*,1992). Stereomicroscopic examination revealed nodules vascular systems are always connected to the adventitious roots but not to stem itself (Figs. 2 & 3). Similar observation was made by James *et al.* (1992) on his work on *Neptunia plena.* Cross section of nodules under

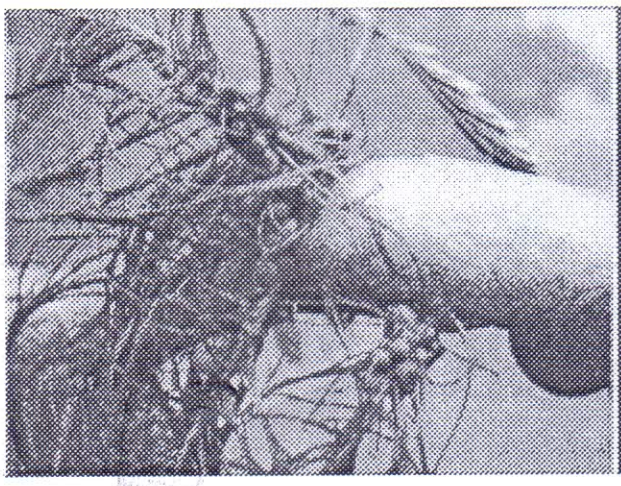

Fig 1a : *Neptunia prostrata* showing clusters of root nodules with floating arenchymatous tissue

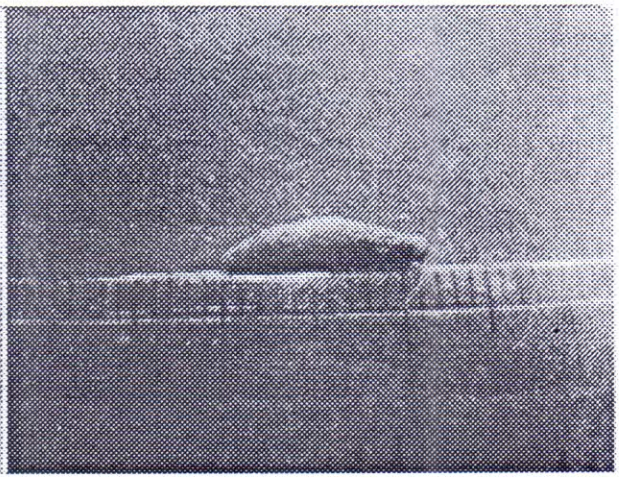

Fig 1b : A mature indeterminate root nodule of *Neptunia prostrata*

Fig 2 : Sterio-micrograph of a young root nodule primordial formed at the base of lateral root

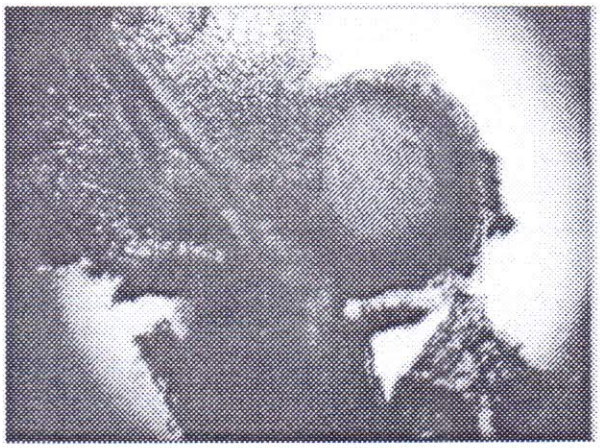

Fig 3 : Brightfield micrograph of an excised root nodule showing nodule vascular connection with the root vascular system 150x

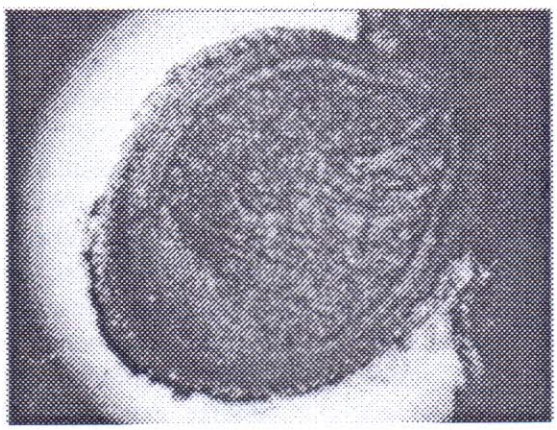

Fig 4a : Brightfield micrograph of a longitudinal section with large central infected zone surrounded by an uninfected peripheral nodule cortex containing vascular elements and a layer of cells with coloured bodies. 150x

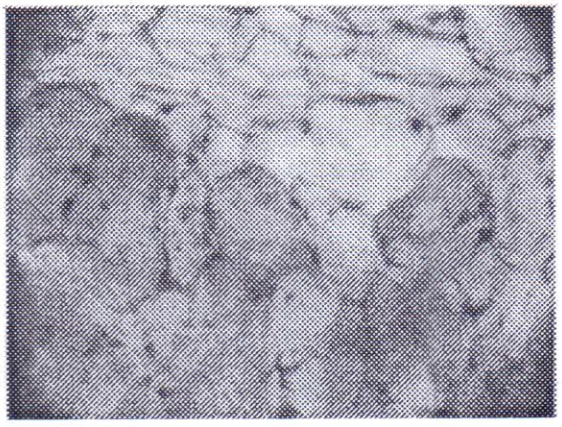

Fig 4b : Section of root nodule showing infected and uninfected cells. 600x

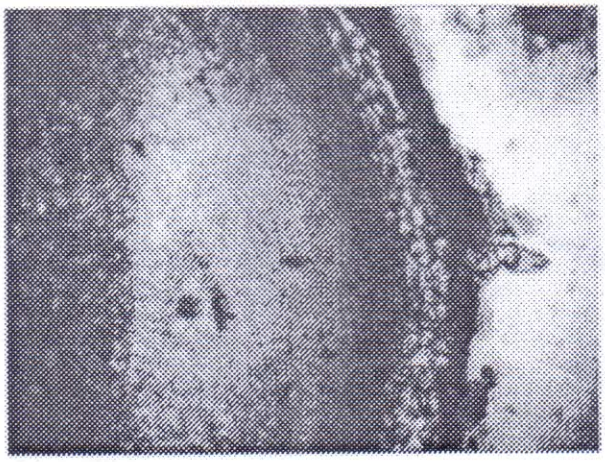

Fig 4c : Coloured bodies of the root nodules showing the typical colour of the nodule and root of *Neptunia prostrata* 300x

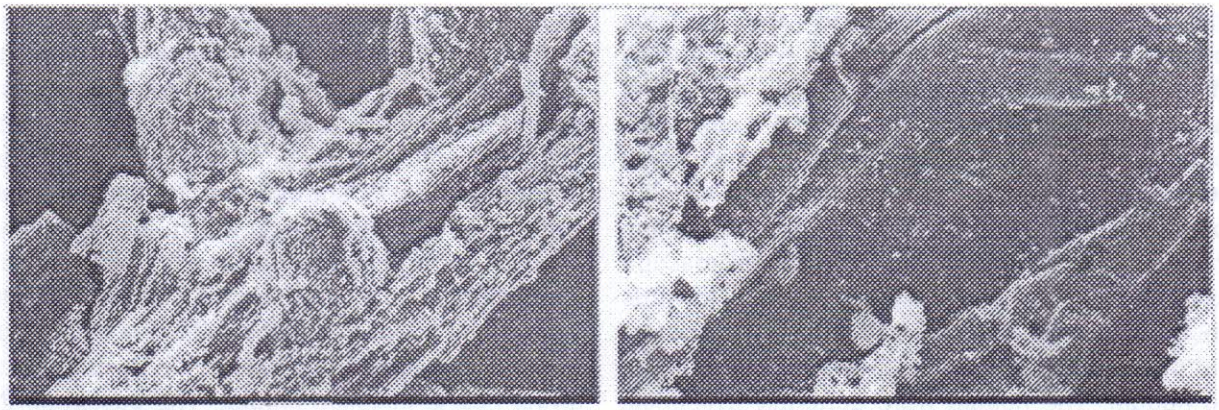

Fig 5 : (a) Scanning electron micrograph of the natural ruptured wound on the surface of a primary root caused by emergence of lateral root. 100x (b) bacterial colonization on the surface of the ruptured tissue. 2500x

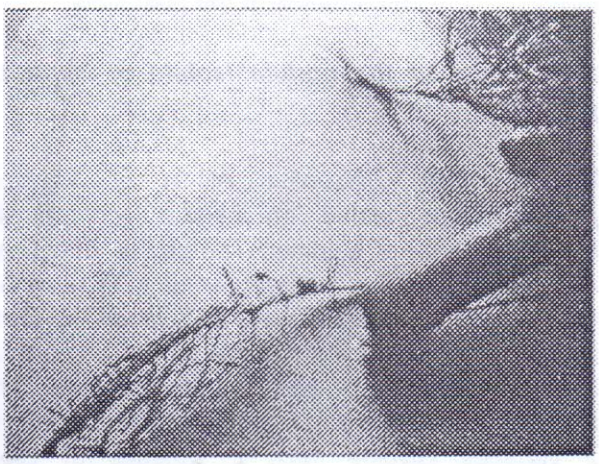

Fig 6 : Root nodule formed on lateral root far from the base of the adventitious root and stem

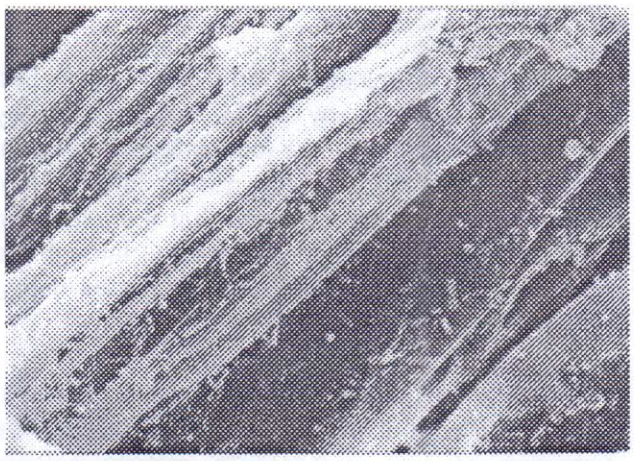

Fig 7 : Scanning electron micrograph showing bacterial colonization deep within the root tissue of *Neptunia prostrata* 2000x

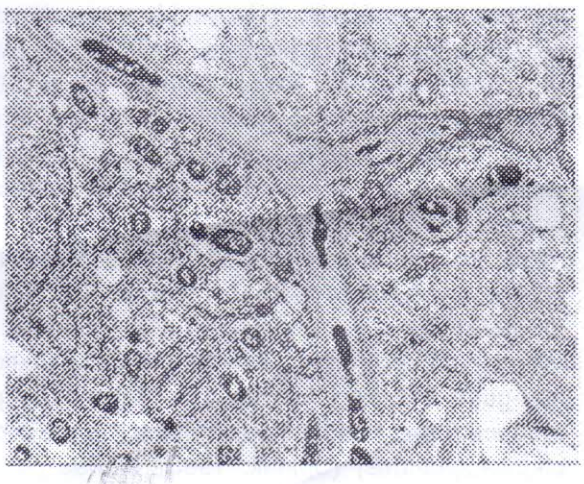

Fig 8 : Transmission electron micrograph of root nodule section showing multibranched infection thread; endosymbiotic bacteroid; infection droplet. 13100x.

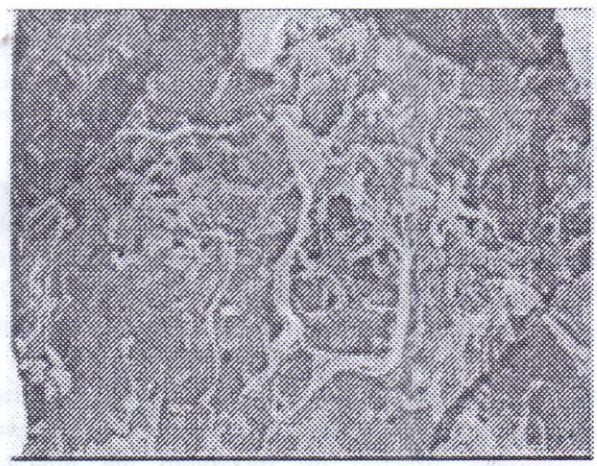

Fig 9 : Scanning electron micrograph of section of root nodule showing endosymbiotic bacteria occupying the whole matrix of the host nodule cell. 3000x.

brightfeild microscope revealed the nodules contains large central infected zone surrounded by uninfected peripheral nodule cortex containing vascular elements (Fig. 4). A layer of 5-6 cells thick coloured bodies were also observed which gave the typical bright red colour to the roots and the nodules of *Neptunia prostrata.* Transverse section of root nodule under brightfield microscope revealed large zone of uninfected areas towards the tip of nodule showing active apical meristem that produced new cells for growth over the life of the nodule. The nodule were generally cylindrical shaped.

Process of root nodule formation in Neptunia prostrata

Microscopic examination on one weak old inoculated seedling by SEM revealed heavy bacterial colonization on the root surface of the plant including epidermal cells and deeper into the root tissue. The microscopy showed that the bacterium colonized the root surface and entered through the wound cavity of the natural fissure created during emergence of lateral roots (Figs. 5a, 5b).

The bacterium entered through the intracellular spaces of the root system and then initiated the formation of root nodules. In microbiologically controlled condition, nodules were not formed on primary root until and unless there was emergence of secondary root. In natural habitat nodules primordial were formed on the emerged young root from the stem but not at the stem itself. In case of adventitious root emergence which emerged from the lateral root, nodules primordial were formed on the adventitious root as well as the lateral root. Nodules formed on lateral root far from the site of adventitious root emergence (Fig. 6). Scanning Electron microscopy revealed that the bacterium entered deeply through plant root tissue and formed root nodule at another site of the root (Fig. 7). The bacterium multiplied within the fissure/dead cells and infected the developing meristematic tissue in the incipient root cortex. The nodule primordial then developed by an apical meristem tissue and was continually infected by rhizobium via infection threads as the nodule grows.

After entering through the intracellular spaces the bacterium colonized open pockets at the periphery of the young nodule. As the bacterial colonization continued deeper into the nodule, it formed a tubular structure giving rise to infection threads penetrating the adjacent host cells. Transmission electron micrograph (Fig 8) showed that the bacterium after colonizing and multiplying within the intracellular spaces between two adjacent cells infected intracellularly and produced a tubular structure intracellular infection threads within the nodule cells. The infection thread enlarged at the tip of the tubule giving rise to infection droplet. The bacterium then migrated from the lumen of the infection droplet into the cytoplasm of the host nodule cells. The bacterium then multiplied by binary fission occupying the entire matrix of the host cells.

Host range of the nodule isolate of Neptunia prostrata

Nodule isolate *Rhizobium* AN-01 of *Neptunia prostrata* was tested on certain economically important crops of Manipur. The strains effectively nodulated *Phaseolus vulgaris* var las early as within two weeks. The strain was also found to incite nodule formation on roots of *Phaseolus vulgaris* var 2, *Vigna sinensis* S and *Vignia cylindrica.* The inoculation test on 4 local varities of *Pisum sativum,* 2 varieties *Glycine max,* 2 varieties of *Cicer aretinum* and *Vigna umbellata* were found to be negative (Table 1).

Several workers had reported the rhizobial infection process on the aquatic mimosoid legumes *Neptunia* sp. (Schaede, 1940; Debellera, 1986; Subha Rao *et al.,* 1995) and *N. plena* (James *et al.,*1992). However, a potential problem with an aquatic environment is that the plant-bacterial recognition signals necessary for the induction of the nodulation process (Sprent, 1989; Brewin, 1991; Hirsch. 1992) might be too greatly diluted. Subha Rao *et al.,* (1995) suggested that the cracks or wounds produced by aquatic legume with non-root hair infection process might allow for the accumulation of nod-gene inducing factors (eg. flavanoids; plant produced) and nodule inducing factor (eg. Lip-chitin oligosaccharides) (Spaink, 1995). It was possible that the dense mats of vegetation of the plant will allow for the accumulation of these chemicals. This was supported by the work of James *et al.,* (1993)

Table 1 : Response to some economically important leguminous crops of Manipur by the inoculation of *Neptunia prostrata* nodule isolate *Rhizobium* AN-01

Name of the plants	No. of weeks				
	1st	2nd	3rd	4th	5th
Cicer arietinum L. var1 (local name: Chana achouba)	–	–	–	–	–
Cicer arietinum L. var 2 (local name Chana)	–	–	–	–	–
Glycine max var 1 (Local name : Nunghawai macha)	–	–	–	–	–
Glycine max var 2 (Local name : Nunghawai achouba)	–	–	–	–	–
Phaseolus vulgaris var 1 (Local name : Hawai kalandri)	–	+	+	+	+
Phaseolus vulgaris var 2 (Local name : Rajma)	–	–	–	+	+
Pisum sativum var 1 (Local name : hawaithrak makhayatmubi)	–	–	–	–	–
Pisum sativum var 2 (local name : hawaithrak arangbi)	–	–	–	–	–
Pisum sativum var 3 (local name : hawaithrak asangba)	–	–	–	–	–
Pisum sativum var 4 (local name : Mangal)	–	–	–	–	–
Vicia faba (local name : Hawai mubi)	–	–	–	–	–
Vigna umbellata (local name : Chawai)	–	–	–	–	–
Vignia cylindrica (local name : Pong hawai)	–	–	+	+	+
Vigna sinensis (local name : Hawai asangbi)	–	–	+	+	+

which showed that stem extract from *N. plena* had substaintial nod-gene inducing activity. Interestingly, root nodule were not observed when the plants were grown in moving water bodies. The signalling molecule for the nodule inducing factors might probably be washed and carried away by the moving water.

Characterisation of nodule isolate of *Neptunia prostrata* showed that they were probably a new, hitherto unrecognized, species of *Rhizobium.* The unusual characteristics of the *Rhizobium* isolates of *Neptunia* sp. and the ability to fix atmospheric nitrogen to the aquatic ecosystem makes a positive approach to an eco-friendly farming technique. Further studies should be carried out for greater understanding of the biology of *Rhizobium-Neptunia prostrata* symbiosis and to exploit the potential of these unusual bacterium.

ACKNOWLEDGEMENT

The authors thank Dr. B.B. Gupta, Head, SAIF, for providing necessary facilities to carry out the experiement and Dr. Sudip Dey (Scientific Officer), Nari K. Rynjah (Scientific Technical Assistant), Dannis A. Bareh (Technical Assistant), Rahul Chakraborty (Technical Assistant), SEM Unit, SAIF, North East Hill University, and Dr. Begonia Dkhar (Scientific Officer), Anjali Haloi (Senior Technical Assistant), Aly Shodap (Senior Technical Assistant), Joston Paul Nongkynoih (Senior Technical Assistant), TEM Unit, SAIF, North Eastern Hill University, Shillong, for their technical assistance in conducting this research work. Thanks are also to CSIR, New Delhi for providing fellowship to M. Babita Devi and to Manipur University for Romesh Sagolshemcha.

REFERENCES

Allen, Ethel K. and Allen, O.N. 1950. Biochemical and Symbiotic properties of the Rhizobia. *Bacteriol Rev.;* **14(4):** 273-330.

Arora, R.K. 1981. Native food plants of North-Eastern India Tribes. In "*Glimpses of Indian Ethnobotany*", Edited by S. K. Jain. Published by Oxford and IBH. Pub. New Delhi-60.

Balatti, A.P. 1982. Culturing *Rhizobium* in large-scale fermentors. In: Graham, P.H and Harris, S. C. ed. *Biological nitrogen fixation technology for tropical agriculture.* Cali, Colombia, *Centro Internacional de Agricultura Tropical,* pp. 127-132.

Boddey, R.M. 1987. Methods for quantification of nitrogen fixation associated with Gramineae. *CRC Critical Reviews in Plant Sciences,* **6,** 209-266.

Brewin, N.J. 1991. Development of the legume root nodule. *Annual Review of Cell Biology* 191-226

Caetano-Annolles, G. and Gresshoff, P.M. 1991. Plant genetic control of nodulation. *Annl Rev Microbiol,* **11,** 793-804.

Chopra, R. N., Nayar S.L. and Chopra, I.C. 1956. *Glossary of Indian Medicinal Plants*. Council of Scientific and Industrial Reserch, New Delhi.

D'Souza Marie. 1985, *A Book on Tribal Medicine*. Pub. Soc. For Promotion of Waste land Development. New Delhi.

De Lajudie, P.; Laurent-Fulele, E.; Willems, A.; Torck, U.; Coopman, R.; Collins, M.DI.; Kersters, K.; Dreyfus, B., and Gillis, M. 1998. *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *Inter Jour Syst Bacteriol,* **48,** 1277-1290.

Deb. D.B. 1961. Dicotyledonous Plants of Manipur Territory. *Bull. Bot. Survey. India*. Vol. 3 (3&4) pp. 253-350.

Debelle, E; Rosenberg C.; Vasse J.; Maillet F.; Martinez E.; Denarie J. and Truchet G. 1986. Assignment of Symbiotic Developmental phenotypes to common and specific nodulation *(nod)* genetic loci of *Rhizobium meliloti. Journal of Bacteriology*. **168:** 1075-1108

Gibson, A.H. 1963. Environment affecting nodulation and nitrogen fixation by legumes. *Plant and Soil,* special volume, 139-152.

Hirsch, A.M. 1992. Developmental biology of legume nodulation. *New Phytologist,* **122:** 211-237.

James, E.K.; Sprent, J.T.; Sutherland, J.M.; Mc Inray, S.G. and Minchin F.R. 1992. The structure of nitrogen fixing root nodules on the aquatic mimosoid legume *Neptunia plena. Annals of Botany*. **69:** 173-180.

James, Euan K; Lourciro, Mde Fatima; Pott. A; Pott, V.J.; Martin, C.M.; Franco, A.A. and Sprent, J.I. 2001. Flooding tolerant legume symbiosis from Brazilian Pantanal. *New Phytologist* **150**(3): 723-738.

Jugindro Singh, A. 1982. Vegetables in Manipur. Published by A. Ibomcha Singh, Mongsangei, Imphal. Distributed by Punshi Marup, Chingamakha, Imphal-8, Manipur, India.

Kreig, N.R. and Holt, J.G 1984. *Bergey's Manual of determinative bacteriology,* Vol. I. Baltimore, MD, Williams and Wilkins.

Kuykendall, L.D., and Dazzo F.B. 1998. *Allorhizobium* De Lajudie, Laurent-Fulele, Willems, Torck, Coopman, Collins, Kersters, Dreyfus & Gillis, (1998). *Allorhizobium. In* D. Brenner, N. Krieg, J. Staley, and G. Garity (ed.), The Proteobacteria. *Bergey's mannual of systematic bacteriology,* 2nd ed., Vol. 2, in press. Springer-Verlag, New York, N.Y.

Nakamura, Y.; Murakami, A.; Koshimizu, K. and Ohigashi, H, 1996. Identification of pheophorbide a and its related compounds as possible anti-tumor promoters in the leaves of *Neptunia oleracea. Bioscience Biotechnology and Biochemistry* **60**(6): 1028-1030.

Loureiro, M.F.; James, E.K. and Franco, A.A. 1998. Nitrogen fixation by legumes in flooded region. pp. 195-233. In Scarano, F.R. & A.C. Franco (eds.). *Ecophysiological strategies of xerophytic and amphibious plants in the neotropics*. Series Oecologia Brasiliensis, Vol. IV. PPGE-UFRJ. Rio de Janeiro, Brazil.

Raul, Rivas; Encarna Velazquez.; Anne Willems.; Nieves Vizcaino.; Najappa S. Subha-Rao.; Pedro F. Mateos.; Monique Gillis.; Frank B. Dazzo and Eustoquio Martinez-Molina 2002. A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce. *App. Environ. Microbiol.* **68**(11): 5217-5222.

Schaede, R. 1940. *Dieknollchen deradvertiven, Wassenwurzeln. Von Neptunia oleracea und ihre Bakterien symbiose. Planta* **31**: 1-21.

Shah, G.L. and Rangayya, S. 1984. Pericarpial studies in *Neptunia oleracea. Acta Botanical Indica* **12**(1): 50-56.

Spaink, H.R. 1995. The molecular basis of infection and nodulation by rhizobia; the ins and outs of sympathogenesis. *Ann. Rev. Phytopathol.* **33**: 345-368.

Subha-Rao, N.S.; Mateos, P.F.; Baker, D.; Pankrazt, H.; Palma, J.; Dazzo, F.B. and Sprent, J.I. 1995. The unique root-nodule symbiosis between *Rhizobium* and the aquatic legume *Neptunia natans* (L.f.) Druce. *Planta* **196:** 311-320.

Subramanyam, K. 1974. *Aquatic Angiosperms.* Council of Scientific and Industrial Researh, New Delhi.

Windler, D.R. 1966. A revision of the genus *Neptunia* (Legumenosae). *Australian Journal of Botany,* 14:37.

(Accepted for publication July 30, 2010)