J. Phytol. Res. **19**(1) : 95-100, 2006

EFFECTS OF KH_2PO_4 AND PLANT GROWTH REGULATORS ON GROWTH AND NITROGEN METABOLIZING ENZYMES AND SOLUBLE PROTEIN IN *RHIZOBIUM* SP.

BARNALI DEY and R.C. SRIVASTAVA
Plant Physiology and Biochemistry Research Laboratory, Department of Life Sciences, Tripura University, Suryamaninagar, West Tripura-799130, India.

The symbiont *Rhizobium* sp. was isolated from the root nodules of *Vigna radiata* cultivated in the field. The isolate was sub-cultured in Yeast Extract Mannitol Broth (YEM) supplemented with optimum concentration of KH_2PO_4, three plant growth hormones (IAA, GA_3 and kinetin) and their different combinations. In free-living cultures, the growth behaviour, nitrate reductase (NR) activity, nitrite reductase (NiR) activity, nitrogenase (N_2-ase) activity and soluble protein were found to be stimulatory with the application of KH_2PO_4, hormones and their various combinations.

Keywords: GA_3; IAA; KH_2PO_4; Kinetin; N_2-ase; NiR; NR; *Rhizobium* sp.

Introduction

Reduction of inorganic nitrate to nitrite by nitrate reductase (EC:1.6.6.2) in higher plants[1] is of common occurrence. Its presence in free-living rhizobial culture, however, is rare[2]. Manhart and Wong[3] isolated bacteroids from various species of *Rhizobium* and found no NR activity in *R. phaseoli, R. leguminosarum, R. lupini and R. trifoli,* but found in bacteroids of *R. meliloti, R. japonicum and R.* spp. of cowpea strain. Siddiqui *et al.*[4] also reported NR activity in *Rhizobium leguminosarum* grown in free-living culture.

Asymbiotically rhizobia are known to produce indole-acetic acid[5] and cytokinins[6] and a relatively high amount of these hormones have also been detected in the root nodules[7]. Fine balancing of the phytohormones triggers the initiation and development of nodules[8]. Exogenous application of hormones has also been shown to enhance the nodulation of pea[9].

Phosphorus plays a major role in the build up and maintenance of soil fertility through its effect on legume growth. Rhizobia differ in their nodulating capacity at low phosphate levels and a delay in infection was observed for nodulation of subclover[10] and soybean[11]. The impaired nodulation frequency and nodule development among phosphate limited strains could be attributed to reduction in Nod metabolite excretion in *R. leguminosarum* bv. *trifolii*[12] or to alteration in the cell surface antigens in *R. etli*[13]. Competition studies between two serogroups of *R. trifolii* demonstrated that limiting of soil to increase the available phosphates replaced the dominant serogroup by a minor serogroup. The addition of phosphate alone had little effect on the out come of competition between the two strains. However, the addition of phosphate and lime restored the dominance of the original dominant serogroup[14]. Renwick and Jones[15] showed that increasing levels of lime significantly influenced the relative proportion of nodules formed on white clover by inoculating with two strains applied at equal rates.

The present study embodies work on cumulative effects of KH_2PO_4 and plant growth hormones on growth, activities of NR, NiR and N_2-ase and soluble protein in the free-living culture of *Rhizobium* isolated from the nodules of *Vigna radiata* (mung-bean).

Materials and Methods

The *Rhizobium* was isolated from nodules of field grown green-gram plants. The *Rhizobium* was purified by standard microbiological technique[16] and its culture was raised in Yeast Extract Mannitol (YEM) broth for 120 h. The effect of optimum concentration of KH_2PO_4, IAA, GA_3 and kinetin and their different combinations, *viz.*, 1mM KH_2PO_4 (T_1), 1µg/ml IAA (T_2), 1µg/ml GA_3 (T_3), 1µg/ml kinetin (T_4), 1mM KH_2PO_4+ 1µg/ml IAA (T_5), 1mM KH_2PO_4 + 1µg/ml GA_3 (T_6), 1mM KH_2PO_4 + 1µg/ml kinetin (T_7) and 1mM KH_2PO_4 + 1µg/ml IAA + 1µg/ml GA_3+ 1µg/ml kinetin (T_8) were used to study rhizobial population. For this study, 1.0 ml rhizobial suspension of uniform turbidity (Absorbance, 0.1) was added to 30 ml of culture broth medium. This 31 ml suspension was maintained at 28 ± 1°C in an incubator-cum-shaker. The absorbance of the growing culture was noted at 610 nm at an interval of 24 h and onward up to 120 h. Six replicates of culture were used for each treatment. Only one concentration of KH_2PO_4 and of plant growth regulators was used because we have already established optimum concentration of KH_2PO_4 (1 µM) and each PGR

Table 1. Time course of the growth behaviour (in terms of optical density at 610 nm) of *Rhizobium* sp. in free-living culture (YEM broth). The culture was raised in presence of optimum concentration of KH_2PO_4 or IAA orGA_3 or KIN and their various combinations.

Treatments		Rhizobial growth (O.D. at 610 nm) Time periods (Hours after inoculation)				
		24	48	72	96	120
T_0	Control	0.24±0.002	0.40±0.003	0.51±0.004	0.63±0.001	0.62±0.004
T_1	1mM KH_2PO_4	0.30±0.001	0.46±0.001	0.60±0.001	0.70±0.003	0.75±0.002
T_2	1µg/ml IAA	0.26±0.000	0.45±0.001	0.55±0.006	0.67±0.002	0.75±0.007
T_3	1µg/ml GA_3	0.28±0.000	0.52±0.000	0.56±0.002	0.70±0.001	0.73±0.001
T_4	1µg/ml KIN	0.26±0.002	0.51±0.001	0.60±0.002	0.68±0.001	0.72±0.000
T_5	1mM KH_2PO_4 + 1µg/ml IAA	0.25±0.000	0.55±0.001	0.57±0.000	0.68±0.002	**0.81±0.002**
T_6	1mM KH_2PO_4 + 1µg/ml GA_3	0.32±0.000	0.41±0.001	0.61±0.002	0.63±0.002	0.66±0.008
T_7	1mM KH_2PO_4 + 1µg/ml KIN	0.27±0.000	0.41±0.003	0.63±0.001	0.61±0.001	0.80±0.001
T_8	1mM KH_2PO_4 + 1µg/ml IAA + 1µg/ml GA_3 + 1µg/ml KIN	0.24±0.000	0.54±0.000	0.62±0.001	0.70±0.001	**0.81±0.002**

Values are mean (N=6) ± error.

Table 2. Nitrate reductase (NR) activity in the free-living culture of *Rhizobium* sp. The measurement was done at different hours (24 to 120 h) of growth. The culture was supplemented with optimum concentration of KH_2PO_4 and plant growth hormones and their different combinations.

Treatments		NR activity (µ mol $NO_2^-h^{-1}ml^{-1}$ rhizobial suspension) Growth periods (Hours after inoculation)				
		24	48	72	96	120
T_0	Control	0.09±0.017	0.12±0.001	0.15±0.001	0.18±0.003	0.13±0.006
T_1	1mM KH_2PO_4	0.09±0.003	0.15±0.008	0.15±0.006	0.20±0.009	0.15±0.002
T_2	1µg/ml IAA	0.09±0.003	0.15±0.001	0.17±0.001	0.21±0.000	0.16±0.004
T_3	1µg/ml GA_3	0.09±0.004	0.12±0.013	0.15±0.010	0.22±0.002	0.18±0.003
T_4	1µg/ml KIN	0.09±0.002	0.16±0.000	0.20±0.019	0.23±0.026	0.15±0.000
T_5	1µM KH_2PO_4 + 1mg/ml IAA	0.09±0.004	0.13±0.000	0.19±0.005	0.27±0.026	0.18±0.004
T_6	1mM KH_2PO_4 + 1µg/ml GA_3	0.10±0.007	0.14±0.012	0.15±0.003	0.25±0.021	0.16±0.003
T_7	1mM KH_2PO_4 + 1µg/ml KIN	0.10±0.002	0.14±0.004	0.19±0.030	0.19±0.004	0.19±0.006
T_8	1mM KH_2PO_4 + 1µg/ml IAA + 1µg/ml GA_3 + 1µg/ml KIN	0.09±0.002	0.17±0.003	0.26±0.074	0.41±0.079	0.20±0.001

Values are mean (N=3) ± error.

Table 3. Nitrite reductase (NiR) activity in the free-living culture of *Rhizobium* sp. The measurement was done at different hours (24 to 120 h) of growth. The culture was supplemented with optimum concentration of KH_2PO_4 and plant growth hormones and their different combinations.

Treatments		NiR activity (μ mol NH_3 produced $h^{-1}ml^{-1}$ rhizobial suspension) Growth periods (Hours after inoculation)				
		24	48	72	96	120
T_0	Control	0.40±0.059	0.87±0.010	0.92±0.066	1.00±0.024	0.68±0.007
T_1	1mM KH_2PO_4	0.55±0.010	1.05±0.010	1.07±0.024	1.49±0.003	1.16±0.007
T_2	1μg/ml IAA	0.54±0.004	1.03±0.034	1.41±0.073	1.74±0.007	0.96±0.031
T_3	1μg/ml GA_3	0.60±0.007	1.07±0.009	1.16±0.042	1.28±0.014	0.79±0.013
T_4	1μg/ml KIN	0.78±0.003	1.03±0.013	1.26±0.074	1.56±0.099	0.84±0.036
T_5	1mM KH_2PO_4 + 1μg/ml IAA	0.98±0.017	1.18±0.049	1.50±0.008	**2.55±0.014**	0.73±0.002
T_6	1mM KH_2PO_4 + 1μg/ml GA_3	0.85±0.005	1.12±0.024	1.21±0.005	1.38±0.007	0.96±0.022
T_7	1mM KH_2PO_4 + 1μg/ml KIN	1.00±0.013	1.44±0.012	1.50±0.051	1.88±0.025	1.11±0.021
T_8	1mM KH_2PO_4 + 1μg/ml IAA + 1μg/ml GA_3 + 1μg/ml KIN	0.70±0.015	0.96±0.002	0.98±0.020	1.07±0.039	0.87±0.019

Values are mean (N=3) ± error.

Table 4. Nitrogenase activity (N_2-ase) in the free-living culture of *Rhizobium* sp. The measurement was done at different hours (24 to 120 h) of growth. The culture was supplemented with optimum concentration of KH_2PO_4 and plant growth hormones and their different combinations.

Treatments		N_2-ase activity (μ mol NH_3 produced $h^{-1}ml^{-1}$ rhizobial suspension) Growth periods (Hours after inoculation)				
		24	48	72	96	120
T_0	Control	0.38±0.008	0.76±0.001	1.10±0.007	1.39±0.049	0.96±0.021
T_1	1mM KH_2PO_4	0.64±0.021	1.01±0.085	1.41±0.117	1.58±0.088	0.94±0.020
T_2	1μg/ml IAA	0.92±0.008	1.15±0.010	1.33±0.014	1.35±0.003	0.92±0.006
T_3	1μg/ml GA_3	0.58±0.020	1.14±0.002	1.76±0.002	2.37±0.009	0.97±0.004
T_4	1μg/ml KIN	0.80±0.029	1.08±0.010	1.24±0.032	1.31±0.025	0.92±0.010
T_5	1mM KH_2PO_4 + 1μg/ml IAA	1.13±0.014	1.36±0.028	1.43±0.078	1.56±0.015	1.35±0.002
T_6	1mM KH_2PO_4 + 1μg/ml GA_3	0.57±0.007	1.72±0.003	1.85±0.010	**2.67±0.146**	1.15±0.002
T_7	1mM KH_2PO_4 + 1μg/ml KIN	0.43±0.005	1.24±0.007	1.27±0.007	2.14±0.060	1.52±0.085
T_8	1mM KH_2PO_4 + 1μg/ml IAA + 1μg/ml GA_3 + 1μg/ml KIN	0.92±0.023	0.98±0.010	1.15±0.003	1.99±0.157	0.63±0.007

Values are mean (N=3) ± error.

Table 5. Level of soluble protein in the rhizobial culture of control set and of those receiving optimum concentration of KH_2PO_4, PGRs and their various combinations. The measurement was done at different hours (24 to 120 h) of growth.

Treatments		Level of soluble protein (mg/ml rhizobial suspension) Growth periods (Hours after inoculation)				
		24	48	72	96	120
T_0	Control	0.43±0.026	0.51±0.041	0.66±0.011	0.74±0.029	0.70±0.034
T_1	1mM KH_2PO_4	0.46±0.028	0.60±0.011	0.67±0.017	0.83±0.007	0.81±0.012
T_2	1µg/ml IAA	0.44±0.008	0.61±0.037	0.65±0.026	0.81±0.031	0.81±0.022
T_3	1µg/ml GA_3	0.49±0.020	0.66±0.010	0.75±0.034	0.83±0.003	0.80±0.017
T_4	1µg/ml KIN	0.45±0.032	0.61±0.028	0.68±0.039	0.81±0.025	0.80±0.000
T_5	1mM KH_2PO_4 + 1µg/ml IAA	0.50±0.019	0.74±0.038	0.80±0.026	0.91±0.018	0.84±0.028
T_6	1mM KH_2PO_4 + 1µg/ml GA_3	0.65±0.012	0.74±0.014	0.79±0.027	0.96±0.035	0.86±0.008
T_7	1mM KH_2PO_4 + 1µg/ml KIN	0.53±0.012	0.68±0.012	0.80±0.059	0.88±0.026	0.79±0.006
T_8	1mM KH_2PO_4 + 1µg/ml IAA + 1µg/ml GA_3 + 1µg/ml KIN	0.36±0.017	0.76±0.017	0.77±0.025	**0.97±0.033**	0.78±0.014

Values are mean (N=3) ± error.

(1 µg/ml) in our previous experiments[17].

The *in vivo* NR activity was assayed by the method of Hageman and Hucklesby[18] and the *in vivo* NiR activity was determined by the method of Srivastava *et al.*[19]. The *in vivo* N_2-ase activity was estimated by a modified micro-diffusion method[20]. The soluble protein of the rhizobial cells was estimated by the method of Lowry *et al.*[21].

Results and Discussion

The effects of optimum concentration of KH_2PO_4 (T_1), IAA (T_2), GA_3 (T_3), KIN (T_4) and their various combinations showed an increasing trend over a growth period of 24 to 120 h. However, in the case of control set all these parameters showed increasing trend up to 96h followed by a slight decrease on 120h of growth. Certain variation was noticed in the optimum period for maximum level of various enzymes and soluble protein in the treated sets. Compared to control, the rhizobial growth, activities of NR, NiR and N_2-ase and level of soluble protein all were much higher in various treatments (T_1 to T_8).

Rhizobial growth showed an increasing trend over a period of 120 h. The growth was maximum on 120 h in the treated sets, and 96 h in the control set. The most effective combination was noticed with 1 mM KH_2PO_4 +1 µg/ ml IAA+1 µg/ ml GA_3+1 µg/ml KIN on 72, 96 and 120 h after inoculation. The data is shown in the Table-1.

The *in vivo* NR activity in free-living culture of *Rhizobium* showed an increasing trend over a period of 96 h after inoculation. The most effective combinations was found to be 1 mM KH_2PO_4 +1 µg/ ml IAA+1 µg/ ml GA_3+1 µg/ml KIN that resulted maximum enzyme activity. The data is shown in the Table-2. *In vivo* NiR activity in free-living culture of *Rhizobium* showed an increasing trend over a period of 96 h after inoculation in almost all combination sets. The most effective combination was found to be with 1 mM KH_2PO_4+ 1 µg/ ml IAA. The data is shown in the Table-3. *In vivo* N_2-ase activity in the free-living culture of *Rhizobium* showed an increasing trend over a period of 96 h after inoculation in almost all sets. The most effective combination was found to be with 1 mM KH_2PO_4+ 1 µg/ ml GA_3. The data is shown in the Table-4.

The level of soluble protein in the free-living culture of *Rhizobium* showed an increasing trend in all sets over a period of 96 h after inoculation and the levels slightly decreased on 120 h. The most effective combination was found to be 1 mM KH_2PO_4 +1 µg/ ml IAA+1 µg/ ml GA_3+1 µg/ml KIN that resulted maximum level of soluble protein. The data is shown in the Table-5.

In a nutshell it can be said that rhizobial growth, *in vivo* NR activity and soluble protein were maximum with T_8, whereas, the *in vivo* N_2-ase and NiR activities were maximum with T_5 and T_6 respectively. The results on growth, NR, N_2-ase and NiR activities and soluble protein indicated that application of KH_2PO_4 with plant growth

hormones caused enhancement in these parameters. Increase in rhizobial growth and N_2-ase activity for longer time during incubation with KH_2PO_4 and PGRs indicated that the survivability and N_2-fixing efficiency of rhizobia was prolonged with this treatment. From this observation it is expected that in the soil application of KH_2PO_4 would prove beneficial for the growth of free-living N_2-fixers. Increased nitrogen fixation by applied phosphorous which in turn supplies nitrogen for the biological activity of the plant (*Sesbania rostrata*) was noticed by Helepyati and Sheelavantan[22]. Phosphorous also plays a major role in the build up and maintenance of soil fertility through its effect on legume growth. Rhizobia differ in their nodulating capacity at low phosphate levels and a delay in infection was observed for nodulation of subclovar[10] and soybean[11]. Phosphorous application increased *Rhizobium* population by 2.6-fold and increased soybean nodulation significantly. Total N content of the soil also increased significantly. Biological nitrogen fixation (N_2-ase activity) increased by the application of P. This is because biological N_2 fixation is an energy-requiring process and P is directly involved in energy synthesis[23]. An adequate supply of phosphorous is necessary for proper root development and functioning of the nodules[24, 25]. Sheoram *et al.*[26] and Taneja *et al.*[27] observed good response of berseem to P fertilization. Halepyati and Sheelavantar[22] reported increased nitrogen fixation by applied phosphorous, which in turn supplies nitrogen for the biological activity of the plant. Tiwari *et al.*[28] reported beneficial effect of P nutrition on N_2 fixation by many pulse crops in P-deficient soils.

The genus *Rhizobium* is well characterized by its novel association with legumes for fixing atmospheric nitrogen. Nitrogen fixation by *Rhizobium*. sp has been reported in asymbiotic cultures[29]. The presence of active NR which reduces NO_3^- into NO_2^- in *Rhizobium* bacteroids has been well documented[3,30], but no correlation between NR activity and nitrogen fixation has been obtained. Antouch *et al.*[31] studied NR activity in 41 strain of *R. meliloti*, but found no correlation with dinitrogen fixation. Singh *et al.*[32] showed that NR deficient mutant of *R. japonicum* have more nitrogen fixing capacity. However, in the present investigation an increasing trend in the soluble protein and *in vivo* activities of NR, NiR and N_2-ase was noticed with treatments of KH_2PO_4 and PGRs which indicated that all these parameters are favourably affected with these treatments.

References

1. Beevers L and Hageman R H 1962, Nitrate reductase in higher plants. *Ann. Rev. Plant Physiol.* **206** 495-522.
2. Daniel R M and Appleby C A 1972, Anaerobic nitrate, symbiotic and aerobic growth of *Rhizobium japonicum*: Effect on Cytochrome P_{450}, other haemoproteins, nitrate and nitrite reductases. *Biochemica et Biophysica Acta* **275** 347-354.
3. Manhart J R and Wong P P 1979, Nitrate reductase activitiy of rhizobia and the correlation between nitrate reduction and nitrogen fixation. *Can. J. Microbiol.* **25** 1169-1174.
4. Siddiqui M H, Mathur A and Mathur S N 1982, Significance of nitrate reductase in nitrate assimilation in free-living *Rhizobium* culture. *Experientia* **38** 1208-1210.
5. Ernstesen A, Sandberg G, Crozier A and Wheeler C T 1987, Endogenous indoles and the biosynthesis and metabolism of indole-3-acetic acid in culture of *Rhizobium phaseoli. Planta* **171** 422-428.
6. Taller B J and Sturtevant D B 1991, Cytokinin production by rhizobia. In: *Advances in Molecular genetics of Plant-Microbe Interaction* (Eds.) H. Hennecke and D.P.S. Verma, Kluwer Academic Publishers. The Netherland **I** 215-221.
7. Badenoch-Jones J, Rolfe B G and Letham D S 1984, Phytohormones, *Rhizobium* mutants and nodulation in legumes. IV. Auxin metabolites in pea root nodules. *J. Plant Growth Regul.* **3** 32-39.
8. Verma D P S 1992, Signals in root nodule organogenesis and endocytosis of *Rhizobium. The Plant Cell* **4** 373-382.
9. Nandwal A S, Bharti S, Garg O P and Ram P C 1981, Effect of indole acetic acid and kinetin on nodulation and nitrogen fixation in pea (*Pisum sativum* L.). *Indian J. Plant Physiol.* **24** 47-52.
10. Leung K and Bottomley P J 1988, Influence of phosphate on growth and nodulation characteristics of *Rhizobium trifolii. Appl. Environ. Microbial.* **53** 2098-2105.
11. Mullen M D, Israel D W and Wollum A G.II 1988, Effects of *Bradyrhizobium japonicum* and soybean (*Glycine max* (L.) Merr.) phosphorus nutrition on nodulation and dinitrogen fixation. *Appl. Environ. Microbiol.* **54** 2387-2392.
12. Mc Kay IA and Djordjevic M A 1993, Production and excretion of Nod metabolites by *Rhizobium leguminosarum* bv. *trifolii* are disrupted by the same environmental factors that reduce nodulation in the field. *Appl. Environ. Microbiol.* **59** 3385-3392.
13. Tao H, Brewin N J and Noel K D 1992, *Rhizobium leguminosarum* CFN42 lipopoly-saccharide antigenic changes induced by environmental conditions. *J. Bacteriol.* **174** 2222-2229.
14. Almendras A S and Bottomley P J 1997, Influence of lime and phosphate on nodulation of soil –grown *Trifolium subterraneum* L. by indigenous *Rhizobium trifolii. Appl. Environ. Microbiol.* **55** 2090-2097.

15. Renwick A and Jones D 1986, The manipulation of white clover "host preference" for strains of *Rhizobium trifolii* in an upland soil. *Ann. Appl. Biol.* **108** 291-302.
16. Vincent J M 1970, In: *A Manual for the Practical Study of Root- Nodule Bacteria.* IBH Handbook No. 15, International Biological Programme (IBP), 7, Marylebone Rode, London, NWI, Blackwell Scientific Publn, Oxford and Edinburg.
17. Dey B 2004, Studies on nitrogen metabolism in *Vigna radiata* (L.) Wilczek in relation to application of certain mineral nutrients and plant growth regulators. Ph. D. Thesis (Life Sciences), Tripura University, Agartala, India.
18. Hageman R H and Hucklesby D P 1971, Nitrate reductase from higher plants. In: *Methods in Enzymology.* (Ed.) A. San Pietro, Academic Press London. **23A** 491-503.
19. Srivastava R C, Bose B, Mukherji D, Mathur S N and Srivastava H S 1979, Measurement of nitrite reductase in leaf tissue of *Vigna mungo. Planta* **147** 196-198.
20. Srivastava R C, Mukherji D and Mathur S N 1980, A freeze/thaw technique for estimation of nitrogenase activity in detached nodules of *Vigna mungo. Annals of Appl. Biol.* **96** 235-241.
21. Lowry O H, Rosenbrough N J, Farr A L and Randall, R J 1951, Protein measurement with the Folin-Phenol Reagent. *J. Biol. Chem.* **193** 265-275.
22. Halepyati A S and Sheelavantan M M 1992, Influence of plant densities and phosphorus level on dry matter production by *Sesbania rostrata. J. Maharashtra Agric. Univ.* **17** 324-325.
23. Raychaudhuri M, Kumar K and Raychaudhuri S 1997, Effect of *Rhizobium japonicum* and phosphorus on nutrient uptake and yield of soyabean (*Glycine max*) in an Ultisol of Manipur hills. *Indian J. Agricultural Sciences* **67** 459-462.
24. Keshikar P B, Jadha OS L, Turphede AB and Shivde V V 1991, Irrigation and phosphorus requirement of barseem. *Forage Research* **17 (1)** 89-92.
25. Rana D S, Sheoram R S, Joon R K and Yadav B D 1991, Effect of sowing dates, seed rates and phosphorus levels on fodder and seed production of egyption clover. *Forage Research* **18(1)** 134-136.
26. Sheoram R S, Rana D S, Joon R K, Yadav B D and Jatasra D S 1992, Forage and seed production of berseem varieties under varying phosphorus level. *Forage Research* **18(1)** 37-41.
27. Taneja K D, Sharma H C and Singh D P 1994, Weather yield relationship in egyption clover. *Forage Abstract* **64(6)** 1-567.
28. Tiwari V N, Lehri L K and Pathak A N 1989, *Rhizobium* inoculation of legumes as influenced by phosphorus and molybdenum fertilization. *Journal of Indian Society of Soil Science* **37** 712-716.
29. Van Brussel A A N, Costerton J W and Chitd J J 1979, Nitrogen fixation by *R.* sp. A morphological and ultrastructural composition of asymbiotic and symbiotic nitrogen fixing form. *Can. J. Microbial.* **25** 352-361.
30. Kennedy I R, Rigaud J and Triuchant J C 1975, Nitrate reductase from bacteroids of *R. japonicum* enzyme characteristics and possible interaction with nitrogen fixation. *Biochem. Biophys. Acta* **397** 24-35.
31. Antouch H, Bordeleau L M, Prevost D and Lachance R A 1980, Absence of correlation between nitrate reductase and symbiotic nitrogen fixation efficiency in *Rhizobium meliloti. Can. J. Plant Sci.* **60** 209-219.
32. Singh R K, Singh R B, Singh R M and Singh B D 1980, Nitrogen fixation by nitrate reductase deficient mutant of *R. japonicum. Indian J. Exp. Biol.* **18** 1165-1167