EFFECT OF HEAVY METALS ON GROWTH OF *RHIZOBIUM* STRAINS AND SYMBIOTIC EFFICIENCY OF TWO SPECIES OF TROPICAL LEGUMES

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Abstract: Study on the effect of three heavy metals (Al, Fe, Mo) were studied on two strains of rhizobia isolated from root nodules of two tropical legume species, *Mucuna pruriens* and *Trigonella foenumgraecum*. The effects were assessed for bacterial growth in culture and in symbiotic parameters such as biomass production and nodulation in host plant. Aluminium was found to have detrimental effect in both in-vitro and in-vivo condition in all its concentration. Iron was found to support bacterial growth and symbiotic parameters such as biomass production and nodulation up to 25 µM. Above 25 µM iron was found to have negative effect. Molybdenum was beneficial up to the 75 µM concentration in culture of both strains of bacteria while in plant productivity and nodulation it was beneficial only up to 20 µM.

Key words: Bacterial growth; Mucuna; Nitrogen fixation; Nodulation; Plant growth; Trigonella.

INTRODUCTION

It has been already established that *Rhizobium* has symbiotic N_2 fixation with leguminous plants. The symbiotic relation, as it occurs in economically important plants, has deep concern with human interest. The symbiosis not only is beneficial for the associates but ultimately when the legume get benefited the human economic concern is benefited. Several scientific researches have been conducted to improvise the symbiotic relation. Several researchers have been devoted to identify suitable and harmful conditions for the symbiosis to be improved to maximum limit. Various environmental factors effecting symbiosis such as salinity (Chen *et al*., 1992), pH (Brockwell, 1991), has already been assessed. Optimum level of physiological function can be achieved in suitable environment and adequate nutrient supplies. Some elements, such as heavy metals, though essential for organisms, are harmful if present in excess. Aluminium has adverse effect on nodule initiation (Kim *et al*., 1985; Brady *et al*., 1990). According to Giller *et al*. (1998), effect of heavy metals depends upon duration of exposure, dose and type of metal used. Kinkle *et al*. (1994) reported that *Sinorhizobium meliloti* strains have high tolerance ability to various heavy metals. In legume-*Rhizobium* symbiosis, maximum yield is possible when there is suitable condition for both partners. Present investigation aims to analyze the effect of aluminium, iron and molybdenum on rhizobial growth, crop productivity and nodulation parameters of two tropical legume plant *Mucuna pruriens* and *Trigonella foenumgraecum*.

METERIALS AND METHODS

Effect of three metals; Al, Fe, and Mo were investigated on two strains of rhizobia (MPR_8 and TFR_3), nodulating two tropical legume species *Mucuna pruriens* and *Trigonella foenumgraecum* respectively. Effects of these metals were studied on 0ìM, 25 ìM, 50 ìM, 75 ìM and 100 ìM concentrations of their corresponding salts ($AlCl_3.6H_2O$, $FeCl_3$ and $Na_2Mo_4.2H_2O$). The effects were assessed on bacterial growth *in vitro* and host plant productivity and nodulation *in vivo*.

i) In bacterial culture:

A loopful of log phase culture of each strain was inoculated separately in flasks containing Yeast Extract Mannitol (YEM) broth medium amended with different salt concentrations of Al, Fe and Mo. The inoculated flasks were then placed at 28±1°c on a shaker at speed 150 rpm. To obtain growth rate, the optical density of each flask was measured after every 4 hours up to 40 hours at wavelength 610 nm. Specific growth rate of both the strains MPR_8 and TFR_3 were calculated using the formula given by Stanier *et al*. (1985)

$$\text{Specific growth} = \frac{\text{Log OD}_1 - \text{Log OD}_0}{T_1 - T_0} \times 2.303$$

Where:

Log OD_1 = Log value of O. D. of culture at time t hour

Log OD_0 = Log value of O. D. of culture at time t_0 hour

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T_1-T_0 = Difference between time interval or duration of incubation.

ii) In host plant productivity:

Surface sterilized seeds of two cultivars of tropical legume *Mucuna pruriens* and *Trigonella foenumgraecum* were inoculated with log phase rhizobial culture and sown in earthenware pots containing sterilized soil amended with different concentration of Al, Fe and Mo salts. Each pot was watered with sterile distilled water up to 45 days. After 45 days the plants were uprooted and the nodule number, nodule weight, plant height and plant weight were recorded for both species in different concentration of heavy metal treatment.

RESULTS

i) Effect of aluminium on bacterial culture and plant productivity

Application of Al was found inhibitory for both strains of rhizobia at all concentrations. Specific growth rate of bacteria at culture was found to decreased form 0.27 in control to 0.17 in 100 ìM of aluminium in MPR_8 (Fig. 1) and form 0.34 to 0.16 for the same condition in TFR_3 (Fig. 2). Similarly, aluminium was observed to have negative effect in all concentration in symbiotic plant productivity and nodulation of both plants (Table 1 and 2).

ii) Effect of iron on bacterial culture and plant productivity

Iron was found beneficial up to the 25 ìM concentration in culture of both strains of bacteria and in plant productivity. Specific growth rate of MPR_8 increased form 0.27 in control to 0.29 in 25 ìM of $FeCl_3$ and beyond 25 ìM it starts to decline reaching 0.17 at 100 ìM of the same (Fig 3). Similarly, in case of TFR_3 the specific growth rate increased form 0.28 to 0.31 for 0 ìM to 25 ìM respectively and reached to 0.18 at 100 ìM (Fig 4). The crop productivity and nodulation of both plants was also found increased on supplying $FeCl_3$ form 0 ìM to 25 ìM and above 25 ìM it was found to have negative effect (Table 3 and 4).

iii) Effect of molybdenum on bacterial culture and plant productivity

Molybdenum was found beneficial up to the 75 ìM concentration in culture of both strains of bacteria while it was beneficial only up to 20 ìM in plant productivity and

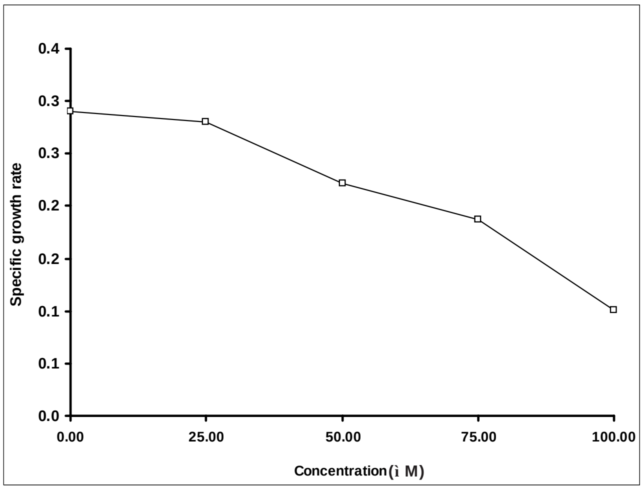

Fig. 1: Effect of Al on specific growth rate of TFR_3

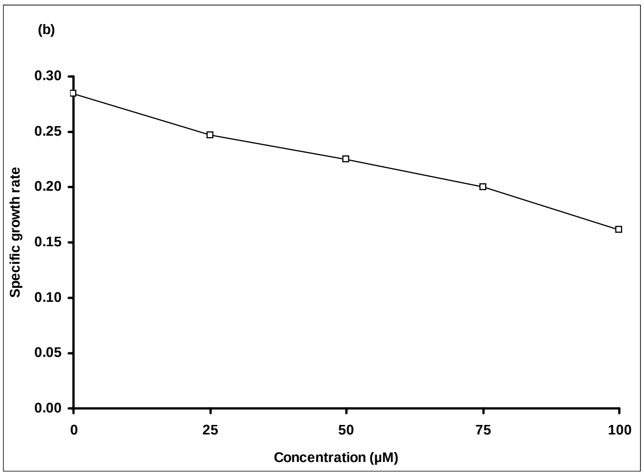

Fig. 2: Effect of Al on specific growth rate of MPR_8

Table 1: Effect of Al on crop yield and nodulation of *Mucuna pruriens.*

Conc.(µM)	Plant dry weight (gm)	Plant height (cm)	Nodule number per plant	Nodule fresh wt. per plant (gm)
0	3.418	341	29	1.024
25	0.541	170	9	0.045
50	0.409	158	3	0.005
75	0.336	152	0	0
100	0.258	90	0	0

Table 2: Effect of Al on crop yield and nodulation of *Trigonella foenumgraecum.*

Conc.(µM)	Plant dry weight (gm)	Plant height (cm)	Nodule number per plant	Nodule fresh wt. per plant (gm)
0	0.720	40	25	0.921
25	0.634	17	17	0.215
50	0.323	15	10	0.102
75	0.222	12	5	0.027
100	0.178	8	2	0.009

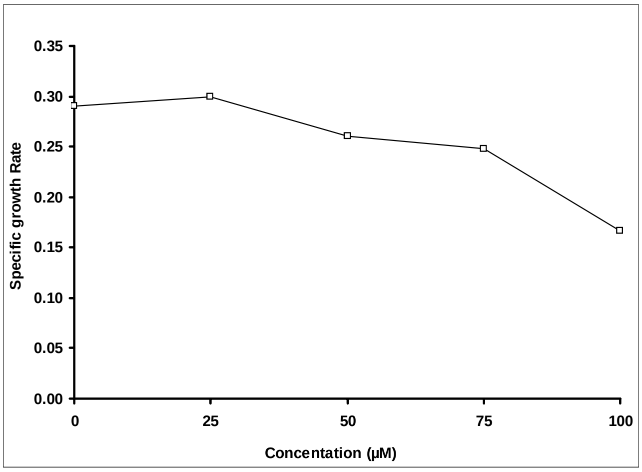

Fig. 3: Effect of Fe on specific growth rate of TFR_3

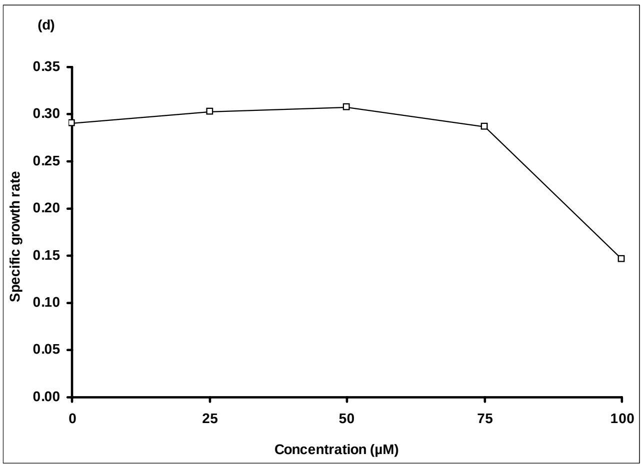

Fig. 5: Effect of Mo on specific growth rate of TFR_3

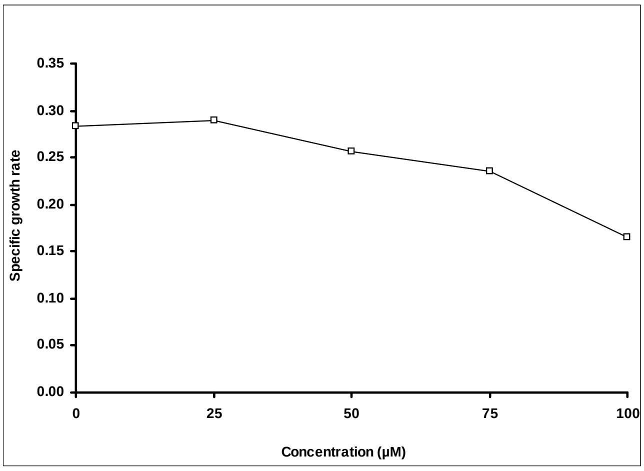

Fig. 4: Effect of Fe on specific growth rate of MPR_8

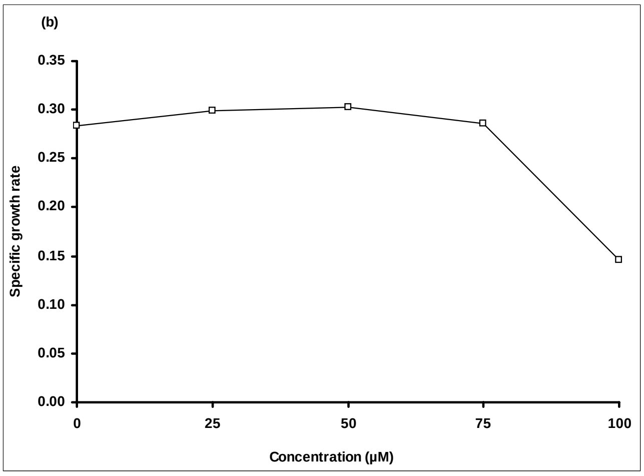

Fig. 6: Effect of Mo on specific growth rate of MPR_8

nodulation. Specific growth rate of MPR_8 increased form 0.27 in control to 0.31 in 75 ìM of Na_2Mo_4 and beyond 75 ìM it starts to decline reaching 0.16 at 100 ìM of the same (Fig 5). Similarly, in case of TFR_3 the specific growth rate increased form 0.28 to 0.30 for 0 ìM to 75 ìM respectively and reached to 0.15 at 100 ìM (Fig 6). The crop productivity and nodulation of both plants were also found increased on supplying Na_2Mo_4 form 0 ìM to 20 ìM and above 20 ìM it was found to have negative effect (Table 5 and 6).

DISCUSSION

The result of this study revels that aluminium, even in small concentration, has negative effect on rhizobial growth kinetics in cultural condition (Fig 1 and 2). Wood and cooper (1988)

Table 3: Effect of Fe on crop yield and nodulation of *Mucuna pruriens.*

Conc.(µM)	Plant dry weight (gm)	Plant height (cm)	Nodule number per plant	Nodule fresh wt. per plant (gm)
0	3.418	341	29	1.024
25	3.740	349	31	11.23
50	3.152	303	22	0.529
75	1.336	287	11	0.296
100	0.670	135	6	0.041

Table 4: Effect of Fe on crop yield and nodulation of *Trigonella foenumgraecum.*

Conc.(µM)	Plant dry weight (gm)	Plant height (cm)	Nodule number per plant	Nodule fresh wt. per plant (gm)
0	0.720	40	25	0.921
25	0.917	47	30	1.006
50	0.780	41	27	0.974
75	0.452	32	10	0.089
100	0.215	26	6	0.008

Table 5: Effect of Mo on crop yield and nodulation of *Mucuna pruriens.*

Conc.(µM)	Plant dry weight (gm)	Plant height (cm)	Nodule number per plant	Nodule fresh wt. per plant (gm)
0	3.418	341	29	1.024
10	4.01	257	33	1.248
20	3.88	353	26	0.983
30	2.66	204	13	0.502
40	2.25	181	7	0.049
50	1.48	106	4	0.007

Table 6: Effect of Mo on crop yield and nodulation of *Trigonella foenumgraecum.*

Conc.(µM)	Plant dry weight (gm)	Plant height (cm)	Nodule number per plant	Nodule fresh wt. per plant (gm)
0	0.72	40	25	0.921
10	0.82	48	29	1.001
20	0.75	33	25	0.887
30	0.48	30	19	0.549
40	0.39	25	15	0.218
50	0.28	19	10	0.046

reported inhibition of multiplication of rhizobial strain at 50 ìM Al concentration. Al toxicity is great problem under acidic medium as solubility of free Al ion (Al^{+++}) increases rapidly under acidic condition (Mc Lean, 1976). As rhizobial strains studied here are fast growing acid producing bacteria, it promotes Al to show its full toxicity. Since the distribution of various ionic species of Al is pH dependent (Martin, 1991), slight change in pH may significantly affect the relative concentration of the various charged Al species and their ligands and hence the toxicity of aluminium. Further, slight change in pH alone can significantly affect the growth of root nodule bacteria (Thornton and Davey, 1983; Richardson and Simpson, 1989). In combination with acidity, Al is detrimental to root nodule bacteria in those cases where growth occurs to longer log periods, decreases growth rates and lowers final cell densities (Whelan and Alexander. 1986). The toxic effect of aluminium is due to its effect on bacterial DNA (Johnson and Wood, 1990; Martin, 1988).

The plant productivity and nodulation was much affected by application of Al salts (Table 1 and 2). Presence of aluminium in the soil reduced the plant productivity and nodulation significantly. Aluminium toxicity in acid soils hampers root development of many crops, and thus reduces the ability of crop plants to acquire phosphorous from the soil (Edwards, 1991). P in the soil is strongly absorbed to aluminium (Al) or iron (Fe) at low pH (Blamey and Edwards, 1989). Phosphorous may be precipitated as $Al_2(PO_4)_3$ either on root surface or in root cell walls (Barlett and Riego, 1972; McCormick and Bordem, 1974). Storage of P depresses legume production and nitrogen fixation and thus strongly effect N supply to food crops (Van Noordwijk *et al.*, 1992). Co-inoculation of phosphate solubilizing fungi (arbuscular mycorrhizal fungi) with *rhizobium* has significant increase in plant biomass accumulation (Al-Garni, 2006). Soil acidity is a complex problem due in part to H^+ ion concentration and especially as the pH drops below 5.0, to toxicity of aluminium and manganese and limited availability of calcium, molybdenum and phosphate (Coventry and Evans, 1986). The growth and nodulation of many legumes has been shown to be affected by aluminium (Kim *et al.*, 1985). In addition to effects on nodule initiation (Brady *et al*., 1990) and root hair formation (Hecht-Buchholz *et al.*, 1990), it has been suggested that a further factor in the sensitivity of nodulation to aluminium may be due to direct effects of aluminium on the growth and survival and numbers of root nodule bacteria in the soil (Coventry and Evans, 1989). As stated earlier, acid soil contains high concentration of H^+ ion and free aluninium and low concentration of calcium and available phosphorus. Some acid soils contain Mn at phytotoxic levels, while in others Mo is not available. The poor growth of legumes in acid soils is frequently the result of an inhibition of nodulation rather than effects solely on the host plant (Munns, 1979; Edwards *et al.*, 1981). The greater concentration of H^+ ions increases the solubility of Al, Mn and Fe and these elements provide toxicity on plant growth and development.

Aluminium has been shown to reduce or inhibit nodulation (Hallsworth, 1958; de Carvalho *et al.*, 1981), the extent of the effect depending on concentration in solution and the legume species concerned. Reduction in the growth of *Medicago sativa* was found at only 8-18.5 ìM Al, but other N-fertilized tropical legumes showed no growth inhibition at 74 ìM Al (Munns, 1965;). Nodulation appears more sensitive to Al than is plant growth (de Carvelho *et al*., 1981; Murphy *et al.*, 1984) each reporting inhibition of nodulation at 25 ìM Al.

During the present study, it has been found that iron and Molybdenum enhanced the bacterial growth up to certain concentration (25 ìM Fe and 75 ìM Mo) (Fig 3-6) and symbiotic efficiency (25 ìM Fe and 20 ìM Mo) (Table 3-6). Both iron and molybdenum being the key components of enzyme nitrogenase and nitrate reductase. Apart from being and important co-factor of enzyme complex, iron is also an inevitable component of various other cellular components and products like leghaemoglobin (Bergersen, 1978; Dudeja *et al.*, 1997). Mo is also an important component of nitrogenase enzyme complex (Solaiman, 1999). Fu and Tabatabai (1989) observed the inhibitive effects of Fe and Mo on nitrate reductase activities of soil microorganisms beyond a certain limit.

It is well known that iron is specially required for N_2 fixing system: it is involved in the synthesis of the nitrogenase, leghaemoglobin, ferredoxin, hydrogenase and cytochromes. Consequently, iron deficiency may affect symbiotic fixation by impairing *Rhizobium* survival, establishment of functional nodules or host photosynthesis and energy transfer to the bacteroids (Johnson and Barton, 1993). Although extensive research has been directed to correct chlorosis (iron deficiency) by the application of available iron compounds to the soil and by selective plant breeding to produce Fe cholorosis resistant cultivars, only during the last decades the possible implications of siderophore producton by rhizobial strains has been considered as a potential way to improve nodulation and N_2 fixation in iron deficiency conditions. The beneficial effect of using siderophore-producing strains of *Bradyrhizobium* sp. and *Rhizobium meliloti* were reported by Gill *et al.* (1991) and Arora *et al.* (2001) respectively. Moreover siderophore porducng ability might favour the persistence of rhizobia in iron deficient soils (Lesueur *et al.*, 1995).

Soil, deficient in Mo, produces poor and ineffectively nodulated legumes. Extremes of pH affect nodulation by reducing the colonization of the legume rhizosphere by rhizobia. Highly acidic soils (pH<4.0) frequently have low levels of phosphorous, calcium and molybdenum and high aluminium and manganese, are often toxic for both partners; nodulation is more affected than host growth and nitrogen fixation (Bordeleau and Pervost, 1994).

CONCLUSION

The isolated strains of *Rhizobium* from *Mucuna pruriens* and *Trigonella foenumgraecum* showed enhancement of growth treated with Mo and Fe to certain limit whereas Al was completely toxic to both the strains. The application of iron and molybdenum promotes growth of bacteria and host because these metals are necessary components of some physiologically important compounds such as enzymes and other cofactors. But aluminium was harmful even in small concentration due to its negative effect on bacterial multiplication and precipitation of phosphorous, an essential nutrient to the plant. The excessive presence of heavy metals is harmful to crop production. Thus excessive accumulation of these heavy metals in soils must be prevented. On the other hand supplementation of optimum amount of Mo and Fe in the soils along with biological formulation so as to increase the processes of symbiotic nitrogen fixation will be beneficial.

REFERENCES

Al-Garni, S.M.S. 2006. *Increased heavy metal tolerance of cowpea plants by dual inoculation of an arbuscular mycorrhizal fungi and nitrogen-fixer Rhizobium bacterium*. African Journal of Biotechnology. **5**(2):133-142.

Arora, N.K., Kumar, V. and Maheshwari, D.K. 2001. *Constraints, development and future of the bio-inoculants with special reference to rhizobial inoculants*. In: D.K. Maheshwari and R.C. Dubey (Eds.) *Innovative Approaches in Microbiology*. Singh and M.P. Singh Publishers, Dehradun, India. Pp 241-254.

Barlett, R.J. and Riego, D.C. 1972. *Effect of chelation on the toxicity of aluminium*. Plant and Soil. **37**:419-423.

Bergersen, F.J. 1978. *The intracellular environment of Rhizobium japonicum in soybean root nodules*. In: M.W. Loutit and J.A.R. Miles (Eds.), Molecular Ecology. Spronger-Verlag, Berlin, Pp 376-383.

Blamey, F.P.G. and Edwards, D.G. 1989. *Limitation to food crop production in tropical acid soil*. In: J. Van der Heide (Ed.), Nutrient Management for Food Crop Production in Tropical Farming System. Institute of Soil Fertility, Haren, The Netherlands. Pp 73-94.

Bordeleau, L.M. and Pervost, D. 1994. *Nodulation and nitrogen fixation in extreme environments*. Plant and Soil. **161**: 115-125.

Brady, D.J., Hetch-Buchholz, C.H., Asher, C.J. and Edwards, D.G. 1990. *Effect of low activities of aluminium on Soybean (Glycine max L.) early growth and nodulation*. In: M. L. Van Blusichem (Ed.), Plant Nutrient Physiology and Application. Kluwer, Dordrecht. Pp 329-334.

Brockwell, J. 1991. *Experiments with crop and pasteur legumes-principles and practice*. In: *Methods for Evaluation Biological Nitrogen Fixation*. John Wiley and Sons, New York. Pp 417-488.

Chen, C.T., Maundu, J. Cavaness, J., Daudurand, L.M. and Orser, C.S. 1992. *Characterization of salt tolerant and salt sensitive mutants of Rhizobium leguminosarum bv viciae strains C1204b*. Fems. Microbiol. Lett. **90**:135-140.

Coventry, D.R. and Evans, J. 1989. *Symbiotic nitrogen fixation and soil acidity*. In: A.D. Robson (Ed.) Soil Acidity and Plant Growth. Academic Press Sydney. Pp 103-137.

de Carvelho, M.M., Edwards, D.G., Asher, C.J. and Andrew, C.S. 1981. Aluminium toxicity, nodulation and growth of *stylosanthes sp. Agron. J.* **73**:261-265.

Dudeja, S.S., Suneja, S. and Khurana, A.L. 1997. *Iron acquisition system and its role in legume Rhizobium symbiosis*. Ind. J. Microbiol. **37**:289-301.

Edwards, D.G., Kang, B.T. and Danso, S.K.A. 1981. *Deferential response to six cowpea (Vigna unguiculata (L) Walp.) cultivars to limiting in a ultisol*. Plant and Soil. **59**:61-63.

Edwards, A.C. 1991. *Soil acidity and its interaction with phosphorous availability for a range of different crop types*. In: R.J. wright , V.C. Baligar and R.P. Murrman. (Eds.). Plant-Soil Interactions at Low pH. Pp 299-305. Kluwer Academic Publisher. Dordrecht, The Netaerlands.

Fu, M.H. and Tabatabai, M.A. 1989. *Nitrate reductase activity in soils: effects of trace elements. Soil.* Biol. Biochem. **21**:943-946.

Gill, P.R., Barton, L.L., Scobbe, M.B. and Neilands, J.B. 1991. *A high affinity iron transport system of Rhizobium meliloti may be required for efficient nitrogen fixation in plants*. Plant and Soil. **130**:211-217.

Giller,K.E., Witter, E. and McGrath, S.P. 1998. *Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils A review*. Soil Biol. Biochem. **30**:1389-1414.

Hallsworth, E.H. 1958. *Nutritional factors affecting nodulation*. In: E.G. Hallsworth (Ed.), Nutrition of the Legumes. Pp 183-201. Butterworths Scient. Publ. London.

Hecht-Buchholz, C.H., Brandy, D.J., Asher, C.J. and Edwards, D.G. 1990. *Effects of low activities of aluminium on soybean (Glycine* *max) root cell structure and root hair development*. In: M.L. Van Beusichem (Ed.), Plant Nutrition-Physiology and Applications. Pp 335-343. Kluwer, Dordrecht.

Johnson, G.V. and Barton, I.L. 1993. *Selected physiological response associated with Fe (III) and Fe (II) metabolism*. In: I.L. Barton and B.C. Hemming (Eds.), Iron Chelation in Plant and Soil Micro-organisms. Pp 371-386. New York Academic Press.

Johnson, A.C. and Wood, M. 1990. *DNA as a possible site of action of aluminium in Rhizobium sp.* Appl. Environ. Microbiol. **56**:3629-3633.

Kim, M.M., Asher, C.J., Edward, D.G. and Date, R.A. 1985. *Aluminium toxicity, effect on growth and nodulation of subterranean clover*. In: H. Kuna, T. Kitahara, T. Okuba, M. Shiyonu, K. Sugawata, A. Tajimi and H Yamaguchi (Eds.), Proceedings of the 15th International Grassland Congress (Tokyo). Pp 501-503. Science society of Japan and Japanese Society of Grassland Science, Japan.

Kinkle, B.K., Sadowsky, M.J., Johnston, K. and Koskinen, W.C. 1994. *Tellurium and Selenium resistance in rhizobia and its potential use for direct isolation R. meliloti from soil*. Appl. Environ. Microbiol. **60**:1674-1677.

Lesueur, D., Carrodel Rio, M. and Diem, H.G. 1995. *Modification of the growth and the competitiveness of Bradyrhizobium strain obtained through affecting its siderophore producing ability*. In: J. Abadia (Ed.). Iron Nutrition in Soils and Plants. Pp 59-66. Dordrecht, Netherlands.

Martin, R.B. 1988. *The chemistry of aluminium as related to biology and medicine*. Clinical Chemistry. **23**:1797-1806.

Martin, R.B. 1991. *Aluminium in biological systems*. In: M. Nicolini, P.F. Zatta and B. Corain (Eds.), Aluminium in Chemistry, Biology and Medicine. Aseries of advances. Pp 3-20. Raven Press, New York.

McCormick, L.H. and Bordem, F.J. 1974. *The occurance of aluminium phosphate precipitate in plant roots*. Soil. Sci. Soc. Am. Proc. **38**:931-934.

McLean, E.O. 1976. *Chemistry of soil aluminium*. Communications Soil Sci. Plant Annals. **7**: 619-636.

Munns, D.N. 1965. Soil acidity and growth of a legume I. Interactions of lime with nitrogen and phosphate on growth of *Medicago sativa* L. and *Trigonella subterraneum* L. *Aust. J. Agric. Res*. **16**:733-741.

Munns, D.N. 1979. *Mineral nutrition and nodulation*. In: F.T. Corbin (Ed.), Proceedings of the World Soybean Research Conference II. Pp 47-56. Westview Press, Boulder, Colorado.

Murphy, H.E., Edwards, D.G. and Asher, C.J. 1984. *Effects of aluminium on nodulation and early growth of four tropical pasture legumes.* Aust. J. Agric. Res. **35**:663-673.

Richardson, E.A. and Simpson, R.J. 1989. *Acid tolerance and symbiotic effectiveness of Rhizobium trifolii associated with a trifolium subterraneum L. based pasture growing acid soils*. Soil Biol. Biochem. **21**:87-95.

Solaiman, A.R.M. 1999. *Effects of Bradyrhizobium japonicum inoculation and molybdenum on soybean*. Bangaladesh J. Bot. **28**:181-183.

Stanier, R.Y., Adelberg, E.A. and Ingraham, J.L. 1985. *Microbial growth*. In General Microbiology MacMillan Publication London. Pp 273-293.

Thornton, F.C. and Davey, C.B. 1983. *Acid dolerance of Rhizobium trifolii in culture media*. Soil Sci. Soc. Am. J. **47**:496-501.

Van Noordwijk, M., Widianto, S.S.M., Hairiah, K. and Gurinto, B. 1992. *Nitrogen management under high rainfall conditions for shallow rooted crops: Principals and Hypothesis*. Agrivita. **15**:10-18.

Whelan, A.M. and Alexander, M. 1986. *Effects of low pH and high Al, Mn, and Fe, levels on the survival of Rhizobium trifolii and the nodulation of subterranean colver*. Plant and Soil. **92**:363-371.

Wood, M. and Cooper, J.E. 1988. *Acidity, aluminium and multiplication of Rhizobium trifolii: Effect of initial inoculum density and growth phase*. Siol Biol. Biochem. **20**:83-87.