Bull. Environ. Contam. Toxicol. (2004) 73:424–431

DOI: 10.1007/s00128-004-0446-5

Bulletin of Environmental Contamination and Toxicology

Role of *Rhizobium* (CA-1) Inoculation in Increasing Growth and Metal Accumulation in *Cicer arietinum* L. Growing Under Fly-Ash Stress Condition

D. K. Gupta,[1] U. N. Rai,[1] S. Sinha,[1] R. D. Tripathi,[1] B. D. Nautiyal,[2] P. Rai,[1] M. Inouhe[3]

[1] Ecotoxicology and Bioremediation Group, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226 001, India
[2] Department of Botany, University of Lucknow, Lucknow 226 007, India
[3] Department of Biology, Earth Science, Ehime University, Matsuyama, Japan

Received: 8 April 2003/Accepted: 13 May 2004

In India, power is mostly generated by thermal power stations using coal as a fuel (Khan and Khan 1996). Indian coal has high moisture and low sulphur content due to which fly-ash produced is alkaline in nature. Fly-ash is generated in huge quantity and nearly 70% of coal is converted into fly-ash during combustion (Srivastava et al. 1995). The disposal of fly-ash is a great problem and most of the ash generated is being dumped in nearby areas as fly-ash landfills or dykes. Although, fly-ash contains many essential nutrients for plant growth and establishment, due to volatilization of N in the form of oxides during coal combustion and loss of P due to high solubility, it is deficient in N and P content. Besides, fly-ash also contains high levels of many toxic metals (Mehra et al. 1998; Gupta et al. 2002), which inhibits survival and growth, thus subsequently leads to the death of the plant (Wong and Wong 1986). Many plant species have been grown on fly-ash landfills, however, the successes rate varies (Wong and Wong 1990; Cheung et al. 2000). Application of various nitrogen fixing cynobacteria and leguminous trees inoculated with *Rhizobium* have been used to enhanced N and P status and in reducing metal toxicity of fly-ash landfills (Banerjee and Deb 1993; Rai et al. 2000, 2004).

Cicer arietinum L. (family, Leguminoceae), an important pulse crop in India, has the ability to grow under nitrogen deficient conditions because of its symbiotic association with *Rhizobium* which form nodules in the plant roots, and binds free nitrogen from the air to meet the N requirement of the plant. *C. arietinum* is an annual herbaceous plant with a life span of 4-5 months attaining a maximum height of 50 cm. Due to its vigorous growth and uniform germination rates the plant is suitable for short term experiment in laboratory and long term experiments in the field. The main aim of the present study was to isolate fly-ash tolerant *Rhizobium* strain from *C. arietinum* grown in fly-ash and inoculate it in *C. arietinum* seedlings raised in Jensen nitrogen free medium and study the effect of fly-ash tolerant *Rhizobium* strain on the metal uptake and growth performance of the plant.

MATERIAL AND METHODS

Unweathered fly-ash was collected from National Thermal Power Corporation,

N.B.R.I. Publication No. NS
Correspondence to: U. N. Rai

Unchahar, Raebareli, Uttar Pradesh (India). Metal analysis of fly-ash were done by oven drying fly-ash samples. Fly-ash samples (1g) were digested with a mixture of nitric, sulphuric, perchloric acids (3:1.5:1; v/v) at 100°C . Iron, copper, zinc, cadmium and chromium contents in the diluted digests were quantified, using Perkin Elmer- 2380 atomic absorption spectrophotometer. Electrical conductivity and pH were determined using conductivity meter and pH meter, respectively by diluting fly-ash with distilled water in 1:2 ratio. For determining EC, fly-ash samples were air dried for 7 days (Piper 1942). Total organic carbon was estimated by the method of Walkley and Black (1934); Total nitrogen was estimated by Micro Kjeldahl digestion method (Nelson and Sommer, 1972); total phosphorus by molybdenum blue method described by Allen et al. (1974).

Rhizobium (CA-1) was isolated on yeast extract mannitol agar (YEMA) medium (Vincent 1970). The plants of *C. arietinum* growing in fly ash were carefully uprooted, washed thoroughly and healthy nodules were detached from the roots. Nodules were surface sterilized with sterile 5% H_2O_2 (5 min), rinsed with sterile distilled water and crushed in double distilled water. Serial dilutions (1:10 to 1:1000) of nodule extract were plated on YEMA plates and incubated for 10 d at 37±2°C. Large gummy colonies of bacteria that emerged within four or five d were selected, isolated subsequently transferred on fresh nutrient plates and sub cultured into yeast mannitol broth with continuous agitation.

For inoculation experiment, seeds of *C. arietinum* procured from Indian Institute of Pulse Research, Kanpur and were surface sterilized with H_2O_2 (5%) for 10 min then rinsed five times with sterilized deionised water. Seeds were soaked over night in sterile tap water, and then germinated on a filter paper. After germination seedlings were transferred to 150 ml conical flask (1 each) containing 25 ml Jenson nitrogen free medium (Roughley 1976). Fifteen days old seedlings were inoculated with either 1ml (2.6×10^8 cell ml^{-1}) or 2 ml (5.2×10^8 cells ml^{-1}) of *Rhizobium* (CA-1) culture. Inoculated seedlings were placed under control conditions (light/ dark cycle 14:10 h, temperature 28± 2°C, 115µmol$^{-2}s^{-1}$ illumination provided through day florescent tube light) and observed for initiation of nodulation. After 7d of inoculation, nodulated sapling were transferred to 15 cm diameter plastic pots (each pot containing 2 plants) in triplicate containing 2 kg of fly-ash and were kept under natural conditions. The plants were irrigated with tap water at regular interval avoiding leakage of water from pots. Uninoculated plants grown and kept under similar conditions served as control. Plants were harvested after 30 and 60 d after transplanting, and used for the determination of growth parameters and metal uptake. Plants were blotted dry and biomass of the entire plant were measured by single pan (Sartorious make) electronic balance. Length of the shoot and entire plant was measured by vernier calipers and nodule numbers per plant were counted manually with the help of a hand lens. Chlorophyll content was estimated after extraction in chilled 80% acetone following the method of Arnon (1949) and carotenoid content was determined by the method by Duxbury and Yentsch (1956). Protein content in the leaf was estimated by the procedure of Lowry et al. (1951) using bovine serum as a standard. Metal content (Fe, Cu, Zn, Cd and Cr) in both root and shoot of the plant were determined in oven-dried (80°C) samples by digesting them with a mixture of nitric and perchloric acid

(3:1; v/v) at 100°C as described in case of fly-ash.

Analytical data quality of metals was ensured through repeated analysis (n=6) of EPA quality control samples in water and the results were found to be within ± 3.05 % of certified values. For plants, recoveries of metal from the plant tissue were found to be more than 98% as determined by digesting 3 samples each from an untreated plant with known amount of metal. The blanks were run in triplicate to check the precision of the method with each set of samples.

A two-way and one-way analysis of variance (ANOVA) in complete randomized block designed involving two treatments and two durations was performed to confirm the validity of data (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

Physico-chemical analysis of the fly-ash used in the experiment showed high pH and low nitrogen and available phosphorus content (Table 1) and was enriched with high concentration of metals viz., Fe, Cu, Zn, Cd and Cr. For studying the effect of *Rhizobium* inoculation on metal accumulation potential of the plant, inoculated plants with two inoculum size ($2.6x10^8$ and $5.2x10^8$ cell ml^{-1}) of fly-ash tolerant *Rhizobium* strain (CA-1) were allowed to grow on raw fly-ash for 60 d. The plants were harvested and accumulation of metals were studied in root and shoot parts of both the varieties. The data presented in Figures 1 a & b show the accumulation of Fe, Zn, Cu, Cr and Cd by *C. arietinum* var. CSG-8962 and C-235 after 60 d of growth. The plant showed maximum accumulation of Fe followed by Cu, Zn, Cr and Cd. The accumulation of metal (Cu, Zn, Cr and Cd) were more in shoot than the root part of the plants, however, inoculation of *Rhizobium* enhanced accumulation of these metal into the plant tissue, which was more pronounced in shoot than the root part of the plant. The accumulation of Fe was more in root part of both the varieties in uninoculated control plants. However, the inoculum size has a great effect on accumulation of these metals as high metal accumulation was found in the plant inoculated with 2 ml *Rhizobium* culture. These findings may be significant while using the plant for metal decontamination of fly-ash landfills.

The data presented in Figures 2 a & b show the effect of *Rhizobium* inoculation on root-shoot growth and biomass yield of both varieties of the plants grown on 100% fly-ash. Result showed that *Rhizobium* inoculation increased plant growth at both the durations (30 and 60 d), however, the inoculation of 2 ml culture resulted into more increased growth of root and shoot resulting into more biomass than 1 ml inoculated plants. These parameters were approximately 1.5 times more than the uninoculated plants. The response of *Rhizobium* inoculation in both the varieties were similar. By inoculation of *Rhizobium*, a significant improvement was found with regards to root-shoot growth and biomass yield in both the varieties in comparison to the plants growing in 100% fly-ash. The effect of *Rhizobium* inoculation on nodule number of *C. arietinum* var. CSG-8962 and var. C-235 after different days of growth on 100% fly-ash showed that inoculation of plants with 2 ml of *Rhizobium* resulted in higher numbers of nodule in both the varieties.

The effect of *Rhizobium* inoculation on photosynthetic pigment of *C. arietinum* var. CSG-8962 and var. C-235 at different growth period is shown in Figures 2 c & d.

Table 1. Physico-chemical properties of fly-ash used in the study.

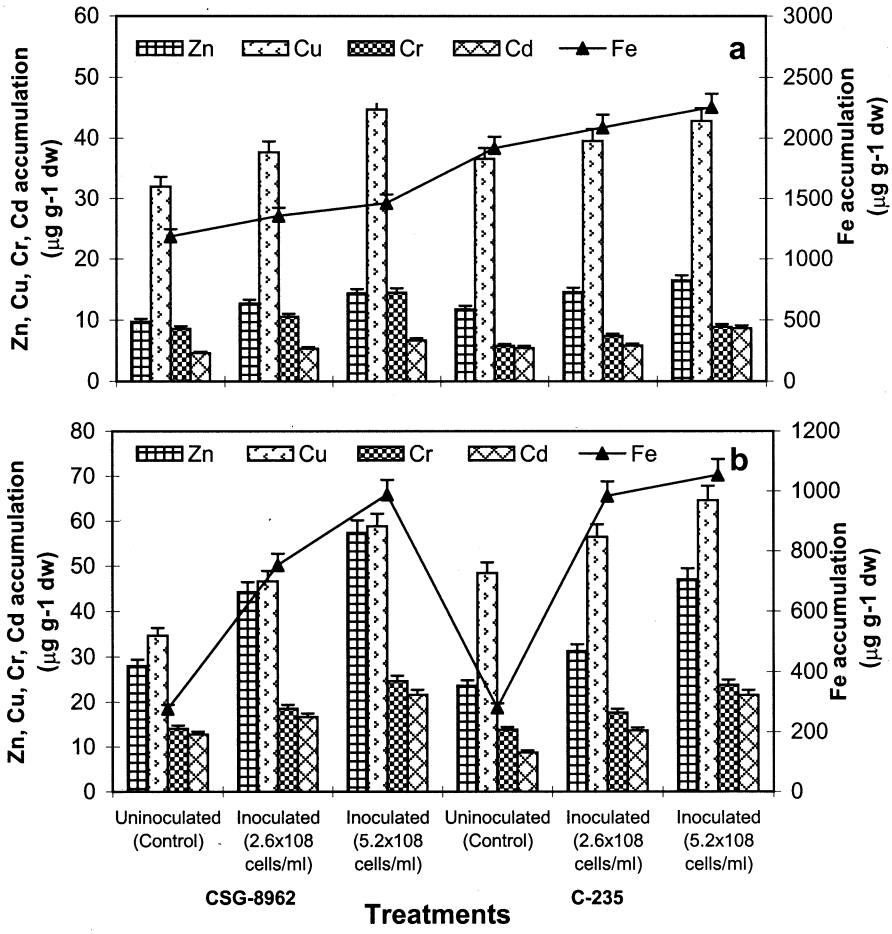
Parameters	Fly-ash
pH	9.6±0.42
Electrical conductivity (dSm^{-1})	7.6±0.38
Cation exchange capacity [meq $(100\ g)^{-1}$]	1.3±0.11
Total nitrogen (%)	0.0±0.00
Total phosphorus (%)	0.0±0.00
Organic carbon (%)	1.2±0.05
Metals ($\mu g\ g^{-1}$ dw)	
Fe	4150±207
Zn	82.0±4.12
Cu	58.6±2.83
Cr	40.3±1.98
Cd	42.3±2.13

Values are mean ±SD (n=3)

Data revealed significant enhancement in the contents of chlorophylls and carotenoids in both varieties at different treatment durations after *Rhizobium* inoculation. In this case also, inoculation of plant with 2 ml of *Rhizobium* culture resulted into more synthesis of photosynthetic pigments in comparison to 1 ml inoculated and uninoculated plant. These results are suggestive of increased tolerance in the plant due to the presence of *Rhizobium*. Both the varieties of the plant exhibited maximum protein content in the plant inoculated with 2 ml of fly-ash tolerant *Rhizobium* culture (CA-1). However, there was no significant difference between these two varieties without *Rhizobium* inoculation.

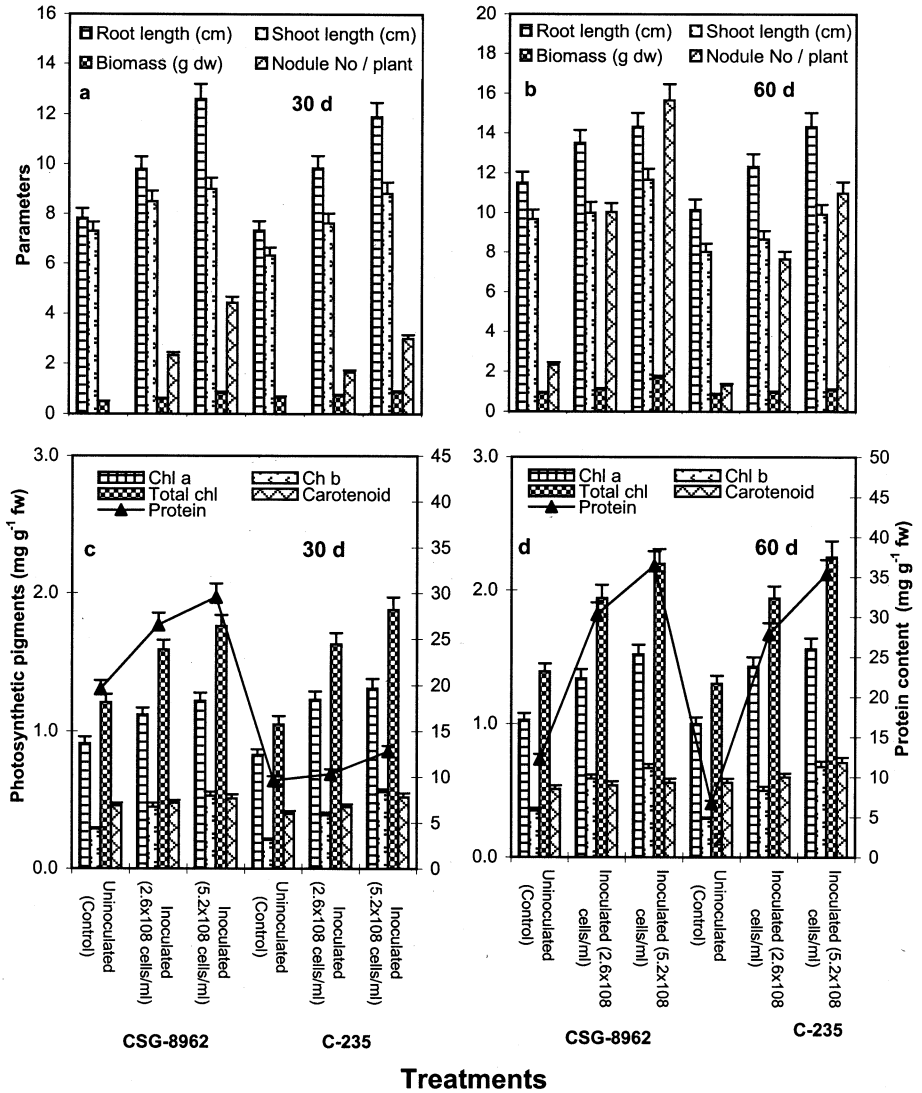
Although the survival rate of both the varieties of *C. arietinum* in different treatment was similar, the growth and metal accumulation potential was increased by inoculating plant with fly-ash tolerant *Rhizobium* strain (CA-1). The increase in metal accumulation potential of *C. arietinum* after *Rhizobium* inoculation may be accounted for more accumulation of metals by *Rhizobium* present in the root nodules of the plants and creation of favorable rhizospheric zone to facilitate metal uptake by the plant.

The two inoculum sizes used in the present study showed significant variation with regards to growth potential and metal accumulation of the plant. Giller et al. (1989) also reported that inoculums of an effective strain of *R. legominosarum biovar trifolii* into soils at metal rich site (Woburn) resulted in the loss of N_2 fixation in clover over 92-month period, unless large densities of cells were inoculated. However, the nodules formed by plants grown in fly-ash were found effective as they were able to form nodules in saplings grown in Jensen nitrogen free medium. Obbard and Jones (1993) also found that rhizobial populations of metal contaminated sites were able to form an effective symbiosis with *Trifolium repens* when grown on the soil in the laboratory. In the present study, the accumulation of metals was enhanced in shoot part of the plant showing more translocation of metals from root to shoot, this findings may be significant while considering use of this plant for phytoremediation of fly-ash landfills. The plants inoculated with *Rhizobium* seem to be more tolerant than the uninoculated plant, which may be due to increased N_2 fixation. Plant inoculated with *Rhizobium* shows more growth than the uninoculated plant. Similar



Figures 1 a & b. Metal accumulation (µg g^{-1} dw) by *C arietinum* var. CSG-8962 and C-235 innoculated with or without 1 and 2 ml of *Rhizobium* culture. ANOVA ; $P < 0.01$

results have been reported in case of *Cassia surattensis* inoculated with *Rhizobium*, which proved to be successful candidate in vegetating fly-ash lagoons (Vajpayee et al. 2000). Growth promotion of canola and lettuce by application of *R. leguminosarum* under stress conditions was also reported by Noel et al (1996). However, failure of nodulation by *Rhizobium* has also been reported due to high pH and metal enrichment of fly-ash (Cheung et al. 2000). Additions of fly-ash to soil decreased CO_2 evolution from the soil bacteria (Wong and Wong 1986; Pichtel and Hayes 1990) and nitrification in the soil (Cervelli et al. 1986). Pichtel and Hayes (1990) indicated that application of unweathered alkaline fly-ash to soil partially inhibited microbial activity. The adverse effects of alkaline fly-ash on microbial respiration and nitrification activity were attributed to high alkalinity (Cervelli et al. 1986; Wong and Wong 1986). The addition of fly-ash to soils could inhibit the development of a functional microbial community. Waterlogged soil causes oxygen



Figures 2 a-d. Effect of *Rhizobium* on growth performance, photosynthetic pigments (mg g^{-1} fw) and protein contents (mg g^{-1} fw) of *C. arietinum* at different durations. ANOVA ; $P < 0.01$

deficiency, which is a limiting factor for the survival of *Rhizobium* (Barnet et al. 1985). Nitrogenase enzyme synthesis by *Rhizobium* for N_2-fixation needs oxygen for oxidative phosphorylation within *Rhizobium* cells (Vance 1991). The high silt content of lagoon ash results in a greater tendency to cement the soil. Poor aeration and water logging may cause inadequate oxygen supply for *Rhizobium* strains, which eventually result in nodulation fluxes. Less numbers of nodule were found due to fly-ash application into soil and saw dust in case of *C. aretinum* (Gupta 2002). Toxic metals, therefore, inhibit the growth of *Rhizobium* (Chaudri et al. 1993; Obbard and

Jones 1993). Zhang et al (1998) found that the EC_{50} values for Zn (effective concentration to reduce the activity by 50%) are 115.6 mg l^{-1} for nodulation, 38.6 mg l^{-1} for N_2-fixation, 638.9 mg l^{-1} for the growth of *Rhizobium* strain AA9108 and 18.3 mg l^{-1} for *Acacia auriculiformis*, respectively.

It could be concluded from the present study that *C. arietinum* is an ideal crop plant for revegetating amended fly-ash landfills deficient in nitrogen, however, inoculation of fly-ash tolerant *Rhizobium* strain may prove to be more beneficial in such rehabilitation programs.

Acknowledgments. We thank the Director, National Botanical Research Institute, Lucknow, India for necessary facilities and encouragement. Dr. D.K. Gupta and P. Rai are indebted to Council of Scientific and Industrial Research; New Delhi, India for providing Senior and Junior Research Fellowships, respectively.

REFERENCES

Allen SE, Grimshaw HM, Parkensin JA, Quarmby C (1974) Chemical Analysis of Ecological Materials. Oxford, Blackwell Scientific

Arnon DI (1949) Copper enzyme in isolated chloroplast polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24: 1-15

Banerjee M, Deb M (1993) Fly-ash induced changes in growth and protein content of *Spirulina platensis*. Chem Environ Res 2(1-2): 151-154

Barnet YM, Catt PC, Hearue DH (1985) Biological nitrogen fixation and root nodule bacteria (*Rhizobium* sp. and Brady *Rhizobium* sp.) in two rehabilitating sand dune areas planted with *Acacia* sp. Australian Jour Bot 33: 593-610

Cervelli S, Petruzzelli G, Perna A, Menicagi (1986) Soil nitrogen and fly-ash utilization: A laboratory investigation. Agrochim 30: 27-35

Chaudri AM, McGrath SP, Giller KE, Rietz E, Saverbeck DR (1993) Enumeration of indigenous *Rhizobium leguminosarum biovar trifolii* in soil previously treated with metal contaminated sewage sludge. Soil Biol Biochem 25: 301-309

Cheung KC, Wong JPK, Zhang ZQ, Wong JWC, Wong MH (2000) Revegetation of lagoon ash using the legume species *Acacia auriculiformis* and *Leucaena leucocephala*. Environ Poll 109: 75-82

Duxbury AC, Yentsch CS (1956) Plankton pigment monograph. Jour Mar Res 15: 92-101

Giller KE, McGrath SP, Hirsch PR (1989) Absence of nitrogen fixation in clover grown on soil subject to long-term contamination with heavy metals is due to survival of only ineffective *Rhizobium*. Soil Biol Biochem 21: 841-848.

Gomez KA, Gomez AA (1984) Statistical Procedures for Agricultural Research, John Wiley and Sons, New York

Gupta DK (2002) Ecophysiological studies on *Cicer arietinum* L. growing under fly-ash stress condition, Ph.D. Thesis, Lucknow University, Lucknow, India

Gupta DK, Rai UN, Tripathi RD, Inouhe M (2002) Impacts of fly-ash on soil and plant responses. Jour Plant Res 115: 401-409.

Khan MR, Khan MW (1996) The effect of fly-ash on plant growth and yield of tomato. Environ Pollut 92: 105-111

Lowry OH, Rosenbrought NJ, Farr AC, Randal RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275

Machlachlan S, Zalik S (1963) Plastid structure, chlorophyll concentration and free amino acid composition of a chlorophyll mutant of barley. Canadian Jour Bot 41: 1053-1062

Mehra A, Farago ME, Banerjee DK (1998) Impact of fly-ash from coal fired power stations in Delhi, with particular reference to metal contamination. Environ Monitor Assess 50: 15-35

Nelson DW, Sommers LF (1972) A simple digestion procedure for estimation of total N_2 in soils by extraction with sodium bicarbonate. US Dep Agricult Circle, 939: 1-19

Noel TC, Sheng C, Yost CK, Pharis RP, Hynes MF (1996) *Rhizobium leguminosarum* as a plant growth promotion of canola and lettuce. Canadian Jour Microbiol 42: 279-283

Obbard JP, Jones KC (1993) The effect of heavy metals on dinitrogen fixation by *Rhizobium* white clover in a range of long term sewage sludge amended and metal contaminated soils. Environ Pollut 79: 105-112

Pichtel JR, Hayes JM (1990) Influence of fly-ash on soil microbial activity and populations. Jour Environ Qual 19: 593-597

Piper CS (1942) Soil and Plant Analysis. New York: Inter-Science

Rai UN, Pandey K, Sinha S, Singh A, Saxena R and Gupta DK (2004) Revegetating fly-ash with *Prosopis juliflora* L. Impact of different amendments and *Rhizobium* inoculation. Environmental International (In press)

Rai UN, Tripathi RD, Singh N, Kumar A, Ali MB, Pal A, Singh SN (2000) Amelioration of fly-ash by selected nitrogen fixing blue green algae. Bull Environ Contam Toxicol 64: 294-301

Roughley RJ (1976) The production of high quality inoculants and their contribution to legume yields. In: Nutman PS (ed), Symbiotic Nitrogen fixation, Cambridge. Uni. Press, Cambridge, pp 125-136

Srivastava K, Farooqui A, Kulshreshtha K, Ahmad KJ (1995) Effect of fly-ash amended soil on growth of *Lactuca sativa* L. J Environ Biol 16(2): 93-96

Vajpayee P, Rai UN, Choudhary SK, Tripathi RD, Singh SN (2000) Management of fly-ash landfills with *Cassia surattensis* Burm: A case study. Bull Environ Contam Toxicol 65: 675-682

Vance CP (1991) Root bacteria interactions. In: Waisel Y, Amram U, Kafkafi E (eds). Plant Roots – the Hidden Half Marcel Dekker, New York, pp 671-701

Vincent JM (1970) A manual for the practical study of the root nodule bacteria. International Biological Programme Handbook, Vol. 15, Blackwell, Oxford

Walkley YA, Black IA (1934) An examination of the Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Sci 37: 29-38

Wong JWC, Wong MH (1990) Effects of fly-ash on yields and elemental composition of two vegetables, *Brassica parachinensis* and *B. chinensis*. Agric Ecosyst Environ 30: 251-264

Wong MH, Wong JWC (1986) Effect of fly-ash on soil microbial activity. Environ Pollut (Ser A) 40: 127-144

Zhang ZQ, Wong MH, Nie XP, Lan CY (1998) Effects of $ZnSO_4$ (zinc sulfate) onrhizobia-earleaf Acacia (*Acacia auriculiform*) symbiotic association. Bioresource Technol 64: 97-104