3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Organisms

The ammonia excreting strains of *Azotobacter chroococcum* and *Rhizobium* were obtained from the department of Microbiology, Haryana Agricultural University, Hissar. Other strains of *A. chroococcum* were isolated from the local soils.

3.1.2 Chemicals

Chemicals used for the present investigations were of AnaL R grade (from Sigma Chemicals, Sarabhai Chemicals, E. Merck and Sisco Laboratories). Antibiotics and substrates for ammonia assimilatory enzymes and soil enzymes were obtained from Sigma Chemicals, USA.

3.1.3 Seeds

Seeds of mung bean (*Vigna radiata* variety K-851), chickpea (*Cicer arietinum* variety H208), wheat (*Triticum vulgare local variety*) were obtained from plant breeding department, Haryana Agricultural University, Hissar. Maize (*Zea mays local variety*) and barley (*Hordeum vulgare local variety*) were obtained from National Seed Corporation of India, Chandigarh.
3.2 Methods

3.2.1 Isolation of free living nitrogen fixing bacteria (Azotobacter)

Soil samples were air dried and sieved according to the method used by Brown and Jackson (1962). Strains of Azotobacter were isolated by plating technique using Jensen's medium consisting of Sucrose- 20 g; K$_2$HPO$_4$, 1 ; MgSO$_4$, 0.5; NaCl, 0.50; FeSO$_4$, 0.10; Na$_2$MoO$_4$, 0.005; CaCO$_3$, 2.0; distilled water- 1000 ml; pH 7.0-7.2). The plates were incubated for 3-4 days at 29 ± 1°C. After 48 hrs mucilagenous colonies showing blackish brown pigment were selected, purified and transferred on Jensen's agar slants and identified as detailed in Bergy's manual of determinative Bacteriology (1974).

3.2.2 Isolation of UV-mutants

The mutants of A. chroococcum AC2 were isolated by the method used by Terzaghi(1980). The culture was chilled, centrifuged, washed with 0.1 M MgSO$_4$ irradiated in 9 cm diameter glass petridishes for varying times in 10 ml quantities with protection from photoreactivation. Samples were plated on Burk's medium ( Sucrose 20 g; KH$_2$PO$_4$, 0.20; K$_2$HPO$_4$,0.20; MgSO$_4$.7H$_2$O, 0.20 ; CaCl$_2$, 0.09 ; FeSO$_4$, 0.10; Na$_2$MoO$_4$, 0.05; Distilled water 1000 ml, pH 7.01 to determine percent survival. The irradiated cultures were then centrifuged resuspended and grown in Burk and Burk-ammonium acetate medium (Burk medium + .03% ammonium acetate) at 25°C until growth
ceased and then plated on Burk and ammonium acetate, bromo-thymol blue (0.001 % w/v) and bromocresol purple (0.001 % w/v) plates, incubated at 28 ± 2°C and then examined. Colonies which formed yellow colour were picked up randomly and tested for ammonia excretion ability.

3.2.3 Media used for ammonia excretion and for enzymatic studies

For studying ammonia excretion under the influence of various factors, Jensen's medium (3.2.1) was used. For enzymatic studies, Burk's medium was used because it is less turbid due to the buffering action of the salts.

3.2.4 Growth conditions

Growth under stationary conditions was measured by withdrawing samples at 4 days interval up to 20 days and determining the optical density at 540 nm using Spectronic-21. Growth under shake conditions (90 rev/min) was measured in a similar way, by withdrawing the samples at 24 hrs intervals up to 72 hrs of incubation.

3.2.5 Cyst count

For determining the number of cysts, culture of *A. chroococcum* AC2 was grown in Jensen's medium. Samples were drawn at various time intervals diluted and suspended in equal volumes of special stain described by Vela and Nyss (1964) of the following composition: Glacial acetic acid- 8.5 ml; Na$_2$SO$_4$- 3.25 g; Neutral red-200 mg; Light green SF yellowish- 200 mg, Ethyl alcohol- 50 ml & Distilled water-100ml.
The number of vegetative cells and cysts were determined using a haemocytometer. The results are expressed as percent of cyst.

3.2.6 **Ammonia excretion by the isolates of A. chroococcum**

The cultures were initially cultivated in 30 ml of Jensen's nitrogen free medium in 100 ml Erlenmeyer flask at stationary conditions for 48 hrs at 30°C. The composition of Jensen's media was the same, except that calcium carbonate was replaced by 100 mg/L of calcium chloride. The inoculum (5%) was seeded in 30 ml of medium in 100 ml Erlenmeyer flasks and incubated for 20 days under stationary conditions. The ammonia released in the culture media was estimated after 4, 8, 12, 16 and 20 days using Indophenol and ammonia electrode method. All experiments were done in six replications and the results are mean values.

3.2.7 **Estimation of ammonia**

3.2.7.1 **Indophenol method**

Ammonia was determined colorimetrically by indophenol method (Burris, 1972) using Chaykin's reagent (Chaykin, 1969) after microdiffusion. Five ml of culture broth was centrifuged at 4000 g for 25 minutes. One ml of the supernatant was taken in 15 ml vaccine bottle and inactivated with 1 ml of 50% potassium carbonate. Each vaccine bottle was then fitted with a rubber stopper number 1 carrying a glass rod, whose tip was broadened and dipped in SN $\text{H}_2\text{SO}_4$. The glass rod was inserted on the vaccine bottle immediately after
the addition of potassium carbonate and incubated for 24 hrs at room temperature (Bergerson, 1980) as to facilitate diffusion. Stopper of the vaccine bottle was carefully removed and broadened end glass rod was dipped in indophenol reagent used by Chaykin. Standard curve was prepared by using 2-10 ug/ml of ammonium sulphate (analytical grade) and ammonia content (Fig.4) was expressed as ug of ammonia per ml of the supernatant.

3.2.7.2 Ammonia electrode

Commercially available ion selective electrode (Kleinschmidt and Kleiner, 1981; Drawert, 1976; Hill, 1982) (Orion Model 95-10) sensitive to ammonia was used in the studies to determine ammonia. The membrane of ammonia electrode is penetrated only by ammonia and not by ammonium ions. Therefore, it was necessary to raise the pH values of the samples. Ammonium ion concentration could be measured over a wide range because there is a logarithmic dependence between the measured voltage and the ammonium ion concentration. Details of the method are given in Appendix.

3.2.8 Dry weight of the cell

Sample withdrawn on 4, 8, 12, 16 and 20 days of incubation, were centrifuged, washed twice with water by centrifugation and dried on planchets at 80°C till constant weight was obtained. Centrifugation was done at 10,000 x g for 15 min at 4°C.
3.2.9 **Factors affecting ammonia excretion**

Following compounds were used to determine their effect on ammonia excretion. All compounds were filter sterilized using GS-sintered glass filter and then added separately to the medium.

3.2.9.1 *Growth factors*

Nicotinic acid, biotin, riboflavin, thiamine and indole acetic acid were studied. 0.5, 10, 15 and 20 μg/ml concentrations were selected according to the reports (Karasevich, 1962 and Zak, 1965) that growth factors increase nitrogen fixation.

3.2.9.2 *Amino acids*

Amino acids like asparagine, glutamine, L-tryptophan, aspartic acid and glutamic acid were chosen because they help in nitrogen fixation (Jones, 1943). They were used at 0, 5, 10, 15 and 20 μg/ml concentrations.

3.2.9.3 *Organic acids*

The sterilized solution of barium acetate, citric acid, sodium citrate, pyruvic acid, sodium pyruvate, acetic acid, sodium acetate, oxalic acid, sodium oxalate, α-ketoglutaric acid, succinic acid and sodium succinate were used to see their effect on the leakage of bacteria, in the amounts so as to make the final concentration of these compounds as 0.005 and 0.01 M except in the case of barium acetate.
where the concentration used was 0.001 and 0.005 M.

3.2.9.4 Sugars

Pentose (xylose), hexoses (glucose, fructose), disaccharides (sucrose, lactose), polysaccharide (starch) and sugar alcohols (mannitol, glycerol) were used at 1 and 2% concentrations.

3.2.9.5 Nitrogen source

Urea, potassium nitrate and sodium nitrate were used at 25 and 50 ppm concentrations respectively. The concentrations were chosen according to the recommended doses for soil and crops.

3.2.9.6 Inorganic salts (trace elements)

Concentration of Co^{2+} (CoCl₂), Cu^{2+}, Zn^{2+} (ZnSO₄) and Mn^{2+} (MnCl₂) were chosen according to their quantity present in the soil. Concentrations used were Cobalt: 10, 12.5 and 15 ppm; Copper: 25, 50 ppm; Zinc: 25, 50 ppm and Manganese 5 x 10⁻⁵, 1 x 10⁻⁵ M.

3.2.9.7 Antibiotics

Bacitracin, carbenicillin, erythromycin and chloramphenicol were used at 20 µg/ml each.

3.2.9.8 Pesticide

TMTD was used at 0.1 to 20 ppm concentrations.
3.2.9.9 **Inhibitors**

Sodium fluoride and sodium azide were used at concentrations of 0.5, 100 and 1000 µg/ml.

3.2.9.10 **Effect of soil texture on ammonia release by Azotobacter chroococcum**

Sand, soil and sand mixed with different amounts of bentonite clay in 250 ml conical flask was prepared and the release of ammonia by *Azotobacter* was studied as follows: 100 g of soil in 250 ml conical flask was incubated with 5% of freshly grown culture. The incubation was carried out for 20 days at 30°C. After 20 days soil was extracted with 10 M NaCl and ammonia was measured by ammonia electrode method. The experiment on release was performed under both sterilized and unsterilized conditions.

3.2.11 **Estimation of ammonia assimilatory enzymes**

For the studies of ammonia assimilatory enzymes, *A. chroococcum* strains were grown in 100 ml Erlenmeyer flask containing 30 ml of Burk's medium under shake and stationary conditions of incubation. Cells growing under stationary conditions were collected after 4 days interval during a total period of 20 days and those growing on shaker were harvested at 24, 48 and 72 hrs of incubation. Cells were collected by centrifugation at 10,000×g for 20 min at 4°C in a refrigerated centrifuge. The cell pellet was washed with 0.02 M (pH 7.0) imidazole HCl buffer and was recentrifuged.
3.2.11.1 Preparation of cell free extracts (CFE)

Cell free extracts were prepared by suspending 200 mg fresh weight of washed cell pellet in four ml of ice cold 0.2M Tris-HCl (pH 7.0) buffer and four sonication treatments of 15 seconds each under cold conditions were given using a ultrasonicator (Chandana, 1982). Two minutes gap was given between each sonication treatment to avoid overheating. After the sonication, the suspension was centrifuged in refrigerated centrifuge at 10,000 x g for 20 min at 4°C. The supernatants were removed and immediately used for enzyme assay. Extracts prepared in 0.2 M Tris-HCl buffer were used for glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). Extracts prepared in 0.2 M imidazole-HCl were used for glutamine synthetase (GS) assay.

3.2.11.2 Enzyme assays

3.2.11.2.1 Glutamine synthetase (GS)

It was measured by the transferase hydroxamate assay described by Shapiro and Stadman (1970). 10 ml of reaction mixture was prepared by mixing 2 ml of imidazole HCl buffer, 4 ml of 0.2 ml glutamine, 0.6 ml of 0.1 M MnSO₄, 0.8 ml of 0.01 M ADP, 0.4 ml of 1.0 M sodium arsenate and 2.2 ml of glass distilled water. 0.5 ml of reaction mixture was mixed with 0.2 ml of enzyme extract and water to give total volume of 0.9 ml. The contents were maintained at 30°C for 5 min. The reaction started by the addition of 0.1 ml
of the hydroxylamine solution (1M NH$_2$OH.HCl and 1M NaOH) and incubated at 30°C for 20 min. Reaction was stopped by the addition of 2 ml of ferric chloride reagent (10% w/v FeCl$_3$, 24% w/v TCA, 6M HCl and distilled water (8:2:1:13). In control assay, hydroxylamine was omitted. The optical density was measured at 540 nm using spectronic-21 colorimeter (Bausch and Lamb). L-glutamyl monohydroxamate was used as standard. One unit of enzyme activity is defined as that amount of enzyme liberating 1.0 ug of L-glutamyl-monohydroxamate/min of incubation at 30°C.

3.2.11.2.2 Glutamate synthase (GOGAT)

This enzyme was assayed by monitoring the oxidation of NADH to NAD$^+$ (Misra et al., 1981). The reaction mixture contained 0.7 ml of 0.2 M-Tris HCl buffer pH 8.6 containing 2% w/v mercaptoethanol; 0.2 ml of 0.1 M -ketoglutarate neutralised; 0.02 ml of 0.01 M EDTA 0.2 ml of 0.3 M KCl and 0.2 ml of 1 mM NADHNa$_2$. The reaction mixture was incubated at 35°C and 0.2 ml of enzyme extracts was added to start the reaction. The decrease in O.D. was measured at 340 nm using Gilford Spectrophotometer. One unit of enzyme activity is defined as that amount of enzyme which catalyses the oxidation of 1.0 n mole of NADH/min of incubation at 35°C.

3.2.11.2.3 Glutamate dehydrogenase (GDH)

GDH was assayed by the method of Duke and Ham(1976) following the oxidation of NADH to NAD$^+$. 2.8 ml of reaction
mixture which contained 2.4 ml of 0.1 M Tris-HCl buffer pH 7.5; 0.1 ml of 3 M NH₄Cl; 0.1 ml of 0.33 M \( \alpha \)-Ketoglutaric acid (pH 7.0); 0.2 ml of 1 mM of NADHNa₂ was mixed with 0.2 ml of the enzyme extract to initiate the reaction. The reaction was carried out at 35°C and the decrease in O.D. was monitored at 340 nm. One unit of enzyme activity is defined as that amount of enzyme which catalyses the oxidation of 1.0 nmole of NADH per min of incubation at 35°C.

3.2.11.3 Total proteins in CFE was estimated according to Lowry et al. (1951) and Herbert et al. (1971).

3.2.12 Analysis of cell walls of A. chroococcum

Cell walls of A. chroococcum were analysed for amino acids, proteins, sugars and lipids according to the method of Forrester and Wicken (1966). A. chroococcum cells were grown in Jensen's nitrogen free medium, centrifuged and washed twice with 0.2 M phosphate buffer in saline (pH 7.2) and finally suspended in a small volume of PBS and sonicated with an ultra sonicator (1.5 K cycles) for 20 min at 4°C. Unbroken cells were separated by centrifugation at 2500 x g for 15 min. Cell walls from broken cells were separated by centrifugation at 30,000 x g for 30 min. The cell walls were then suspended in 2 M NaCl solution and stirred at 4°C for 60 min. Supernatants were discarded and cell wall preparations were washed twice with cold distilled water and lyophilized. Lyophilized cell walls were subjected to different hydrolysis
treatments to detect amino acids, sugar, hexosamine, teichoic acid and lipids.

3.2.12.1 Amino acid

Amino acids in the cell walls were analysed by heating 2 mg of lyophilised cell wall with 200 µl of 6N Hydrochloric acid under nitrogen atmosphere in sealed tubes at 105°C for 16 hrs. Humin was separated by centrifugation. The amino acids were detected in hydrolysate by ascending chromatography using butanol, acetic acid and water (4:1:5) as solvent. 0.25 % ninhydrin (in acetone) was used as the spraying agent.

3.2.12.2 Protein in the cell wall was estimated by the method of Lowry et al. (1951).

3.2.12.3 Sugars

The analysis and detection of sugars in the cell walls were done by heating 3 mg of lyophilised cell walls with 200 µl of 2N H₂SO₄ under nitrogen atmosphere in sealed tubes at 100°C for 2 hrs. Hydrolysates were neutralized with 0.2 N barium hydroxide, the precipitate of barium sulphate was removed by centrifugation and washed twice with water. Supernatant fluids and the washings were passed through Dowex 50 (H⁺) (1-2 ml). The resin was washed with 5 column volumes of water and combined elutes were concentrated by rotary evaporation. Sugars were identified by ascending chromatography using butanol : pyridine : water (6:4:3) as solvent.
and 0.5% benzidine was used as the spraying agent. Rest of the hydrolysate was used for the estimation of total reducing sugars by Dubois-phenol-sulphuric acid method (1956).

3.2.12.4 Hexosamines

Hexosamines were estimated by heating 2 mg of lyophilized cell walls with 200 ul 4 N HCl in sealed tubes for 4 hrs at 100°C. Excess of hydrochloric acid was removed by vacuum over phosphorous pentaoxide and NaOH pellets. Neutral sugars were removed by passing the hydrolysates through short column of Dowex 50 (H') resin (1-2 ml). After washing the resin with water (5 column volume), hexosamine was eluted with 1M NH₄OH and evaporated to dryness in vacuum, diluted and were quantitatively estimated from the hydrolysates by the method of Elson and Morgan (1955). Values were calculated from standard curve of N-acetyl glucosamine.

3.2.12.5 Extraction of teichoic acid

Teichoic acid was extracted from 25 mg portion lyophilized cell with 10% TCA at 5°C for 48 hrs and centrifuged. After centrifugation, teichoic acid was precipitated by addition of 5 volumes of ethanol and phosphorous was estimated by the method of Bartlett (1959).

3.2.12.6 Determination of lipids in cell walls

Lipids from the cell walls were determined by treatment of lyophilized cell walls with chloroform:methanol (2:1)
for 2 hours under cold conditions and identified by Thin Layer Chromatography.

3.2.13 Polysaccharides

Polysaccharides of *A. chroococcum* strains were estimated by the method of Amarger et al. (1967). *A. chroococcum* was grown in Jensen's nitrogen free medium and incubated for 48 hrs. Cells were centrifuged at 10,000 x g for 25 min and polysaccharides present in supernatant were precipitated with cold acetone. Precipitates were treated with concentrated sulphuric acid and was refluxed for 11 hrs in water bath at 85-90°C. Excess of sulphuric acid was removed by precipitating out with barium carbonate and dilutions were made. Exopolysaccharides (EPS) were determined by the method of Mokrasch (1954) (Anthrone's reaction). Individual sugars were detected by ascending paper chromatography using Butanol : pyridine : water (6:4:3) as solvent. Sugars were detected by spraying with 0.5% benzidine (10 ml of 40% TCA diluted to 100 ml with ethanol). *R*<sub>f</sub> values of unknown sugars were compared with the standard *R*<sub>f</sub> values.

3.2.14 Determination of total lipids

3.2.14.1 Preparation of cells

*A. chroococcum* strains were grown in Jensen's nitrogen free medium under stationary conditions at 30°C. Cells were centrifuged washed twice with distilled water, lyophilized and stored at -20°C.
3.2.14.2 Extraction of lipids

Lipids were extracted according to the method of Folch and Lees (1957). For this, 500 mg of lyophilized were mixed with 20 ml of chloroform : methanol (2:1) and extraction was carried out at 4°C with a continuous stirring for a period of 2 to 4 hrs with a magnetic bar over a magnetic stirrer. The extract was filtered through fat free Whatman filter paper. Filtrate was washed twice with chloroform: methanol:water (3:48:47) and kept in refrigerator for 24 hrs. A biphasic system was formed. Washings were given to the lower layer with chloroform : methanol : water (3:48:47) and allowed it to stand for sometime at room temperature. Upper layer was removed and volume of the lower layer was made to twenty ml with chloroform and methanol (2:1).

3.2.14.3 Estimation of total lipids

Total lipids in the above extract were estimated by the method of Frings and Dunn (1970). Total lipids were estimated by the addition of 1.9ml of conc. sulphuric acid to 0.1 ml of extracted sample and kept in the boiling water bath for 15 minute. After cooling it for 5 min, 0.2 ml of this acid treated extract was taken into another tube and 5 ml of 0.6 % vanillin and orthophosphoric acid (20:60) was added, incubated for 30 min and colour measured at 540 nm by using spectronic-21. Readings were converted into μg/ml from the standard values of olive oil. Lipid contents were calculated as μg/mg of cell weight.
3.2.14.4 Phospholipids

Phospholipids were estimated by the method of Bartlett (1959) by measuring the total phosphorus in the samples and the values calculated from $K_2HPO_4$ standard. Qualitative detection of phospholipids was done by thin layer chromatography (Renderath, 1965) using the following solvents (1) petroleum ether : diethyl ether : acetic acid (90:10:1), (2) chloroform : methanol : water (65:25:4). Spots were detected by exposing the plates to iodine vapours. The $R_f$ values were compared to the $R_f$ values of standard phospholipids.

The effect of compounds which stimulate (3% glycerol, 20 ppm sodium oleate and 0.5% sodium acetate) and decrease ($10^{-4}$ M flavin mononucleotide) lipid production (Hugo and Stretton, 1966) were studied on lipids of *A. chroococcum* strains AC2, AC50, and AC45.

3.2.15 Determination of antibiotic resistance of the isolates of Azotobacter chroococcum

All the isolates of *A. chroococcum* were tested for resistance to various antibiotics using Burk's medium incorporated with different antibiotics. One loopful of growth from two days old slant was suspended in 1 ml sterilized distilled water and a loopful of this cell suspension was spotted on the antibiotic plates in check board pattern. Growth was evaluated after 4 days. Concentration of antibiotics used was 10 $\mu$g/ml each of bacitracin, chloramphenicol, ampicillin, erythromycin, polymyxin B, carbenicillin,
vancomycin, neomycin, gentamycin, tetracycline and kanamycin for single as well as for multiple antibiotic resistance.

3.2.16 Curing studies of A. chroococcum strain AC2

The nongrowth inhibitory concentrations of A. chroococcum strain AC2 was determined using different curing agents. Initially the nongrowth inhibitory concentration for various curing agents were prepared as follows: 30 ml of Burk's media was distributed in 100 ml Erhlemeyer flask and curing agents like ethidium bromide (0, 1, 2, 3, 4, 5 and 10 μg/ml), acridine orange (0, 2, 5, 10, 15, 25 and 50 μg/ml), acriflavin (0, 1, 2, 3 μg/ml) and SDS (0, 25, 50, 75, 100, 200, 300, 400 and 500 μg/ml) were added.

Flasks were inoculated with actively growing A. chroococcum cells (10^3-10^4 cells/ml) and incubated at 30°C on shaker. After 24-28 hrs maximum concentration which did not inhibit the growth of the organism was taken as the non-growth inhibitory concentration. Flasks containing determined concentration of curing agent was inoculated and incubated for 18 hrs and then transferred to Burk's + 400 μg N as ammonium acetate and incubated. After 24 hrs culture was plated on Burk + Ammonium acetate plates, and incubated at 30°C. Colonies were picked, suspended in 1 ml sterilized distilled water and spotted on control plates (Burk's nitrogen free medium): Burk + antibiotics, Burk + ammonium acetate (400 μg N/ml), and Burk + ammonium acetate and respective antibiotics and EL-medium.
The colonies which did not grow on nitrogen free media but grew on plates with nitrogen source, were picked up and retested to check their antibiotic resistance property. Finally the nitrogen fixing and ammonia excretion ability of these colonies were checked.

3.2.16.1 Detection of plasmid DNA

Agarose gel electrophoresis was used for detection of plasmid DNA by the method of Ledeboer (1977).

3.2.17 Soil enzymes

Following soil enzymes were estimated from the soil samples taken from soils planted with different crops after 35 days of growth.

3.2.17.1 Amylase

Amylase activity was estimated by the method of Hofmann, (1963) and Cole, (1977). To 5 gm of air dried soil, 1.5 ml of toluene was added and mixture was shaken and allowed to stand for 15 min following the addition of 10 ml distilled water and 5 ml of 2 % (w/v) soluble starch. The flask was then stoppered and incubated at 37°C for 5 hrs. After 5 hrs of incubation, 15 ml of distilled water was added to the flask and contents were mixed. Ten ml of the soil suspension was centrifuged to produce a clear supernatant and was then analysed for reducing sugars by Nelson-Somogyi's method (1944) and expressed as μ mole of glucose released gm⁻¹ soil 24 hr⁻¹.
3.2.17.2 Invertase

Invertase activity was estimated by the method of Hofmann and Seegerer (1951) and Cole (1977). 10 gm of fresh soil was sieved through 2 mm sieve and was mixed with 2.5 ml of toluene in 250 ml conical flask, incubated for 15 min at 20°C and further incubated at 37°C for 2 hrs with 20 ml of 0.5 M acetate phosphate buffer (pH 5.5) and 20 ml of 5% sucrose solution. After incubation, contents were filtered and reducing sugars were estimated in the filtrate by Nelson-Somogyi's method and values were expressed umole of glucose released gm⁻¹ soil 3 hr⁻¹.

3.2.17.3 Cellulase

The cellulase activity of the soils was estimated by the method of Pancholy and Rice (1973). 5 gm of soil was mixed with 0.5 ml of toluene and kept for 15 min followed by the addition of 10 ml of acetate buffer pH 5.9 and 10 ml of 1% carboxymethyl cellulose. The flasks were incubated for 24 hrs at 30°C. After 24 hrs 50 ml of distilled water was added and volume of the filtrate was made to 100 ml. The reducing sugar was determined by Nelson-Somogyi's method (1944).

3.2.17.4 Phosphatase

Phosphatase activity was determined by the method of Tabatabai and Brenner (1969). One gm of soil was taken in a test tube and incubated with 1 ml of 5 mM buffered sodium-p-nitrophenyl phosphate in acetate buffer (pH 5.2) and 0.3 ml of toluene was added and incubated at 37°C for 1 hr. Incubated
soil was treated with 4 ml water, 10 ml of 0.5 M calcium chloride & 4 ml of 0.5 M sodium hydroxide and filtered through Whatman No. 42 paper. Filtrate was shaken and readings were recorded at 450 nm and expressed as umole p-nitrophenol released g⁻¹ soil hr⁻¹.

3.2.17.5 Arylsulphatase

Arylsulphatase activity was measured by the method of Tabatabai and Bremner (1970), 4 ml of 0.5 M sodium acetate (pH 5.8), 0.25 ml of toluene and 1 ml of 0.005 M p-nitrophenyl sulphate solution was added to 1 gm of soil. Flasks were swirled for few seconds, stoppered and placed at 37°C for 1 hr. After 1 hour of incubation, 1 ml of 0.5 M calcium chloride and 4 ml of 0.5 M sodium hydroxide were added and shaken. Finally the soil suspension was filtered and intensity of yellow colour was measured at 400 nm and activity was expressed as umole of p-nitrosulphate released g⁻¹ soil hr⁻¹.

3.2.18 Pot house experiments

3.2.18.1 Garden soil and sand were mixed in 2:1 ratio, sieved to ensure proper mixing and amended with 0.5 % K₂HPO₄ and 0.1 % calcium carbonate. Experiments were done both under sterilized and unsterilized conditions. Soil was sterilized at 15 lbs pressure for 1 hr for three consecutive days and pots were filled.
3.2.18.2 Plant inoculation

Seeds used in these studies were surface sterilized by the method described by Vincent (1970) and germinated on 1% agar. Prior to sowing, seeds were treated with different strains of rhizobia and azotobacter according to the treatments and were planted in sterilized and unsterilized soil. Six seedlings were kept in each pot. Plants were watered twice a day in summer and once a day in winter. Plants were harvested after 35 days of growth.

3.2.18.3 Nitrogen fixing (C$_2$H$_2$ reduction) activity

Nitrogenase activity was measured in all the crops studied by the method of Hardy (1973). The assay was conducted in 100 ml serum bottle with a hole on top but protected by the inner rubber septum. Plants were dug out carefully and adhering soil was gently removed (Hardy et al., 1973). The plants were decapitated and two or four nodulated roots systems were introduced in 100 ml serum bottle. 10 per cent volume of air was removed from serum bottle with the help of a disposable syringe and equal volume of gas was injected. The bottles were buried in the soil under the shade and incubated for 1 hr. All samplings and inoculations were done between 10.00 A.M. to 11.30 A.M. Samples were removed and ethylene determined by gas chromatography using Portable Gas chromatograph. Nitrogenase activity was expressed as nanomoles of C$_2$H$_4$ hr$^{-1}$ plant$^{-1}$ per gm fresh weight of nodules.
3.2.18.4 Determination of nodule number and fresh weight of nodules

Following the assay, the nodules were detached washed, blotted dry and fresh weight of nodular tissue and their number was determined.

3.2.18.5 Determination of dry weight of plants

The shoots were dried to constant weight at 80°C and the dry weight was determined. All values in each crop are mean values of at least six replicates.

3.2.18.6 Total nitrogen

Total nitrogen was determined by conventional microKjeldahl's method.