MATERIAL AND METHODS

The investigations were carried out in ten different desi and kabuli genotypes of chickpea (*Cicer arietinum* L.) belonging to the family Fabaceae. Seeds of different genotypes were obtained from Central Soil Salinity Research Institute, (CSSRI) Karnal. The skeleton of the experiment is outlined below:

<table>
<thead>
<tr>
<th>Year</th>
<th>2000-01</th>
<th>2001-02</th>
<th>2002-03</th>
</tr>
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<tr>
<td>Crop</td>
<td><em>Cicer arietinum</em> L.</td>
<td><em>Cicer arietinum</em> L.</td>
<td><em>Cicer arietinum</em> L.</td>
</tr>
<tr>
<td></td>
<td>(Chickpea)</td>
<td>(Chickpea)</td>
<td>(Chickpea)</td>
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<tr>
<td>Genotypes</td>
<td>CSG 9651</td>
<td>CSG 9651</td>
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<td></td>
<td>BG 267</td>
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<td>Petridish and Pot culture</td>
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<tr>
<td>Replications</td>
<td>30 &amp; 6</td>
<td>30 &amp; 6</td>
<td>30 &amp; 6</td>
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</table>

Since the method of sowing raising of plants, observations recorded and methodology used were similar, following description is applicable to all the genotypes.
1. PETRIDISH CULTURE

Seeds of various desi and kabuli chickpea varieties were surface sterilized with 0.2 percent mercuric chloride (HgCl₂) solution for 2 minutes and then rinsed with sterile distilled water two or three times. They were inoculated with a salt tolerant *Mesorhizobium ciceri* strain PF: 75 before sowing. Ten seeds were put in each sterile petridish provided with Whatman No. 1 filter paper and filled with saline soils (garden loam, sand and farmyard manure) of various concentrations i.e. 4, 6 and 8 deciSemenes per unit meter (dSm⁻¹) electrical conductivity prepared from sodium chloride, calcium chloride and sodium sulphate according to the recommended specifications (Richards, 1954). For control, seeds were maintained in salt free soil. Each treatment was replicated thrice. Germination counts were taken after 48 hours of sowing and continued till the data became constant and there was no further sprouting of the seeds under various treatments.

2. POT CULTURE

Chickpea (*Cicer arietinum* L.) seeds of all the genotypes were subjected to preliminary selection on the basis of their uniformity in size and colour. They were surface sterilized with 0.2 percent mercuric chloride and were inoculated with a commercial preparation of salt tolerant *Mesorhizobium ciceri* strain PF: 75 procured from the Department of Microbiology, I.A.R.I., New Delhi. Pots (320 in number, size 30 x25x25 cm) were lined with polythene bags and a thick wad of loose glass wool was placed at the bottom of each pot that was covered with an inverted watch glass. Each pot was filled with garden loam and soil mixed with farmyard manure (7 kg in each pot) in the ratio of 2:2:1. Ten seeds per pot, after prior inoculation with a proper rhizobial strain were sown at uniform depth and distance, under well
watered conditions. When the seedlings were well established, thinning was done to have only three plants of uniform size in each pot.

For salt treatments the pots were numbered and divided into equal lots of 20 pots each. Once the symbiosis was well established (i.e. 15 days after sowing) the plants were subjected to salt stress by adding saline solutions of various concentrations i.e. 4, 6, 8 dSm$^{-1}$ electrical conductivity, prepared from a mixture of sodium chloride calcium chloride, and sodium sulphate according to recommended specifications (Richards, 1954). Control plants were maintained in salt free soil. Desired salinity levels of the soil were maintained throughout the growing period of the plants by treating the pots with saline solutions of desired concentrations at regular weekly intervals after duly monitoring the conductivity levels of soils with the help of electrical conductivity, (EC) meter.

3. PROCEDURE FOR SAMPLING

The required numbers of pots (2 in each case) were selected from each of the treatment for sampling at appropriate time interval. i.e. 40($S_1$), 70($S_2$), 100($S_3$) days after sowing. Three plants of one pot were sampled, at random, for recording the observation. Since the root systems of the plants were badly entangled and inseparable, the data was taken for all the three plants in the pots and calculated on per plant basis. The plants were carefully separated from the pots by gently washing out the soil with running tap water in order to avoid damage to the nodules. The sticking soil, along with the plants were transferred to the sieve and the roots with attached nodules were washed to clear of adhering soil. The detached roots and nodules were collected from the sieve and combined with the rest of plant material. On the basis of preliminary observations recorded for all the
ten genotypes, four genotypes, two each of desi (DCP 92-3, CSG 8962) and kabuli (BG 267, CSG 9651) differing in their salt sensitivities i.e. former most salt sensitive and the latter most salt tolerant respectively, amongst the two groups, were selected for a detailed study. The data recorded pertains to the four genotypes only. All these four genotypes of chickpea are at present the released cultivars of CSSRI, Karnal.

4. OBSERVATIONS RECORDED

The plant samples from all the cultivars of chickpea were analysed for various physiological and biochemical parameters.

4A. Dry Weights

For dry weight measurements, the samples were dried in an oven at 70°C for a few days till they reached constant weight. The following observations were recorded under each of the experiments:

1. Per cent germination
2. Dry weights of root at the three sampling stages
3. Dry weights of shoot
4. Number of nodules per plant
5. Dry weights of nodules per plant
6. Total number of flowers per plant
7. Pod number per plant
8. Seed weight per plant
9. Hundred seed weight
10. Total plant dry weight at harvest

4Bl. Speed Of Germination Index (SGI):

SGI calculated for the entire period using the formula developed by Maguire (1962):

\[ SGI = \sum_{i=1}^{c} \frac{N_i}{i} \]
Where $i = \text{germination count day}$

$N_i = \text{number of seeds germinating on day } i$

$c = \text{total number of days.}$

4B II. Growth Rate and Relative Growth Rate

The simple growth rate (GR) of plants under various saline treatments was calculated by employing the equation:

$$\text{GR} = \frac{W_2 - W_1}{t_2 - t_1}$$

Relative growth rate (RGR) values were calculated by employing the standard equation as given by Evans (1972) as follows:

$$\text{RGR} = 2.303 \frac{(\log_{10} W_2 - \log_{10} W_1)}{t_2 - t_1}$$

4C Statistical Analysis

The data was analysed statistically following the methods of analysis of variance. All the values are means of six replications per treatment. Standard deviation (S.D.) and standard error (S.E.) of the means were calculated and the values are incorporated into the tables/figures.

5. BIOCHEMICAL ESTIMATIONS

5A Leghemoglobin

The nodules were detached immediately after sampling and their leghemoglobin content was determined by the method of Hartree (1957), which is based upon the conversion of hematin to pyridine hemochromogen.

5A.1 Reagents: 1) 0.05 M phosphate buffer (pH 7.0), 2) 0.2 N NaOH.
5A.2 Extraction

500 mg of fresh nodular tissue was homogenized in 5 ml of 0.05 M phosphate buffer (pH 7.0). The extract was centrifuged at 10,000 rpm for 15 minutes with the discarding of residue.

5A.3 Precipitation

The saturation level of (NH₄)₂SO₄ was raised to 50 per cent by gradual addition of ammonium sulphate and allowing the same to stand for one hour at 4°C. The solution was centrifuged again and the residue was discarded. Subsequently, the saturation percentage of (NH₄)₂SO₄ was further raised to 80 per cent. The precipitates containing all the components of leghemoglobin were dissolved in 2 ml distilled water and to it, 2 ml of 0.2 N NaOH was added. After one hour, 1 ml of pyridine and 50 mg of sodium hydrosulphite were added so that their ultimate concentrations were 20 per cent and 1 per cent respectively. The O.D. was taken at 545 nm after half an hour following centrifugation at 10,000 rpm for 10 minutes. Standard curve was prepared using graded concentrations of hemin.

5B Rate of Nitrogenase Activity

The acetylene – ethylene assay of nitrogenase activity was done by the method of Herdina and Silsbury (1990). The root system with intact nodules was incised from freshly separated plants and was incubated at room temperature in vials containing acetylene (C₂H₂) (10%, v/v) in air and sealed with serum caps. The samples were flushed with acetylene gas by gentle shaking of the bottles and were incubated for 1 hour. The gas chromatography unit was kept on for 15 minutes in order to stabilize the instrument. The sample of 1 ml of gas from the incubation mixture was analysis for ethylene in a Perkin Elmer 8600 gas chromatograph equipped with a Porapak R column.
(Ligero et al., 1986). Room temperature at the time of estimation was also recorded. From the standard values, n. moles of ethylene produced in each case was calculated. The nodules were dried in an oven at 70°C for 24 hours and their dry weights were taken. The rate of enzyme activity was calculated as n.moles of ethylene produced per mg dry weight of nodules per hour.

5 C. Invivo Nitrate Reductase (NR) Activities

NR was assayed by the modified method of Hageman and Hucklesby (1971) and Nair and Abrol (1973).

5C.1 Reagents
a) Phosphate buffer (0.2M, pH 7.5)
b) Potassium nitrate (0.4M)
c) 1 percent (w/v) sulphanilamide
d) 0.01 percent NEDD (N-1 napthyl ethylenediammonium dichloride)
e) Sodium nitrite standard solution.

5C.2 Extraction

The leaves as well as roots were cut into small pieces of about 8-10 mm. After thorough mixing, samples weighing 500 mg were placed in culture vials of 30 ml capacity containing ice cold incubation medium (incubation medium: 3 ml of 0.2 M potassium phosphate buffer (pH 7.5) and 0.4M potassium nitrate solution). The culture vials containing incubation medium and plant tissue were evacuated with the help of a vacuum pump for about 30 second and vacuum was released. The process was repeated 3-4 times till the material settled down in the medium. The tubes were then incubated in the water bath at 33°C for one hour of dark and then placed in the boiling water bath.
for 5 minute to terminate the reaction and for the effective efflux of nitrate accumulated in the tissue. Nitrite formed was estimated by diazotiation using the method of Snell and Snell (1949).

5C.3 Assay

Suitable aliquot (0.2 ml) was taken into test tube and 1 ml each of 1% sulphanilamide in 1ml and 0.01% NEDD in distilled water was added. The color was allowed to develop for 25 minutes after which the volume was made to 6 ml using distilled water. The absorbance was read at 540 nm. The amount of nitrite was calculated with the help of calibration curve prepared with sodium nitrite of known concentrations. The enzyme activity was expressed as μmol NO₃ reduced g- fr wt hr⁻¹.

5D Glutamate Synthase

GOGAT was assayed using method of Tempest et al. (1970).

5D.1 Reagents

Tris HCl buffer, 50 mM pH 7.6

Prepared the following reagents in Tris HCl buffer 50 mM pH 7.6.

Glutamine, 5 mM (36.5 mg/10 ml)

2-oxoglutarate, 5 mM (36.5 mg/10 ml)

NADPH, 0.25 mM (10 mg/10 ml)

5D.2 Extraction

500 mg of nodules were homogenized with 5 ml of 100 mM phosphate buffer pH 7.5 containing 1 mM disodium EDTA, 1 mM dithioerythritol and 1% polyvinyl pyrrolidone (PVP) and then centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was collected for enzyme assay.

5D.3 Assay
The reaction mixture was prepared using 1 ml glutamine, 1 ml 2-oxoglutarate, 1.0 ml NADPH, 0.2 ml enzyme extract and buffer 1.8ml. In the blank instead of 2-oxoglutarate, 1ml of buffer was added. The mixture was incubated for 15-30 minute at 37°C and then the change in absorbance was recorded as n mole of NAD (P) H oxidized per min per mg protein.

5E  Glutamine Synthetase

GS was assayed according to method of Rowe et al. (1970).

5E.1 Reagents

(i)  0.1 M tris HCl buffer (pH 7.6)
(ii) 0.2M imidazole HCl buffer (pH 7.5)
(iii) 0.1 M Mg Cl₂
(iv) Mercaptoethanol (1:100v/v)
(v)  0.5 M sodium glutamate
(vi) 0.5 m NH₂OHHCl (freshly prepared by mixing equal volume of 1.0 M NH₂OHHCl and 1.0 M NaOH)
(vii) 0.1 M ATP.
(viii) FeCl₃ (containing FeCl₃ 0.37M, HCl 0.67M, TCA 0.2M)

5E.2 Extraction

500 mg of nodules were homogenized in a mortar with a pestle using ice cold 0.1 M tris HCl buffer (pH 7.6). The homogenate was centrifuged at 10,000 x g for 40 minutes in a refrigerated centrifuge. All the steps were carried out at 0-4°C. Clear supernatant was decanted and was used as crude extract for enzyme assays.

5 E.3 Assay
Reaction mixture comprised of 0.4 ml 0.2M imidazole HCl buffer, 0.4 ml 0.1 M MgCl₂, 0.4 ml mercaptoethanol, 0.2 ml 0.5 M sodium glutamate, 0.2 ml freshly prepared 0.5 M NH₂ OH HCl, 0.2 ml 0.1 M ATP and 0.3 ml enzyme extract. The reaction mixture was then incubated at 37°C for one hour in a shaking water bath. The reaction was stopped by the addition of 3 ml of FeCl₃ reagent. The contents were centrifuged at 5000x g for 5 minutes and absorbance of the clear supernatant was recorded at 535 nm against reagent blank. In the control sets, the ATP and substrate was omitted. A standard curve using α-glutamyl hydroxamate (0.2-2.0 μ moles) was prepared and the enzyme activity was expressed on the basis of α-glutamyl hydroxamate formed.

5F Glutamate Dehydrogenase Activity

GDH was assayed according to Pahlich and Joy (1971).

5F.1 Reagents

i) 0.1M phosphate buffer
ii) 0.2 M tris HCl buffer (pH 8.0)
iii) 0.2 M α-ketoglutaric acid, adjusted pH to 7.5-8.0 with NaOH
iv) 3.2 M ammonium sulfate
v) 1 mM NADH.

5F.2 Extraction

The enzyme extract was prepared by crushing 500 mg of nodules in a prechilled pestle and mortar using 5 ml 0.1 M phosphate buffer. After centrifugation at 25000 Xg for 10 minute the supernatant was used for assay.

5F.3 Assay
Enzyme extract was added to 2ml of tris HCl buffer (pH 8.0). In the sequence, 0.2 ml \( \alpha \)-ketoglutaric acid and 0.2 ml of ammonium sulfate were added. The reaction was started by adding 0.5 ml of NADH. The tubes were incubated at 37°C for 30 min. For blank instead of \( \alpha \)-ketoglutaric acid 0.2 ml of water was added. The change in absorbance was measured at 340 nm. The enzyme activity was defined as n mole of NADH oxidized/ min/ mg/ protein.

### 5G Nitrogen Content

The nitrogen content of leaves was determined using colorimetric method of Lindner (1944).

#### 5G.1 Reagents

1. Digestion mixture: Mixture of concentrated H\(_2\)SO\(_4\) and HClO\(_4\) (70 per cent ) in the ratio of 9:1, (ii) 2.5 N NaOH, (iii) 10 per cent sodium silicate, (iv) Nessler’s reagent: Dissolved 100g Hgl\(_2\) and 70 g KI in about 400 ml distilled water, (v) Dissolved 100g NaOH in about 500 ml distilled water, cooled added to solution (iv) and made the volume to one litre. Filtered the solution and stored the reagent in a brown bottle.

#### 5G.2 Digestion

50 mg of dried and well ground plant material was taken in a 25 ml conical flask with 3 ml of digestion mixture. The flasks were heated gently over a heating plate until the solution became colourless. The digest was cooled and added 0.5 ml of 30 per cent H\(_2\)O\(_2\). The solutions were heated gently until the same became clear and colourless. The solutions were cooled and diluted to 100 ml with distilled water.

#### 5G.3 Estimation
0.5 ml aliquot of the diluted digest was taken and to it 0.3 ml of 2.5 N NaOH was added to partially neutralize the excess acid. Another amount of 0.1 ml of 10 per cent sodium silicate was added to the same to avoid turbidity and the final volume was made to 5 ml with distilled water. After shaking thoroughly, 5 drops of Nessler’s reagent were added. The mixture was allowed to stand for 30 minutes at room temperature. The O.D. was recorded at 420 nm on Double Beam UV-190 spectrophotometer against reagent blank. The standard curve was prepared using graded concentrations of (NH₄)₂SO₄.

5H Total Free Amino Acids

Method of Lee and Takahashi (1966) was adopted for the estimation of total free amino acids.

5H.1 Reagents

1) Ninhydrin reagent (pH 6.0): This reagent was prepared by mixing the following constituents (A., B, and C) in the ratio of 5:12:2.
A) 1 per cent Ninhydrin in 0.5 M citrate buffer pH 5.5, B) Pure Glycerol, C) 0.5 M citrate buffer pH 5.5.

5H.2 Extraction

100 mg of dried material was homogenized in 5 ml of 80 per cent ethanol, refluxed for 15 minutes on a steam bath and centrifuged. The residue was further refluxed twice with 80 per cent ethanol. The supernatants were pooled together for free amino acid estimations.

5H.3 Procedure

0.2 ml of extract was added to 3.8 ml of ninhydrin reagent. The contents were heated in boiling water bath for 12 minutes and cooled to room temperature. The purplish blue colour was read at 570 nm. The quantity of total free amino acids was calculated from the
reference curve prepared by using glycine (5-50 μg) and expressed as μg amino acids per mg tissue dry weight.

51. Proteins

The total proteins were estimated by the method of Bradford (1976).

51.1 Reagents

1) 85 per cent (w/v) O-phosphoric acid, 2) Coomassie Brilliant Blue G-250, 3) 95 per cent ethanol, 4) Protein reagent: Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml of 95 per cent ethanol and to it added 100 ml of 85 per cent (w/v) O-phosphoric acid. The resulting solution was diluted to a final volume of 1 litre. Final concentrations in the reagent were 0.01 per cent (w/v) Coomassie Brilliant Blue G-250, 4.7 per cent (w/v) ethanol and 8.5 per cent (w/v) O-phosphoric acid.

51.2 Extraction

100 mg of plant tissue (leaves) was homogenized in 5 ml phosphate buffer (pH 7.0). The extract was centrifuged at 15000 rpm for half an hour. The residue was re-extracted with 3 ml phosphate buffer and the supernatant were pooled together.

51.3 Procedure

0.1 ml of the above extract was added to 5 ml of protein reagent and the contents were mixed thoroughly. The O.D. was taken at 595 nm against reagent blank prepared from 0.1 ml of appropriate buffer and 5 ml of protein reagent. The weight of protein was plotted against corresponding absorbance resulting in a standard curve used to determine the protein in unknown sample.
5J  **Estimation of total soluble Sugars**

The sugars were estimated by the method of Yemm and Willis (1954) with some modification as follows:

5J.1 **Reagents**

0.2 gm anthrone in 100 ml concentrated sulphuric acid

5J.2 **Extraction**

100 mg dried material was homogenized in 5 ml of 80% ethanol, refluxed for 15 minutes on a steam bath and centrifuged. The residue was further refluxed twice with 80% ethanol. The supernatants were pooled together for estimation.

5J.3 **Estimation**

0.1 ml of ethanol extract was evaporated to dryness in a test tube with a spirit lamp. On cooling, the residue was dissolved in 1 ml of distilled water. Added to it, 4 ml of anthrone reagent and heated in a water bath for 10 minutes. After cooling, O.D. was read at 620 nm against reagent blank. Standard curve was prepared using graded concentrations of glucose.

5K  **Chlorophyll Content**

Extraction of chlorophyll was done in the dimethyl sulphoxide (DMSO) from the leaf discs of plants following the method of Hiscox and Israelstam (1979).

5K.1 **Estimation**

Fully expanded leaves of treated plants were finely cut into uniform discs. (100 mg fresh weight) and suspended in 10 ml of DMSO and then incubated at 65°C for one hour. The DMSO was recovered by decantation and the final volume was corrected to 10 ml with fresh DMSO. The extinction value of chlorophyll in DMSO that recovered
was measured at dual wavelength of 645 and 663 nm on spectrophotometer using DMSO as blank. Dry weight equivalent of each of the fresh weight discs in oven maintained at 100°C for 12 hour. The amount of total chlorophyll was calculated from the extinction values following the equation of Arnon (1949).

$$\text{Chla} = 10.63 \times A_{663} - 2.39 \times A_{645}$$

$$\text{Chlb} = 20.11 \times A_{645} - 5.18 \times A_{663}$$

$$\text{Total chl} = 6.45 \times A_{663} + 17.72 \times A_{645}$$

5L **Ribulose 1-5 Biphosphate Carboxylase (RuBCase)**

RuBCase activity was determined according to method of Fair *et al.*, (1973)

5L1. **Reagents**

<table>
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<th>Reagent</th>
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<td>Tris- HCl Buffer</td>
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<tr>
<td>pH 8.0</td>
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<tr>
<td>EDTA</td>
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<tr>
<td>PMSF</td>
<td>1mM</td>
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<tr>
<td>BSA</td>
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</table>

5L2 **Extraction**

500 mg of leaf material were homogenized in a precooled pestle and mortar using 5 ml of grinding medium (50 mM tris HCl buffer, pH8.0, 1mM ethylenediamine tetraacetic acid (EDTA), 1mM Phenylmethane sulfonyl fluoride (PMSF) and 0.1% Bovine serum albumin (BSA). A pinch of PVP was added during grinding. The homogenate was filtered through four layers of cheese cloth and centrifuged at 900 g for 15 minute at O°C. The supernatant was used for assay.

5L3 **Assay**

The enzyme present in 1 ml of supernatant was activated by incubating at room temperature with 0.1 ml each of 10 mM
NaHCO₃, 10 mM MgCl₂, 5 mM glutathione, EDTA 0.1 mM, Tris HCl buffer (0.6ml) (50 mM, pH8.0) for 10 minute. The assay medium comprising of 0.2 ml tris HCl buffer, 0.10 ml NaH¹⁴CO₃, 0.1 ml NaHCO₃, 0.01 ml MgCl₂, 0.01 ml EDTA, 0.10 ml activated enzyme extract and 0.10 ml RuBP. All these components except RuBP were added into scintillation vials. Assay medium without RuBP served as blank. The reaction was started by the addition of RuBP in vials and terminated after 10 min with the addition of 0.2 ml 6N acetic acid. The contents of vials were then evaporated to dryness at 65°C. The acid stable ¹⁴C was subsequently counted in a liquid scintillation counter (Packard TRICARB, 1600 TR, USA) after adding 10 ml of scintillation fluid containing 4 g of 2,5 diphenyloxazole (PPO) and 100 mg 1,4-bis[5-Phenyl -2oxazoly ] benzene; 2,2'-p-phenylene-bis [ 5-phenyloxazole] (POPOP) dissolved in 100 ml toluene. Activity was expressed as ¹⁴CO₂ fixed 10³ DPM per g per h.

5M Invitro Phosphoenol Pyruvate Carboxylase Activity
Invitro PEPC was assayed by the method of Christaller et al. (1977).

5M.1 Extraction
Washed nodules were grounded in ice cold extraction medium (1 gm nodule /5ml buffer) in pre cooled mortar with a pestle. The extraction medium contained 50mM tris HCl, 20 mM MgCl₂, 5 mM dithioethreitol with pH adjusted to 8.0 The homogenate was centrifuged at 35,000xg for 20 minute. The supernatant obtained was assayed according to procedure of the Maruyama et al. (1966)
5M.2 Assay

The Mg\(^{2+}\) dependent carboxylation of Phosphoenolpyruvate (PEP) resulted in the formation of oxaloacetate and orthophosphate. The reaction rate in the presence of NADH and malic dehydrogenase was determined by following the rate of incorporation of \(^{14}\)CO\(_3\) into malate. The reaction mixture consisted of following components in a total volume of 1.0 ml tris buffer (pH 7.8) 80 \(\mu\) mole, NaH\(^{14}\)CO\(_3\) 10 \(\mu\) mole, PEP, 2.0 \(\mu\) mole, MgCl\(_2\) 2.0 \(\mu\) mole, NADH 2.0 \(\mu\) mole, malate dehydrogenase 14 units and enzyme extract 0.2 ml. The reaction was initiated by the addition of PEP. Assay without PEP was treated as control. The incubation was carried out for 15 min at 30\(^\circ\)C in scintillation vials. The reaction was terminated by the addition of 1 ml 2NH\(_4\)Cl. The entire mixture was taken to dryness at 85\(^\circ\)C in an oven. After the addition of 1 ml water, 10 ml of liquid scintillator was added (prepared from 0.25g POPOP, 10g of PPO and 100 g of recrystallised naphthalene per litre of dioxane). The acid stable \(^{14}\)C was counted in liquid scintillation spectrometer (TriCab, Packard, 3320).

5N. Invivo Phosphoenolpyruvate Carboxylase Activity

Dark \(^{14}\)CO\(_2\) fixation was estimated by exposing nodules to \(^{14}\)CO\(_2\) in a plexiglass chamber using method of Kar et al. (1990).

5N.1 Estimation

Radioactive NaHCO\(_3\) and 5 ml HCl were taken in petridish and placed in the chamber. To start the reaction some air was circulated in the chamber (for moving the stagnant air in the chamber). Pump was switched on and off periodically after every 10 minute for 20 seconds. After one hour, KOH was added to radioactive NaHCO\(_3\) solution to stop the reaction and to utilize all left CO\(_3\). Nodules were kept in paper packets and dried at 500\(^\circ\)C and taken in ground form. 10 mg of nodules
were taken in 200 ml of silica powder (carbonized) in a scintillation vial. On that 5 ml of scintillator was added. In the scintillator vials, incorporated radioactivity ($^{14}$C) was determined using liquid scintillation counter (Packard –TRICARB).

50 Potassium and Sodium Content

Potassium and sodium content were estimated using flame photometry by method of Chapman and Pratt (1961).

50.1 Predigestion

2-5 g grinded sample (roots and shoots) were taken in 100 ml conical flask and 10 ml of acid mixture was added (consisting of nitric acid, sulphuric acid and perchloric acid in ratio 9:4:1). Funnels (30 mm diameter) were put on the mouth of the conical flask and this was kept at 120°C for the night.

50.2 Digestion

Next morning, the samples were maintained at 70°C on hot plate for 30 min, then temperature was increased to 120°C for 30 min and finally it was increased to 250°C and samples were kept till white fumes appeared and solution became clear. Hot plate was switched off when 3-4 ml of sample was left and samples were cooled. Using distilled water the samples were transferred to 50 ml volumetric flask and final volume of 50 ml was maintained and it was left over night. Next day, these were filtered using filter paper whatman No. 1 and sodium and potassium content was estimated on flame photometer. Blank was run without plant samples.