CHAPTER 2

MATERIAL AND METHODS
A. Material:

Chickpea (*Cicer arietinum* L.) is a promising pulse crop which is traditionally grown in winter season throughout India. Amongst several cultivars few varieties have been recommended by Agricultural officials. In Maharashtra most common varieties used are Chafa, G-1, G-2, G-4, G-5, JG-62, BG-209 and BG-210. Chickpea cultivar chafa is most popular cultivar preferred throughout this region. The 'chafa' cultivar is widely recommended because of certain advantages such as short crop duration (98-107 days), better yield (1500 kg/ha), drought resistant nature, disease tolerant capacity and high nutritive value. This cultivar has better nodulation capacity which is advantageous for next cropping season. This cultivar needs minimum requirement for better development and yield. Hence chafa cultivar was selected for physiological and biochemical studies in the present investigation. Every time fresh seed stock was obtained through courtesy of Agricultural college, Pune or Mahatma Phule Krishi Vidyapeeth, Rahuri and was used to raise plants for various analytical procedures.

B. Methods:

1. Salt tolerance studies:
   a. Germination Studies:

      To evaluate salt tolerance capacity at germination stage seeds of several promising varieties were obtained through
Healthy seeds were sorted out and surface sterilized with 0.1% aqs. \( \text{HgCl}_2 \) solution for five minutes. The seeds were then thoroughly washed with distilled water and air dried. Twenty seeds were kept in sterilized petridishes for germination over Whatman Filter Paper No.1. The filter paper was moistened with 15 ml of distilled water as control or desired salt solution.

An extensive study at International Rice Research Institute (IRRI) has shown that the salt concentrations (0.4% W/V) gives an ECe of approximately 8-10 mmhos cm\(^{-1}\) at 25°C and this can be considered as the discriminating level of salinity (Ponnampetuma, 1977). Hence for the present studies three concentrations of NaCl namely 0.4%, 0.8% and 1.2% (ECe values 7.6, 15 and 21 mmhos cm\(^{-1}\) respectively) are chosen. The emergence of radicle from seed coat was acknowledged as a criterion for germination counts which were taken after every 24 hours. The experiment was carried out (in triplicate) in germination chamber at 28°C under dark conditions. The germination index (GI) was calculated by using following formula of Czabator (1962) -

\[
\text{Germination index} = \frac{\sum_i \text{TiNi}}{S}
\]

Where, \( \text{Ti} \) = Number of days of sowing,
\( \text{Ni} \) = Number of seeds germinated,
\( S \) = Total number of seeds sown.
At 120 hrs the seedlings were analysed for various growth parameters such as length of plumule and radicle, fresh weight and moisture percentage.

After growth analysis the seedlings were separated into embryo axis and cotyledons (removing testa), washed with distilled water, blotted to dryness and were kept in oven at 60°C for ten days for drying. The dried material was powdered and subjected for further analysis of inorganic constituents like Na⁺ and K⁺ as well as organic constituents like reducing, non-reducing and soluble sugars and free proline.

The activities of various enzyme systems were studied at different stages of germination (0, 24, 48, 72, 96 and 120 h) with two salt treatments (viz. 0, 4% and 0.8% NaCl). As per requirement either whole seedlings after removing testa were subjected for enzyme extraction or seed parts like cotyledons and embryo axis were separated. Each enzyme was assayed in triplicate.

II. Inorganic constituents :

Inorganic constituents in cotyledons and embryo axis were determined from acid digest of oven dried plant material. The method of Toth et al., (1947) was followed for acid digestion process which involved digestion with conc. HNO₃ and perchloric acid.
Sodium and Potassium :

Na\(^+\) and K\(^+\) were estimated flame photometrically using standard procedure on flame-photometer (model - Elico CL 22A). For standardization, various concentrations of Na\(^+\) and K\(^+\) were prepared ranging from 1 to 10 ppm by diluting stock solutions of NaCl and KCl (100 ppm). Using these standard solutions standard curve for these elements was prepared using flame photometer with specific colour filters. The plant extract was subjected to same procedure. From galvanometer readings the inorganic elements Na\(^+\) and K\(^+\) were estimated using calibration curves of known concentrations of Na\(^+\) and K\(^+\).

III. Organic constituents :

Soluble sugars and free proline were estimated from powder of oven dried plant material namely cotyledons and embryo axis.

i) Soluble sugars :

The sugars were estimated following the method of Nelson (1944). The soluble carbohydrates were extracted from 0.5 g plant material with 80% neutral alcohol. The extract was filtered through Buchner funnel using Whatman No.1 filter paper. The filtrate thus obtained was condensed on water bath to about 5 ml. To this 2-3 g lead acetate and potassium oxalate (1:1) were added for decolorization. After decolorization, the 50 ml of distilled water were added and aliquot was
filtered. The volume of filtrate was measured and it served as an extract for determination of reducing sugars. A known volume of this extract (20 ml) was hydrolysed with 2 ml conc. HCl by autoclaving at 15 lbs atm. pressure for half an hour. The content was cooled, neutralized with anhydrous sodium carbonate and filtered. The volume of filtrate was measured and this filtrate was used for the estimation of total (reducing + non-reducing) sugars.

The sugars from both the filtrates were estimated by determining the reducing power by employing arsénomolybdate reagent introduced by Nelson (1944) for the colorimetric determination of the cuprous oxide formed in the oxidation of sugars by alkaline copper reagent. For this, 0.5 ml aliquots were taken in test tubes along with different concentrations of standard glucose solution (0.1 mg/ml) in other test tubes. To this required amount of D.W. was added to make final volume 1 ml. In case of blank instead of filtrate or standard glucose, distilled water was added to begin with the reaction. One ml of Somogyi’s alkaline copper tartarate reagent (4 g CuSO₄, 5 H₂O, 24 g anhydrous Na₂CO₃, 16 g Na-K-tartarate and 180 g anhydrous Na₂SO₄ dissolved in 1 litre distilled water) was added in each test tube and all these reaction mixtures were transferred to boiling water bath for 10 minutes. After cooling to room temperature, 1 ml Arsenomolybdate reagent (25 g ammonium molybdate in 450 ml water to which 21 ml conc. H₂SO₄
were added followed by 3 g sodium arsenate, $\text{Na}_2\text{HAsO}_4 \cdot 7 \text{H}_2\text{O}$ dissolved in 25 ml water. These ingredients were mixed well and digested for 48 h at 37°C in incubator before use) was added to each reaction mixture which were further diluted to 10 ml with distilled water. After 10 minutes, absorbance was read at 560 nm on spectrophotometer.

Using calibration curve of standard glucose, the sugar percentage in the above three fractions were calculated. Values of soluble sugars are expressed as 100 g$^{-1}$ dry tissue.

ii. Free proline:

Free proline content from cotyledons and embryo axis was determined according to the method of Bates et al., (1973). For this, 0.5 g of plant material was homogenized in 10 ml sulfosalicylic acid (3%) thoroughly and then filtered through Whatman filter paper. 2 ml from this filtrate was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin reagent (prepared by warming 1.25 of ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid with agitation, cooled and stored at 4°C) in a test tube for 1 hour at 100°C in boiling water bath. Similar procedure was also followed for another set of test tubes containing various concentrations of standard proline solution (0.1 mg/ml proline). After boiling, the reaction was terminated by transferring the test tubes immediately to ice bath. The reaction mixture was extracted with
4 ml toluene and mixed vigorously with test tube stirrer for 15-20 sec. Reaction mixtures were then brought to room temperature and absorbance of toluene chromophore was measured at 520 nm using toluene blank. Proline concentration was calculated from calibration curve and final values were expressed as \( \mu g 100 \text{ g}^{-1} \) dry tissue.

IV. Enzymes:

For extraction of most of the enzymes ten seedlings from each germination stage (0, 24, 48, 96 and 120 h respectively) subjected to different concentrations of NaCl (Control (0), 0.4% and 0.8% respectively) were selected and they were made free from seed coats. For study of dehydrogenase cotyledons and embryo axis were separated and subjected for further analysis. Enzyme activities were determined as follows:

i. \( \alpha \) amylase (E.C.3.2.1.1):

The enzyme \( \alpha \) amylase was studied following modified blue value method of Katsumi and Fukuhara (1969). For enzyme extraction, 10 seedlings at different stages of germination (24 to 120 h and dry seeds as 0 h) without testa were macerated in a chilled mortar with pestle in 10 ml cold acetate buffer (0.1 M, pH 5) and filtered through four layers of cheese cloth. The filtrate was centrifuged at 10,000 g for 20 minutes and supernatant was collected, which served as enzyme source.
The enzyme was assayed in following manner. Two sets of reaction mixtures were prepared one serving as '0' minute control and the other serving as '30 min' reaction mixture. One ml of acetate buffer (pH 5) and one ml of enzyme solution were mixed in test tubes. After equilibration period (10 min) 1 ml of 0.2% amylose (pH 7.0) solution was added in both sets. The reaction in control (0 min) was immediately terminated by adding 10 ml of 0.5 N acetic acid. The reaction in another set was allowed to continue with constant shaking for 30 minutes and after 30 minutes the reaction in second set was terminated by adding 10 ml 0.5 N acetic acid. One ml aliquot from above test tubes was used for determination of unused amylose. This aliquot was mixed with 10 ml of dilute I₂KI solution. The blue colour developed was measured spectrophotometrically at 700 nm using dilute I₂KI solution as blank. The enzyme activity is expressed as Δ O.D. hr⁻¹ seedling⁻¹.

ii. Acid phosphatase (E.C. 3.1.3.2):

For study of Enzyme acid phosphatase the method of McLachlan (1980) was adopted. Ten seedlings from each germination stages (24 to 120 h and dry seeds as 0 h) were homogenized separately in 10 ml of cold acetate buffer (pH 5) in an ice cold mortar and pestle. The resultant homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged at 10,000 g for 20 minutes. The supernatant served as enzyme source.
The assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg p-nitrophenyl phosphate/ml of acetate buffer, pH 5), 2 ml acetate buffer and 1 ml enzyme. The reaction was allowed to proceed for 30 minutes and then was terminated by addition of 1.5 ml of 1.68 N NaOH. The reaction terminated immediately (0 min) served as control. The optical densities of the developed yellow colour complex were read at 420 nm. The enzyme activity is expressed as Δ O.D. h⁻¹ seedlings⁻¹.

iii. Alkaline inorganic pyrophosphatase (EC 3.6.1.1):

Alkaline inorganic pyrophosphatase was assayed according to the method described by Rauser (1971). Ten seedlings of each germination stage (0-120 h) subjected to different salinity treatments (0, 0.4% and 0.8% NaCl) were extracted in chilled mortar and pestle in cold 10 ml 0.1 M Tris-HCl buffer (pH 7.5). The resultant suspension was filtered through four layered cheese cloth and filtrate was centrifuged at 10,000 g for 15 minutes. The supernatant served as enzyme source.

The reaction was assayed by incubating 0.5 ml of enzyme extract with 1 ml reaction mixture containing 2 μmol Na₄P₂O₇ (sodium pyrophosphate), 0.6 μmol Tris-K-phthalate and 0.4 μmol MgCl₂ (at pH 8.7) for 30 minutes at room temperature. The reaction was stopped by addition of 1 ml ice cold 10% TCA (Trichloroacetic Acid). After 15 minutes, assay mixture was centrifuged at full speed for 5 minutes and 1 ml aliquot of the
supernatant was used for estimation of liberated iP by the method of Fiske and Subba Row (1925). For this, to 1 ml of supernatant 1 ml 5 N sulphuric acid was added followed by 1 ml 2.5% ammonium molybdate and 0.1 ml reducing solution (10 ml of distilled water containing 0.25 g of following ingredients: 0.2 g 1-amino-2-Napthol-4 sulfonic acid, 1.2 g sodium bisulfite and 1.2 g sodium sulfite). The volume is made 10 ml with distilled water. After 10 minutes, absorbance of developed blue colour is read at 660 nm. The amount of iP liberated due to enzyme activity was determined from the absorbance difference of 0 and 30 min reaction mixtures and with the help of standard curve of iP plotted in a similar manner. The enzyme activity is expressed as µg iP liberated seedling⁻¹.

iv. Protease (EC 3.4.2.2):

The enzyme protease was assayed according to the method of Penner and Ashton (1967) as described by Chinoy et al., (1969). The plant material from different germination stages (24 to 120 h and dry seeds for 0 h) in different salinity treatments was made free from seed coat and crushed in chilled mortar and pestle in 10 ml of phosphate buffer, pH 7 at 4°C. The homogenate was filtered through four layers of cheese cloth and filtrate was centrifuged in refrigerated centrifuge at 10,000 g of for 10 minutes. Supernatant served as enzyme source.

For assay following ingredients were taken - 1 g casein (pH 7.0), 3 ml (0.2 M) phosphate buffer (pH 7), and 1 ml
enzyme. The reaction was incubated for 1 h at 37°C after which it was terminated by adding 2 ml 5% Trichloroacetic acid. After 20 minutes, reaction mixture was centrifuged and 1 ml aliquot from supernatant was taken for estimation of free tyrosine. To this, 4 ml 0.5 N NaOH and 1.2 ml Folin-phenol reagent was added, mixed thoroughly and absorbance of developed blue colour was read at 660 nm on spectrophotometer.

For blank distilled water was used instead of enzyme source and the same procedure was followed.

The enzyme activity was calculated using following formula described by Chinoy et al., (1969):

\[
\text{Activity} = (142.4 \times A) - 0.13 = \mu g \text{ of Tyrosine liberated h}^{-1} \text{ seedling}^{-1}
\]

Where \( A \) = change in optical density.

v. Nitrate Reductase (EC 1.6.6.1):

The enzyme nitrate reductase in cotyledon and embryo axis was assayed according to the method of Evans (1982). As this enzyme is substrate inducible, the seedlings (10) were raised in 1/2 Hoagland nutrient medium with different concentrations of NaCl (0 {control}, 0.4% and 0.8%) upto 120 h germination stage. At this stage cotyledons and embryo axis of these seedlings were separated removing testa and subjected for further analysis. The separated tissue was suspended in
5 ml of a mixture of 0.1 M phosphate buffer (pH 7.5), 0.02 M KNO$_3$, 5% propanol, and two drops of chloramphenicol (0.5 mg/ml), in sealed jars incubated at 25°C in the dark for 60 min. After 60 min, nitrate reductase activity was measured by determining NO$_2$ production which was traced by treating 0.4 ml of the incubation mixture with 0.3 ml each of 1% sulfanilamide (in 3 M HCl) and 0.02% NEEDA (N-1-Naphthyl - ethyleneamide hydrochloride) for 20 min. The sample was diluted to 2.2 ml with distilled water. The absorbance was measured at 540 nm.

Standard curve was prepared following different concentrations of NaNO$_2$ (10, 20, 30, 40, µg) by using same procedure of NO$_2$ estimation. The enzyme activity is expressed as µmol NO$_2^-$ formed h$^{-1}$ cotyledon$^{-1}$ or embryo axis$^{-1}$.

vi. Glutamic oxaloacetic transaminase (GOT)(EC 2.6.1.1) and Glutamic pyruvic transaminase (GPT)(EC 2.6.1.2):

For study of these transaminases the method of Harper and Paulsen (1969-a) was adopted. Ten seedlings from different germination stages under different salinity treatments (0, 0.4% and 0.9%) were homogenized in chilled medium containing 3.3 mM Tris-HCl buffer (pH 7.2), 3.3 mM cysteine and 0.1 mM sodium salt of EDTA. The resultant suspension was filtered through four layered cheese cloth and centrifuged in refrigerated centrifuge at 10,000 g for 15 minutes at 2°C. Supernatant served as enzyme source.
For GOT, 1 ml of crude enzyme was incubated with 1 ml reaction mixture containing 0.02 M aspartate and 0.02 M \( \alpha \) ketoglutarate in 0.2 M K-phosphate buffer (pH 7.5). After 0 min and 60 min the reactions were stopped with 1 ml of colour reagent (2,4 DNP 1 mg in 1 ml of 1.35 N HCl). After 30 minutes 10 ml of 0.4 N NaOH was added followed by 10 ml distilled water. The absorbance of oxaloacetate - phenyl - hydrazone was read at 504 nm against reagent blank. The enzyme activity is expressed as \( \Delta O.D. \ h^{-1} \) seedling\(^{-1}\).

Activity of glutamic pyruvic transaminase (GPT) was assayed with some modifications in similar manner except that aspartate was replaced by alanine in reaction mixture and of pyruvate phenyl hydrazone was \( \Delta \) absorbance read at 504 nm. The enzyme activity is expressed as \( \Delta O.D. \ h^{-1} \) seedling\(^{-1}\).

vii. **Peroxidase** (EC 1.11.1.7) :

The method of Maehly (1954) was followed for assay of enzyme peroxidase. Enzyme was extracted from 10 seedlings at various germination stages subjected to different NaCl treatments by macerating in cold distilled water in chilled mortar and pestle. The suspension was filtered through four layered cheese cloth and the filtrate was centrifuged at 10,000 g for 20 minutes in refrigerated centrifuge. The supernatant was used as enzyme source.
Enzyme assay was prepared by adding 2 ml 0.1 M phosphate buffer (pH 7), 1 ml 20 mM guiacol and 1 ml enzyme. The reaction was initiated by addition of 0.04 ml of 10 mM H$_2$O$_2$ and the changes in optical density due to oxidation of guiacol were recorded at every 30 sec for 2 minutes at 470 nm with frequent stirring of reaction mixture. Enzyme activity is expressed as $\Delta$ O.D. min$^{-1}$ seedling$^{-1}$.

viii. Catalase (EC 1.11.1.6) :

For study of catalase a modified method of Herbert (1955) was adopted. The enzyme was extracted from seedlings subjected to different salinity treatments at different stages of germination, in a manner similar to the one described for peroxidase.

The assay mixture was prepared by mixing 1 ml of 0.045 M H$_2$O$_2$ in phosphate buffer (pH 6.8) and 1 ml aliquot of enzyme. Assay mixture was incubated at room temperature for 1 minute and then the reaction was terminated by the addition of 5 ml of H$_2$SO$_4$ (5 N). For 0 min the reaction was stopped before addition of enzyme by 5 N H$_2$SO$_4$. To this 1 ml of 10% KI solution and a drop of 2% ammonium molybdate were added. The liberated iodine was titrated against 0.01 M sodium thiosulphate using starch indicator. The difference in titration reading between 0 and 1 min reaction mixtures was taken as a measure of enzyme activity. Activity of enzyme is expressed as mg H$_2$O$_2$ broken down min$^{-1}$ seedling$^{-1}$.
ix. **Dehydrogenase (EC 1.1.1.??)**:

For study of dehydrogenase from cotyledons and embryo axis the tetrazolium method of Kittoeh and Law (1957) was followed. The cotyledons and embryo axis were separated carefully from seedlings at various germination stages (0 to 120 h) and incubated for 4 hours in 4 ml 0.2% 2-3-5 Triphenyl tetrazolium chloride (TTC) solution in dark. After this, embryos and cotyledons were washed 2-3 times with distilled water, surface dried and were treated with 5 ml of 2-methoxyethanol for extraction of the red coloured formazon, which is formed due to activity of dehydrogenase. The optical density of coloured formazon was measured at 470 nm. The enzyme activity is expressed as $A_{O.D. \ h^{-1}}$ cotyledon$^{-1}$/embryo axis$^{-1}$.

b. **Plant growth studies**:

For salt tolerance studies a sand culture technique was employed. 20 seeds were sown in polyethylene culture containers (42 cm diameter with a hole at the bottom) containing acid free silica sand of about 100 mesh in the month of October, which is the ideal season for chickpea plantation in this region. Hoagland nutrient solution (1/2 strength) was supplied to these plants and this nutrient dose was repeated after every 6th day throughout crop duration. Two liters of Hoagland nutrient medium (1/2 strength) contained following ingredients

\[
\begin{align*}
1 \text{ M } \text{KNO}_3 & \quad \ldots \quad 6 \text{ ml} \\
1 \text{ M } \text{Ca(NO}_3)_2 & \quad \ldots \quad 4 \text{ ml}
\end{align*}
\]
<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
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<tbody>
<tr>
<td>1 M MgSO₄</td>
<td>2 ml</td>
</tr>
<tr>
<td>1 M (NH₄)₂HPO₄</td>
<td>1 ml</td>
</tr>
<tr>
<td>5% Iron tartarate</td>
<td>1 ml</td>
</tr>
<tr>
<td>Micronutrients</td>
<td>1 ml</td>
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(2.86 g H₃BO₃; 1.81 g MnCl₂, 4 H₂O; 0.11 g ZnCl₂, 0.05 g CuCl₂, 2 H₂O and 0.05 g Na₂MoO₄, 2 H₂O).

Micronutrient solution in a final volume one litre was prepared by adding ingredients in 100 ml of distilled water.

After one month of establishment, the plants were subjected to treatment of sodium chloride. Following levels of NaCl were selected 0 (Control), 25, 50, 75, 100 and 150 mM. The salt treatment was given along with culture medium twice a week alternating with equal amount of water to avoid evaporation and excess salt accumulation.

Chickpea plants after such treatments (about 65 days old) were analysed for various growth parameters. In every case 10 plants were carefully uprooted, cleaned well in distilled water, surface dried and used for analysis. The parameters selected for growth analysis were, total plant height, shoot height, root length, number of leaves, number of leaflets, leaf area, number of branches and fresh and dry matter (biomass) of the plant and individual plant parts. At this stage plants were
subjected to determination of osmotic potential of the cell sap and leaf succulence following the methods of Janardan et al., (1975). For osmotic potential determination 1 g of fresh leaf tissue was homogenized, filtered through cheese cloth and volume was made to 25 ml by adding distilled water. The electrical conductivity of the cell sap was measured with the help of conductivity bridge (Elico model CM 82 T). The moisture content was measured by weight difference method. The osmotic potential in bars was then calculated as -

$$ OP = \left( \frac{EC \times 0.36 \times df}{0.987} \right) $$

Where

- $EC =$ Electrical conductivity in mmhos cm$^{-1}$ at 25°C of Plant extract.
- $df =$ dilution factor depending upon the moisture content of the tissue and extract volume.
- $\frac{Vol. \times Fr.wt.}{Fr.wt. - Dry wt.}$

$0.987 =$ factor for converting atmospheric pressure into bars.

Succulence index (SI) was calculated using following formula of Kluge and Ting, (1978).

$$ Succulence \ index = \frac{Fresh \ weight}{dry \ weight} $$

After 110 days of crop growth, plants were harvested and some yield parameters like number of pods, number of seeds, number of filled pods and fresh weights and dry weights of pods and seeds were determined. The data was statistically analysed.
c. **Analysis of inorganic constituents**

Various inorganic constituents like Na\(^+\), Cl\(^-\), K\(^+\), Ca\(^{2+}\), P\(^5+\), Mg\(^2+\), Fe\(^3+\), Mn\(^2+\) were estimated from the chickpea plants at two growth stages, one immediately after commencement of NaCl salinity (about 35 days old plants) and second after 5th week of salinity treatment (about 65 days old plants). At these two stages, plants were carefully uprooted, washed in water and blotted to dryness. The different plant parts viz. root, stem and leaf were carefully separated and subjected to drying in oven at 60 °C. Oven dried plant material was powdered and 0.5 g of sample was acid digested following the method of Toth et al., (1948) in concentrated nitric acid and perchloric acid. This acid digest served as sample for analysing various inorganic constituents.

I. **Sodium, potassium and calcium**

These cations were estimated according to standard flame photometric process employing ELICO Flamephotometer model CL 22A. The method described for sodium and potassium in earlier part has been followed for determination of calcium with a special calcium filter and standard calcium solution (200 ppm, prepared by dissolving known amount of CaCl\(_2\) in glass distilled water).
II. Chlorides :

The method of Volhard (1956) was followed for estimation of chlorides. In this method chlorides are extracted from ashed plant material using hot water and dilute nitric acid. Dissolved chlorides are precipitated with standard AgNO₃ (known amount). Unconsumed AgNO₃ is then titrated against standard ammonium thiocynate. Subtracting the value of unconsumed silver nitrate from the amount of silver nitrate added volume for precipitation of dissolved chlorides in plant extract is calculated. The concentration of chlorides in the plant material is then calculated using following formula:

\[ 1 \text{ ml } 0.1 \text{ N } \text{AgNO₃} = 3.55 \text{ mg Cl}^- \]

III. Phosphorus :

For estimation of P⁵⁺ the method of Sekine et al., (1965) was followed. Here phosphorus reacts with "Molybdate Vanadate reagent" to give yellow colour complex. By estimating colorimetrically the intensity of the colour developed and by comparing it with the colour intensity of known standards, phosphorus content was estimated.

To 1 ml of acid digest in a test tube 2 ml of 2 N HNO₃ were added followed by 1 ml of molybdate vanadate reagent (1.25 g of ammonium molybdate in 500 ml 1 N HNO₃ and 25 g of ammonium vanadate in 500 ml distilled water mixed in equal volumes). Volume was made to 10 ml with distilled water. The
ingredients were mixed well and allowed to react for 20 minutes. After 20 minutes colour intensity was measured at 420 nm using a reaction blank containing no phosphorus.

Calibration curve of standard phosphorus was prepared from standard phosphorus solution (0.110 g KH₂PO₄ per litre = 0.025 mg P⁵⁺/ml) taking different concentrations (0.025, 0.05, 0.1, 0.2 and 0.4 mg P); other steps being essentially similar to the one described above. With the help of standard curve amount of phosphorus in the plant material was calculated.

IV. Magnesium:

Mg²⁺ was estimated following the method of Drosdoff and Nearpass (1948). To 5 ml of acid digest in a 50 ml volumetric flask the following reagents were added in a sequential order and mixed thoroughly: 1 ml hydroxylamine (5% w/v), 5 ml starch compensating solution (equal volumes of freshly prepared 2% starch solution and compensating solution: 3.7 g calcium chloride, 0.74 g aluminium sulphate, 0.36 g manganese chloride, 0.6 g of trisodium phosphate all dissolved in distilled water containing 10 ml conc. HCl and then volume made to 1 lit.), 1 ml Thiamale yellow (0.1% aqs) and 5 ml 2.5 N NaOH. Volume was made to 50 ml with distilled water and after 30 minutes colour intensity was measured at 525 nm spectrophotometrically.
Reagent blank was prepared in the same manner as above except that the extract was replaced by distilled water. A standard curve for Mg\(^{2+}\) was also prepared with the help of different concentration of Mg\(^{2+}\) from stock solution of Mg (100 ppm) and with this the Mg\(^{2+}\) content in the sample was calculated.

V. Iron:

Iron was determined by the method described by Durie et al., (1965). In this method ferric ion is reduced to ferrous ion. The colour developed between the latter and 1,10 phenanthroline is read at 510 nm spectrophotometrically.

Five ml of acid digest, 5 ml of standard iron solution (3 \(\mu g\) Fe\(_2\)O\(_3\)/ml) were taken separately in 50 ml volumetric flasks. One more 50 ml volumetric flask was reserved for reagent blank containing no iron solution. To this, 10 ml of hydroxylamine hydrochloride (10% w/v) were added along with a small square of Congo red paper. The contents were mixed well. Acetate buffer (140 g sodium acetate in D.W. 60 ml acetic acid) was added dropwise until the indicator paper changes its colour from blue to just red. 8 ml 1,10-phenanthroline (0.25% w/v) were then added and volume was adjusted to 50 ml with D.W. Optical density of colour developed was read at 510 nm spectrophotometrically against reagent blank.
By comparing the O.D. of the test solution with that of standard Fe solution total amount of Fe$^{3+}$ in the plant material was calculated.

VI. Manganese :

Manganese was determined following the method of Durie et al., (1965). Manganese is oxidized to permanganate with potassium periodate and developed colour of permanganate is measured spectrophotometrically at 525 nm.

25 ml of acid digest (Sample) is pipetted out in 100 ml beaker. 25 ml D.W. in another beaker served as reagent blank. To this 5 ml acid mixture (a mixture of equal volumes of 10% orthophosphoric acid and 25% sulphuric acid) and 0.3 g potassium periodate were added. The beakers are covered and brought to boil, simmered for 30 min maintaining the original volume to complete the reaction. Sample is cooled, transferred to 50 ml volumetric flask and volume is adjusted with D.W. The contents are mixed well and the developed colour of permanganate is read spectrophotometrically at 525 nm against reagent blank.

Standard curve is prepared from different concentrations of Mn from the stock solution (6 $\mu$g Mn$^{2+}$ ml$^{-1}$) and with help of this total manganese content in the sample is calculated.
Several enzymes were extracted and assayed from leaves of chickpea plants raised in various concentrations of NaCl (0 (control), 25, 50, 75, 100 and 150 mM) at second stage of plant analysis, (after 5th week of salinity treatment). Mature leaves (5th, 6th, 7th and 8th leaf) were randomly sampled, washed in distilled water and blotted to dryness. 0.5 g leaf tissue was homogenized in 10 ml chilled 0.1 M Tris-HCl buffer (pH 7.5) containing 1 M KCl, 0.01 M EDTA and 0.4 ml 0.2 M β-mercaptoethanol (Weimberg, 1970) in cold mortar with pestle in Walk-in-Cooler. The resultant suspension was filtered through two layered cheese cloth and filtrate was centrifuged at 20,000 g for 20 min at 4 °C in refrigerated centrifuge. The supernatant served as enzyme source for various enzymes namely GOT, GPT, alkaline phosphatase, alkaline inorganic pyrophosphatase, ATPase, fructose, 1,6-bisphosphatase, aldolase, pyruvate kinase and peroxidase. In case of other enzyme systems where the extraction medium differed from this routine procedure a mention has been made under respective heads.

Enzyme proteins were estimated according to the method of Lowry et al., (1951). 0.05 ml crude enzyme extract was taken in test tube and was diluted to 1 ml with distilled water. To this, 5 ml of reagent C (50 ml of 'A' containing 2% sodium carbonate in 0.1 N aqs. NaOH is mixed with 1 ml of
'B' containing 0.5% copper sulphate in 1% sodium tartarate) was added. Solution was mixed well and allowed to stand for 15 min at room temperature. After 15 min 0.5 ml of Folin and Ciocalteu's phenol reagent was added rapidly with immediate mixing. This was allowed to stand for next 30 min and the developed colour intensity was measured at 660 nm spectrophotometrically. A reagent blank without enzyme source served as control. Protein concentration was calculated by comparing with standard curve of different concentrations of bovine serum albumin prepared in a similar manner.

I. Nitrate reductase (EC 1.6.6.1) and Nitrite reductase (EC 1.6.6.4):

The activity of in vivo nitrate reductase was assayed according to the method described by Evans (1982). This enzyme was assayed from both leaf and root tissue. 0.5 g freshly harvested tissue was cut into small pieces and used for in vivo enzyme assay following the procedure described in earlier section. The enzyme activity is expressed as \( \mu g \text{NO}_2^- \text{liberated h}^{-1} \text{g}^{-1} \text{ fresh tissue.} \)

Enzyme nitrite reductase from leaf tissue was assayed following the method of Rama Rao et al., (1983) with slight modifications. 0.5 g freshly cut leaf tissue was incubated in 5 ml reaction medium containing 0.02 M NaNO\(_2\), 5% propanol and 0.05% Triton-X-100 in light for 60 min. The enzyme activity
was detected by determining consumed NO\textsubscript{2} than in 0 min reaction using sulfanilamide and NEDDA with a small aliquot of R.M. Enzyme activity is expressed as \( \mu g \text{ NO}_2^- \) consumed h\(^{-1} \) g\(^{-1} \) fresh tissue.

II. Glutamine synthetase (EC 6.3.1.2) :

For study of enzyme glutamine synthetase the procedure of Lea (1982) was adopted. 0.5 g of fresh leaf material was homogenized in chilled mortar and pestle with 5 ml 50 mM cold imidazole HCl buffer (pH 7.2) containing 0.5 mM EDTA and 1 mM DTT. Resultant homogenate was filtered through two layered cheese cloth. The filtrate was centrifuged at 10,000 g for 15 minutes at 2 °C and supernatant was used as enzyme source.

For enzyme assay 0.25 ml of crude enzyme source was incubated with 1 ml reaction mixture at 30°C containing glutamate (92 \( \mu mol \)), ATP (18 \( \mu mol \)), MgSO\textsubscript{4} (45 \( \mu mol \)), hydroxylamine (6.0 \( \mu mol \)), and imidazole acetate (50 \( \mu mol \)) with pH 7.2 for 15 minutes. The reaction was terminated by addition of 1 ml ferric chloride reagent (0.37 M ferric chloride, 0.67 M HCl and 0.2 M trichloro acetic acid). Another set was followed with reaction terminated at 0 min. The reaction mixture was centrifuged to remove precipitated proteins. After 5 minutes absorbance was read at 540 nm spectrophotometrically. The absorbance difference between 0 min R.M. and 15 min R.M. served as a measure of enzyme activity which is expressed as \( \mu mol \)
glutamyl hydroxamate formed $h^{-1} \text{ mg}^{-1}$ protein. A standard curve of glutamyl-hydroxamate was prepared with upto 3 $\mu$ mol per 1 ml of assay mixture.

III. Transaminases - glutamic oxaloacetic transaminase (EC 2.6.1.1) and glutamic pyruvic transaminase (EC 2.6.1.2):

Activities of GOT and GPT were determined according to the method developed by Harper and Paulsen (1969-a) with slight modifications. The enzymes were extracted according to method of Weimberg (1970) described earlier and for the assay of enzymes the procedure described in earlier section was followed. Enzyme activity is expressed as change in optical density ($\Delta \text{O.D.}$) $h^{-1} \text{ mg}^{-1}$ protein.

IV. Phosphatases - acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1):

Enzyme acid phosphatase was assayed according to the method of Mc Lachlan (1980) described in earlier section. For study of alkaline phosphatase method adopted by Weimberg (1970) was followed. During assay conditions 0.5 ml crude enzyme was incubated with 1 ml 0.1 M Tris-HCl (pH 7.5), 0.1 ml 0.05 M MgCl$_2$, 0.1 ml 0.02 M p-nitrophenyl phosphate and 1.7 ml D.W. at 30°C. Absorbance was measured immediately after enzyme addition at 410 nm and after 1 h of incubation. From the difference in absorbance value of 60 and 0 min R.M.
enzyme activity was calculated. Enzyme activity of phosphatases is expressed as change in optical density h\(^{-1}\) mg\(^{-1}\) protein.

V. **ATPase (EC 3.6.1.3):**

Activity of ATPase was determined following the method described by Todd and Yoo (1964). Crude enzyme extracted according to the method of Weimberg (1970) described earlier served as enzyme source.

One ml of supernatant was added to 0.5 ml of 0.01 M CaCl\(_2\) and 0.5 ml 0.003 M ATP. The reaction was carried out at 38\(^\circ\)C for 1 h and was stopped by adding 1 ml of 0.1 M NaOH. The liberated phosphorus was estimated by the method of Fiske and Subba Row (1925) as described earlier. Enzyme activity is expressed as \(\mu\) mols iP liberated h\(^{-1}\) mg\(^{-1}\) protein.

VI. **Alkaline inorganic pyrophosphatase (EC 3.6.1.1):**

This enzyme was assayed by the method of Hauser (1971) from crude enzyme extract in Tris-HCl buffer medium following the procedure described in earlier section of germination studies. The enzyme activity is expressed as \(\mu\) mols iP liberated h\(^{-1}\) mg\(^{-1}\) protein.

VII. **Fructose 1,6-bisphosphatase (EC 3.1.3.11):**

Enzyme fructose 1,6-bisphosphatase was assayed according to Racker (1962) from the enzyme extracted in Tris-HCl buffer medium described earlier.
0.5 ml of crude enzyme was incubated with reaction mixture containing 100 μ mol Tris- buffer pH 8.8, 5 μ mol fructose 1,6-biphosphate, 600 μg EDTA and 5 μ mol MgCl₂ at 30°C for 30 minutes and the reaction was terminated by adding 1 ml 10% TCA. The reaction terminated in a similar manner immediately (0 min) served as control. The liberated IP (difference in IP content of 30 min and 0 min reaction mixtures) was estimated following the method of Fiske and Subba Row (1925) described earlier. Corresponding enzyme activity was calculated and expressed as μ mol IP liberated h⁻¹ mg⁻¹ protein.

VIII. Aldolase (EC 4.1.2.13):

Activity of enzyme aldolase was studied following the procedure of Bar-Akiva et al., (1971). Crude enzyme extracted in Tris-HCl buffer medium described earlier served as enzyme source.

The assay mixture containing 1 ml 0.05 M Tris-HCl buffer pH 8.5, 0.2 ml 0.22 M hydrazine sulphate, 0.1 ml enzyme or water (in blank) and 0.2 ml 0.02 M fructose 1,6-diphosphate were incubated for 10 minutes at 37°C and then 2 ml 10% TCA was added to stop the reaction. After 10 minutes, the mixture was centrifuged and 1 ml of DNPH was added to an aliquot of 1 ml supernatant and left again for 10 minutes. After the addition of 1 ml 2-4 dinitrophenyl hydrazine (DNPH) (0.1% in
2 N HCl) and incubation at 37°C for 30 minutes the colorimetric assay mixture was diluted with 7 ml 0.75 N NaOH. The O.D. was measured at 540 nm. Enzyme activity is expressed as change in optical density h^{-1} mg^{-1} protein.

IX. **Pyruvate kinase (EC 2.7.1.40)**:

Pyruvate kinase activity was assayed by the method of Weidner and Salisbury (1974). Crude enzyme extracted in Tris-HCl buffer (pH 7.5), as described earlier, served as enzyme source.

To assay following ingredients were added in test tube 0.5 ml Tris-HCl (50 μ moles), 0.1 ml PEP (1.5 μ moles), 0.1 ml ADP (4 μ moles), 0.08 ml MgCl₂ (8 μ moles) and 0.17 ml KCl (42 μ moles). The reaction was started by adding 0.05 ml enzyme extract and incubated for 10 minutes at 37°C after which 1.0 ml DNPH (0.25% in 2 N HCl) was added as a killing agent. This was followed by 1.8 ml water and 4.5 ml 0.75 N NaOH. The mixture was stirred in and after 15 minutes the ppt. was separated by centrifugation at full speed. Optical density of supernatant at 510 nm was determined colorimetrically against a blank without extract and PEP. Enzyme activity is expressed as change in optical density h^{-1} mg^{-1} protein.
X. Peroxidase (EC 1.11.1.7) :

Activity of peroxidase was determined according to the method of Maehly (1954). The crude extract prepared according to method of Weimberg (1970) served as enzyme source. The method of assay for this enzyme has been already described in earlier section of germination studies.

XI. Polyphenol oxidase (EC 1.10.3.1) :

Enzyme polyphenol oxidase was assayed by the method of Sato and Hasegawa (1976). 0.5 g leaf material was extracted in 10 ml 0.2 M K-phosphate buffer (pH 6.8) and filtered through two layered cheese cloth. Filtrate was centrifuged at 10,000 g for 10 minutes. Supernatant served as enzyme source.

A mixture of 0.5 ml enzyme sample, 0.5 ml 10 mM catechol, 1 ml 10 mM sulfanilic acid and 2 ml 0.2 M K-phosphate buffer (pH 6.8) was incubated at 25°C. Absorbance was read at 0 and 30 minutes at 500 nm colorimetrically and the difference between the two readings served as enzyme activity. Enzyme activity is expressed as change in O.D. h$^{-1}$ mg$^{-1}$ protein.

e. Analysis of organic constituents :

I. Total nitrogen and soluble proteins :

Total nitrogen from various plant parts viz. root, stem and leaf of chickpea plants grown under different salt treatments was estimated following the method of Hawk et al.,
(1948). 0.5 g of oven dried, powdered plant material was taken in kjeldahl flask with a pinch of microsalt (200 g $K_2SO_4$ + 5 g CuSO$_4$, dehydrated) and to it 5 ml $H_2SO_4$ (1:1) was added. Few glass beads were added to avoid bumping and the material was digested on low flame. After complete digestion a faint yellow solution was obtained which was cooled to room temperature, transferred to volumetric flask and diluted to 100 ml with distilled water.

In very clean Nessler's tubes 1 ml of plant extract and different concentrations of standard ammonium sulphate solution (0.236 g of Ammonium sulphate dissolved in water and few drops of $H_2SO_4$ are added. The volume is made 1,000 ml. This solution contains 0.05 mg of nitrogen per ml) are taken. In control tube 1 ml distilled water was taken. To this 1 drop 8% KHSO$_4$ is added and volume is made 35 ml with distilled water. To this 15 ml Nessler's Reagent are added (Reagent A : 7 g KI + 10 g HgI$_2$ in 40 ml distilled water. Reagent B : 10 g NaOH in 50 ml water. A and B are to be mixed in proportion of 4:5 at the time of estimation). After 15 minutes the absorbance is read at 520 nm. Nitrogen value is calculated from the standard curve. Soluble proteins were extracted from fresh tissue in 0.1 M Tris-HCl buffer (pH 7.5) and estimated following the procedure of Lowry et al., (1951). Soluble proteins were calculated using standard protein curve prepared with the help of bovine albumin.
II. Nitrate content:

Nitrates were estimated by the zinc dust method of Wooley et al., (1960). Powdered oven dried plant material (100 mg) was transferred to 100 ml beaker followed by 10 ml distilled water. The mixture was boiled for few seconds on a hot plate and after cooling the material was filtered. Nitrates were estimated from the filtrate as follows:

About 0.6 g Zn dust powder (100 g BaSO_4, 75 g citric acid, 10 g MgSO_4, 4 g sulfanilamide, 2 g powdered Zn and 2 g NEEBA) was taken in test tube. To this 8 ml 20% acetic acid solution containing 0.2 ppm CuSO_4 was added. About 2 ml aliquot of filtrate was added in each test tube and shaken vigorously. The colour was allowed to develop for half an hour after which the contents were centrifuged at 3000 g for 5 min. and the absorbance of supernatant was read at 540 nm.

Standard nitrate curve was prepared according to the method of Wooley et al., (1960). For calibration various dilutions ranging from 0 to 40 \( \mu \) mole were prepared using stock solution (100 \( \mu \) mole nitrate ml\(^{-1}\)).

III. Nitrate from metabolic pool:

The nitrate in the metabolic pool was estimated by the method of Ferrari et al., (1973). 100 mg of the tissue was placed in a test tube containing 2 ml of 0.1 M phosphate buffer
(pH 7.5). The contents in the tubes were flushed with nitrogen for 2 min., stoppered and incubated in darkness for 1 h at 30°C until nitrate production ceased. The produced nitrite was measured spectrophotometrically using sulfanilamide and NEDGA. The amount of nitrite produced under anaerobic conditions was taken as equivalent to that in metabolic pool. The nitrite produced was calculated from calibration curve for nitrite.

IV. Proline:

Proline was estimated according to the method of Bates et al., (1973) as described earlier in section of germination studies.

V. Total carbohydrates:

The method described by Nelson (1944) was followed for estimation of total carbohydrates from different plant parts. Method for estimation of reducing and non-reducing sugars has been covered in earlier section of germination studies. For estimation of starch, the insoluble residue obtained at the beginning after filtering the alcoholic extract was transferred to a 100 ml conical flask with 5 ml distilled water and 5 ml concentrated HCl. The same was hydrolysed at 15 lbs atm. pressure for half an hour and cooled to room temperature, neutralized by addition of anhydrous Na₂CO₃ and filtered. The volume of the filtrate was measured as this contains reducing sugars (mostly glucose) formed as a result.
of hydrolysis of starch. These sugars represent starch content in the residue and they were determined according to the procedure described earlier for estimation of reducing sugars. Total carbohydrates are expressed as g 100 g$^{-1}$ dry tissue.

VI. Oxalate:

Oxalate content in leaf tissue was estimated according to the method of Cooke and Sansum (1976). One gram dried, ground plant material was taken in test tube and 10 ml 1.5 M HCl was added to it. The test tube was placed in boiling water bath for 1 hour and the solution was then filtered through Whatman filter paper No.41, washed several times with distilled water and volume was made. One ml aliquot from this sample alongwith a series of standard oxalic acid (0, 2.25, 4.5, 6.25, 9.0, 13.5 and 18.0 μg ml$^{-1}$) was taken in separate test tubes and to this 3 ml DAC reagent and 2 ml Zr$^{+4}$ solution (5 mg Zr/lit) was added. The DAC reagent was prepared by following procedure: 5.5 g catechol and 18.5 g aluminium sulphate were dissolved in 50 ml deionised water and cooled in ice (solution 1); 7.0 g anthranilic acid and 3-5 g sodium nitrite were dissolved in 50 ml deionised water and 7 ml concentrated HCl added (solution 2). Solution 2 was added to solution 1 with stirring at between 5 and 10°C. The resulting mixture was removed from the ice and stirred (using a magnetic stirrer) throughout the following procedure: 50 ml of a 20% w/v sodium acetate solution was added over 40 min to precipitate aluminium
salt to DAC; after a further 30 min 15 ml of concentrated HCl was added and left over for another 30 min to decompose the aluminium salt. The cherry red precipitate of DAC was then filtered through a Whatman No. 50 filter paper on a Buchner funnel under suction. The filtrate was dried in an evacuated dessicator. Purification was done by fractional crystallization from ethanol in following manner. The crude DAC product was dissolved in a minimum amount (30 ml) of ethanol at 50°C and filtered using a Buchner funnel under pressure. The residue was discarded and filtrate was left to stand at room temperature for about one and half hour. It was refiltered and the residue discarded. The filtrate was retained and the solvent evaporated very slowly from a covered beaker in a dessicator until the product precipitated. The DAC was filtered off and dried at 60°C. (It is stable as the solid when stored in desiccator).

The absorbance of developed colour was read at 520 nm on spectrophotometer using 1 M HCl as blank. (with increasing concentration of oxalate the colour intensity decreases). From standard curve oxalate content was calculated from the plant sample and expressed as g 100 g⁻¹ dry tissue.

VII. Total Polyphenols:

Total polyphenols were determined according to the method of Folin and Denis (1915). Fresh leaf material (0.5 g) was homogenized in 30 ml 80% acetone and filtered through
Buchner funnel. The residue was washed several times with 80% acetone and the final volume was made 100 ml with 80% acetone. Two ml extract along with a series of standards (Std. tannic acid 0.1 mg per ml) is taken in Nessler’s tubes and to this 10 ml 20% Na$_2$CO$_3$ and 2 ml Folin-Denis reagent were added. Final volume is made 50 ml with distilled water. After 20 min absorbance is read at 660 nm using reagent blank. Total polyphenols are calculated from standard curve of Tannic acid and are expressed as g 100 g$^{-1}$ fresh tissue.

f. Leaf exudation studies:

These studies were carried out at 2nd stage of plant analysis (about 65 days old plants). Collection of leaf exudate and estimation of titratable acidity was conducted according the method of Koundal and Sinha (1981). Twenty five fresh leaflets from the plants were randomly selected at 7.00 a.m. (all plants were sprinkled with water on previous day and night to wash out earlier accumulation of exudate) and placed in 150 ml conical flasks containing 50 ml distilled water. The flasks were constantly whirred for 10 min gently. The leaflets were removed through filtration by 2 layered muslin cloth and the known amount of filtrate was titrated against 0.1 N NaOH using phenolphthalein indicator. The NaOH solution was standardized against 0.1 N HCl solution. From titration readings, acidity status of the leaf exudate was calculated. A known volume of the collected exudate was subjected to
analysis of inorganic constituents like sodium, potassium and calcium with the help of flame photometer. The chlorides in the exudate were estimated titrimetrically by the method of Volhard et al., (1956) which has been described earlier.

g. Photosynthesis and photorespiration:

I. Photosynthetic pigments:

Chlorophylls were estimated according to method of Arnon (1949). Chlorophylls were extracted in chilled acetone (80%) from 0.5 g of the plant material in a cold room. The extract was filtered through Buchner funnel using Whatman No.1 filter paper. Residue was washed repeatedly with 80% acetone collecting the washings in the same filtrate. The volume of filtrate was made to 100 ml with 80% acetone. The absorbance was read at 645 and 663 nm.

Chlorophylls (mg 100 g\(^{-1}\) fresh tissue) were calculated using the following formula.

\[
\text{Chlorophyll } 'a' = 12.7 \times A_{663} - 2.69 \times A_{645} = X
\]

\[
\text{Chlorophyll } 'b' = 22.9 \times A_{645} - 4.68 \times A_{663} = Y
\]

\[
\text{Chlorophyll } 'a'/b' = \frac{X/Y \times \text{Vol.of Extract } \times 100}{1000 \times \text{wt.of plant material (g)}} \times \text{Yol.of Extract} \times 100
\]

Carotenoids were estimated by reading the absorbance of acetone extract at 480 nm (Kirk and Allen, 1965). Total
Carotenoids were estimated using the formula of Liaaen-Jensen and Jensen (1971).

\[ C = D \times v \times f \times \frac{10}{2500} \]

Where, \( C \) = total carotenoids in mgs.,  
\( D \) = Optical density,  
\( v \) = total volume in ml,  
\( f \) = dilution factor and  
2500 = Average extinction.

II. Carbon isotope ratio (\( \delta^{13}C \)):  

The value of \( \delta^{13}C \) was determined from the oven dried leaf material with the help of Dr. H.R. Krouse, Calgary University and Dr. Robert Guy, Carnegie Research Institute, Washington, U.S.A. The \( \delta^{13}C \) value was estimated by following the standard established method of Smith and Epstein (1971). In this method a small amount of dried leaf tissue is combusted at 800°C and the CO\(_2\) released is analysed for isotope ratio on mass spectrometer. The results are expressed as -

\[ \delta^{13}C \text{ } ^{\circ}/\text{o} = \left\{ \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right\} \times 1000 \]

Where \( R \) = mass 45 to 44 ratio of sample or standard.

The standard of reference is belemite carbonate from Pee Dee formation of South Carolina (PDB).
III. **Carbon assimilation studies**

The fresh leaf material from various treatments was employed for photosynthetic studies. (For salinity studies leaves from plants subjected 0, 25, 50 and 100 mM NaCl salinity, for senescence studies mature and senescent leaves in control plants, while for K deficiency studies leaves of complete and 'K deficient' plants were chosen). Study of photosynthetic carbon assimilation was performed according to method standardized in our laboratory with numerous plant systems (Joshi, 1976). 1 g of leaves collected randomly were allowed to float in Tris-HCl buffer (pH 7.8) in stoppered Erlenmayer flasks. 14C fixation studies were carried out at 11 a.m. by illuminance under HPLR lamp with the intensity of light at the point of incidence 0.6 cal cm\(^{-2}\) min\(^{-1}\) (approx. 45,000 Lux.). Temperature was maintained at 25 ± 2°C by adding ice in the water bath time to time. The reaction was started by adding 100 \(\text{NaH}^{14}\text{CO}_3\) (specific activity, 49.7 mCi/m mole, obtained from BARC, Bombay, India). The flasks were continuously shaken during the period of experiment. The reactions were terminated after different time intervals by adding boiling hot ethanol. The material was homogenized in 80% alcohol to complete the extraction and filtered. The extracts were condensed to a volume of about 3 ml under reduced pressure. The residue was hydrolysed in conc. HCl and after repeated condensations, sample for insoluble fraction were prepared when maximum acid was removed.
The compounds were analysed by two dimensional paper chromatography using solvent systems Phenol : Water (80:20) and Butanol : Acetic acid : Water (80:22:50). The identity of individual compound was confirmed by specific colour reactions, with detectors such as ninhydrin, aniline pthalate, bromophenol blue and molybdate perchloric acid, co-chromatography and autoradiography. (For autoradiography Sakura X-ray films were used). The radioactivity incorporated in each compound was counted on proportional counting system (EC model No.2541).

IV. L/D ratio :

L/D ratio (Light/Dark ratio) was determined by the method of Kisaki and Tolbert (1970) with slight modifications.

The plant material weighing 1 g was cut into small pieces and placed in a glass jar 6 cm in diameter and 10 cm in height containing 0.1 M phosphate buffer (pH 7.0). A glass vial (10 ml capacity) containing 5 ml of 20% KOH was placed at the centre of the jar to trap the evolved $^{14}$CO$_2$ during the process of photorespiration. After stabilization of the plant material, 50 µl NaH$^{14}$CO$_3$ (specific activity 49.7 mCi/m mole) was added to the phosphate buffer. The jars were made air tight. The temperature was maintained at 25 ± 2°C. One set of the jars was kept under continuous illumination with HPLR lamps which provide approximately 30,000 lux of light intensity for 1 h.
and another set was kept in dark for 1 h. After desired time course small vial containing KOH alongwith trapped $^{14}$CO$_2$ was removed from the experimental jar, 100 µl of the KOH solution was dried slowly on filter paper on a planchet and immediately counted on proportional counting system (EC model No.2541).

V. Photorespiratory enzymes :

i. **Phosphoglycolate phosphatase (EC 3.1.3.18)** :

Phosphoglycolate phosphatase from leaves was assayed following the method of Randall et al., (1971). One g leaf material was homogenized in a chilled mortar with pestle in 5 ml cacodylate buffer (20 mM) containing 1 mM EDTA at pH 6.3. The homogenates were squeezed through four layers of cheese cloth. The extract so obtained was centrifuged at 10,000 g for 10 min at 0°C. The supernatant served as the enzyme source.

The enzyme was assayed for 10 min at 28 ± 1°C using 10 moles of substrate (phosphoglycolate), 100 moles of cacodylate buffer pH 6.3 and 1 mM MgCl$_2$ in a final volume of 0.75 ml and 0.25 ml crude enzyme. The reaction was terminated by the addition of 0.25 ml 10% TCA. The precipitate was removed by centrifugation and the released phosphorus was measured by the method of Fiske and Subba Row (1925). Enzyme activity is expressed as µg Pi liberated min$^{-1}$ mg$^{-1}$ protein.
ii. Glycolate oxidase (EC 1.1.3.1):

Glycolate oxidase activity was estimated according to the method of Hess and Tolbert (1967). One g of leaf tissue was homogenized in 10 ml cold 0.1 M Tris-HCl buffer (pH 7.5) containing 1 M KCl, 0.01 M EDTA and 0.4 ml 0.2 M β-mercapto-ethanol (Weimberg, 1970) in chilled mortar with pestle. The resultant suspension was filtered through four layered cheese cloth and filtrate was centrifuged at 20,000 g for 20 min at 0°C. The supernatant served as crude enzyme source.

The assay mixture (3 ml) contained 2.4 ml 0.1 M phosphate buffer (pH 8.3), 0.1 ml phenylhydrazine hydrochloride (0.1 M, pH 6.6), 0.1 ml 0.1 M cysteine hydrochloride, 0.3 ml sodium glycolate (0.1 M) and 0.1 ml crude enzyme. The enzymatic reaction was initiated by the addition of sodium glycolate. Activity of the enzyme was measured at 324 nm in which the absorbance was increased due to formation of phenylhydrazone. Enzyme activity is expressed as μ moles glycolate oxidised min⁻¹ mg⁻¹ protein.

iii. Catalase (EC 1.11.1.6):

Enzyme catalase was assayed following the procedure of Herbert (1955) with some modifications. Crude enzyme in 0.1 Tris-HCl buffer system similar to glycolate oxidase served as enzyme source.
Assay mixture for catalase contained 1 ml 0.45 M $H_2O_2$ in phosphate buffer (pH 6.8) and 0.5 ml crude enzyme. The reaction was terminated either at 0 min or 5 min by adding 5 ml of 1 N $H_2SO_4$. To this 1 ml 10% KI and a drop of 1% ammonium molybdate were added and the liberated iodine was titrated against 0.01 N sodium thiosulphate using starch as an indicator. A colour change from blue to colourless was taken as the end point. Blank reading was taken by mixing all the ingredients except enzyme. Enzyme activity is expressed as mg of $H_2O_2$ liberated min$^{-1}$ mg$^{-1}$ protein.

h. Composition of Pods and Seeds:

Pods were harvested from plants subjected to various treatments. Total number of pods, number of filled pods, number of seeds, fresh and dry weight of pods and seeds were determined for each treatment so as to have idea about various yield parameters. Pod shells and seeds were separated, oven dried and powdered. Powdered material was subjected for analysis of inorganic and organic constituents according to the procedure described in earlier section.

Study of carbon assimilation in pods was carried out from fresh pod shells collected from plants subjected to NaCl salinity (about 80 days old) according to the procedure described earlier for leaf photosynthetic studies.
2. Leaf senescence studies:

Leaf senescence studies were performed from the senescent leaves (4 weeks old, yellow) as well as fully expanded mature leaves (2 weeks old, green) from the same plants raised in chickpea fields. The fate of various inorganic constituents, organic constituents and enzyme systems during the process of leaf senescence was studied using the analytical methods described earlier under sections. For study of photosynthetic $^{14}$C assimilation mature and senescent leaves in the control plants of salinity experiment were selected. The photosynthetic studies in these two leaf categories were also performed according to the procedure described in earlier section.

To study effect of malic acid on leaf senescence leaves of chickpea plants were collected from one month old plants growing in field. The leaves were washed thoroughly and leaflets were cut halfway. The leaf segments were randomly sampled and 0.5 g of leaf segments were allowed to float in petridishes containing distilled water and different concentrations of L. malic acid (1, 10 and 100 mM) in dark at 27°C. After every 24 h upto 96 h the chlorophyll content in the leaf segments was estimated according to the method of Arnon (1949) while the method of Lowry et al., (1951) was employed for estimation of soluble proteins in the extract of leaf segments in 0.1 M Tris-buffer pH 7.5.
3. **Potassium deficiency studies**:

Chickpea var. chafa plants were raised from seeds in acid free silica sand in 4 litres capacity polyethylene culture containers (42 cm diameter). Plants were grown and well stabilized for 15 days in Hoagland nutrient medium then the treatments were commenced. For the composition of complete and potassium deficient nutrient medium the procedure of Moore (1974), described in his book "Research Experiences in Plant Physiology" was followed. Plants were treated with the respective nutrient media twice a week with alternate watering to avoid excess accumulation of salts and to check the loss of water from the container due to evaporation. Sixty day's old plants were subjected for analysis. The experiment was carried out in triplicate. Chickpea plants raised in two treatments (complete and potassium deficient) were subjected to analysis of plant growth readings with respect to plant height, shoot-length, root length, number of leaves, number of leaflets, number of branches and fresh and dry weights of individual plant parts viz. leaf, stem and roots.

The level of various micronutrients in leaves of complete and potassium deficient chickpea plants was investigated with the help of Dr. G.V. Alexander, University of California, U.S.A. by employing a technique of optical emission spectrometry (Alexander and McAmulty, 1981).
Titratable acid number (TAN) in leaves of plants receiving complete medium and potassium deficient plants was determined by the method of Thomas and Beevers (1949) from fresh leaves. One g plant material was boiled in 60 ml of D.W. for 30 min and filtered. The known volume of filtrate was titrated against N/40 NaOH using phenolphthalein as an indicator. NaOH was standardized against standard N/40 oxalic acid. TAN value was calculated using following formula -

\[
TAN = \frac{\text{Vol. of oxalic acid taken for titration ml}}{\text{Titration reading ml}} \times \frac{\text{Total Vol. of extract ml}}{\text{Wt. of plant material g}} \times \frac{\text{Extract reading ml}}{\text{Vol. of extract taken for titration ml}} \times \frac{100}{4}
\]

The plant analysis with respect to various other biochemical aspects like status of major inorganic constituents, enzyme systems, organic constituents, photosynthesis and photorespiration from fresh or oven dried plant material (wherever necessary) was performed according to the methods described in earlier section.