CHAPTER - II

MATERIAL AND METHODS

A. MATERIALS

Several species of Amaranthus have been cultivated in Maharashtra. The seeds of *A. caudatus* and *A. paniculatus* were obtained from the local stock. The seeds of *A. hypochondriacus* were obtained from National Botanical Research Institute, Lucknow.

B. METHODS

1. Potassium Deficiency Studies

For the deficiency studies, sand culture technique was employed. The seeds were sown in polyethylene culture containers with a small hole at the bottom containing acid free silica sand of about 100 mesh size for first 8 days the seedlings from all containers were supplied with complete nutrient medium. Thereafter one litre complete nutrient solution was added to control plants after every 4 days throughout the crop duration. For plants subjected to K deficiency nutrient solution without potassium was given. The composition of nutrient solution was essentially same as described by Moore (1974) in his book 'Research Experiences in Plant Physiology'.

The nutrient medium contains following ingradients.

	Complete	- K
1 M KNO_3	10 ml	Nil
1 M $Ca(NO_3)_2$	10 ml	10 ml
1 M $MgSO_4$	4 ml	4 ml
1 M KH_2PO_4	2 ml	Nil
FeEDTA	2 ml	2 ml
Micronutrients	2 ml	2 ml
1 M $NaNO_3$	Nil	10 ml
1 M NaH_2PO_4	Nil	2 ml

(The micronutrient contains 2.86 g H_3BO_3, 1.81 g $MnCl_2.2H_2O$, 0.11 g $ZnCl_2$, 0.05 g $CuCl_2.2H_2O$, and 0.025 g $Na_2MoO_4.2H_2O$ in one litre distilled water).

Plants were treated with the respective nutrient media twice a week with alternate watering with tap water to avoid excess accumulation of salts and to check the loss of water from the container due to evapotranspiration. Every care was taken to raise healthy plants. The weed control from the containers was done by hand weeding. The growth abnormalities and viable K deficiency symptoms (if any) were recorded in plants from time to time.

2. Growth

It is essential to mention that life span of *A. caudatus* is of 50 days where as life span of *A. hypochondriacus* and *A. paniculatus* is 70 days. Hence, the growth analysis was made at separate times in these three species. The plant height, average number of leaves per plant, average leaf area. Fresh weight and dry weight were determined in 40 days old plants in case of *A. caudatus* and 60 days old plants in case of *A. hypochondriacus* and *A. paniculatus*. For this analysis 10 plants were employed and average was determined. Leaf area was determined by multiplying the product of length and breath by a factor 0.63. This factor was obtained after extensive graphical leaf area analysis of the three species.

3. Leaf Water Relations

a. Water content (WC) :

The leaves from control and potassium deficient plants were randomly sampled and water content was calculated according to Weatherly (1950) as follows :

$$\underset{(\%\text{ of Dry wt.})}{\text{WC}} = \frac{\text{Fresh wt.} - \text{Dry wt.}}{\text{Dry wt.}} \times 100$$

Dry weight was obtained by keeping the Weighed material in oven at 60°C till a constant weight was obtained.

b. Leaf succulence

The leaf succulence from control and potassium deficient plants was calculated by the method of Kluge and Ting (1978) by calculating the ratio of Fresh weight to dry weight.

c. Relative water content (RWC)

The method described by Slatyer (1955) was employed to determine the relative water content in leaves of three *Amaranthus* species. 19 of leaf discs were floated on distilled water in petridishes under diffused light for 4 hours and turgid weight was obtained. Finally the same leaf discs were kept in an oven at 60°C till a constant dry weight was obtained. The RWC was calculated by using following formula :

$$RWC\ (\%) = \frac{\text{Fresh wt.} - \text{Dry wt.}}{\text{Turgid wt.} - \text{Dry wt.}} \times 100$$

4. Stomatal Behaviour

Stomatal behaviour in 30 days old *Amaranthus* plants was studied at 11.30 a.m. with the help of steady state Porometer (LI 1600, LICOR, U.S.A.). In this study the leaves at identical position on control and potassium deficient plants were selected and various parameters like transpiration rate towards the upper and lower leaf surface were directly determined while diffusive conductance to

water was calculated by using the formula proposed by Jarvis (1971).

$$\text{Diffusive conductance of water} = \frac{1}{\text{Diffusive resistance (DR)}}$$

5. **Estimation of Inorganic Constituents**

a. Preparation of Acid Digest

i. Potassium sodium, calcium, magnesium, iron, manganese, zinc :

The inorganic constituents were estimated from root, stem, old leaves and young leaves of three *Amaranthus* species. Plants were carefully uprooted, washed in water and blotted to dryness. The root, stem, old and younger leaves were carefully separated and kept in oven at 60°C for 12 days and the dried plant material when showed constant weight was taken for further analysis. Acid digest was prepared following the method of Toth et al. (1948) and different inorganic constituents were estimated from the same. 500 mg of oven dried powdered root, stem and leaf materials were transferred to 150 ml capacity beakers to which 20 ml concentrated HNO_3 were added. The beakers were covered with watch glass and kept till the primary reactions subsided. These beakers were heated slowly to dissolve solid particles. Then after cooling to room temperature, 10 ml of perchloric acid (60%) were added to it and mixed thoroughly.

These beakers were heated strongly until a clear and colourless solution (2-3 ml) was obtained. It was then cooled and transferred to 100 ml capacity volumetric flask and diluted to 100 ml with distilled water and kept over night. After 12 hours these extracts were filtered through dry Whatman No.44 filter paper and the filtrates were used as the source of different inorganic constituents, sodium, Potassium, Calcium, Magnesium, Iron, Manganese and Zinc.

These elements were estimated using Atomic Absorption spectrophotometer (Perkin Elmer 3030).

ii. Phosphorus

For the estimation of phosphorus the method described by Sekine et al. (1965) was employed. 2 ml of acid digest were taken in test tubes and to this 2 ml of 2 N HNO_3 and 1 ml of molybdate vanadate reagent (A-25 g Ammonium molybdate in 500 ml of distilled water. B-1.25 g Ammonium vanadate in 500 ml 1 N HNO_3, A and B were mixed at the time of using) were added. Then final volume of each test tube was adjusted to 10 ml with distilled water. The ingredients were mixed well and allowed to react for 20 minutes, and 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 KH_2PO_4 per litre = 0.025 mg $p^{5+}ml^{-1}$) taking different

13090
A

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 in mg 100 g^{-1} on dry weight basis.

6. **Photosynthetic Pigments**

a. Chlorophylls

Chlorophylls were estimated following the method of Arnon (1949). Chlorophylls were extracted in 80% chilled acetone from 0.5 g of fresh plant material in dark. This extract was filtered through Whatman No.1 filter paper using Buchner Funnel. Residue was washed repeatedly with 80% acetone collecting the washings in the same filtrate. The volume of filtrate was made to 50 ml with 80% acetone. The absorbance was read at 663 and 645 nm for chlorophylls a and b respectively.

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

$$\text{Chlorophyll a} - (12.7 \times A_{663}) - (2.69 \times A_{645}) \quad \ldots\ldots X$$

$$\text{Chlorophyll b} - (22.7 \times A_{645}) - (4.68 \times A_{663}) \quad \ldots\ldots Y$$

$$\text{Total Chlorophylls} = (8.02 \times A_{663}) - (20.2 \times A_{645}) \quad \ldots\ldots Z$$

$$\begin{array}{l}\text{Chlorophyll a or} \\ \text{Chlorophyll b or} \\ \text{Total chlorophylls} \\ (\text{mg } 100\ g^{-1} \\ \text{fresh tissue})\end{array} = \frac{X|Y|Z \times \text{Volume of Extracts} \times 100}{1000 \times \text{weight of plant material in g}}$$

b. Carotenoids

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 Liasen-Jonsen and Jensen (1971).

$$C = D \times V.F \frac{10}{2500}$$

where,

C = total carotenoids in mgs, g^{-1} fresh material

D = optical density

V = total volume in ml

F = dilution factor and

2500 = average extinction

c. Betacyanin

Betacyanin content was determined following the procedure described by Wagner (1970). 1 gram freshly harvested leaf discs were placed in 10 ml of 1 % aqueous HCl for 48 h at 4°C in darkness, to extract the betacyanin pigment. Then the optical density of the extract was measured at 525 nm. The values were corrected for dispersion by measuring the optical density at 660 nm.

7. Titratable Acid Number (TAN)

The method described by Thomas and Beevers (1949) was employed for determination of TAN. Fresh leaves of three

Amaranthus species was washed and rinsed with distilled water and blotted to dryness 2 g of blotted leaves were boiled for half an hour in 40 ml distilled water. After cooling it was filtered through cheese cloth and volume was made to 50 ml with distilled water. The filtrate was then titrated against standardized NaOH (N/40) using 3 drops of phenolphthalein as an indicator. NaOH was standardized again with N/40 oxalic acid using 3 drops of phenolphthalein as an indicator. TAN value was calculated using the following formula :

$$\frac{\text{Vol. of oxalic acid taken for titration ml}}{\text{Titration reading ml}} \times \frac{\text{Total volume of extract ml}}{\text{wt. of plant material in g}} \times \frac{\text{Extract titration reading ml}}{\text{vol. of extract taken for titration ml}} \times \frac{100}{4}$$

8. <u>Photosynthetic Products - Carbohydrates</u>

The sugars were estimated following the method of Nelson (1944). The soluble carbohydrates were extracted from 0.5 g fresh leaves 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 1 g lead acetate and 1 g potassium oxalate (1:1) were added for decolourization. Then 40 ml of distilled water was added and aliquote was filtered. The volume of filtrate was measured and it served as an extract for determination of reducing sugars. A 20 ml

aliquote of this extract was hydrolysed with 4 ml concentrated HCl by autoclaving at 15 lbs atm. pressure for ½ an hour. The content was cooled, neutralized with anhydrous sodium carbonate and filtered. The volume of the filtrate was measured and this filtrate was used for the estimation of total sugars.

For starch estimation, insoluble residue along with the filter paper obtained at the beginning after filtering the alcoholic extract was transferred to a 100 ml conical flask with 50 ml distilled water and 5 ml concentrated HCl and hydrolyzed at 15 lbs atm. pressure for ½ an hour. These conical flasks were cooled to room temperature, neutralized by addition of unhydrous sodium carbonate and filtered. The volume of filtrate was measured as this contains reducing sugars (glucose) formed as a result of hydrolysis of starch. These sugars represent the starch content in the residue.

The reducing sugars (glucose equivalents) present in the extracts were estimated with the help of dinitrosalicylic acid (DNS) reagent (Miller, 1972).

For preparation of DNS reagent 1 g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite were dissolved with continuous stirring in 100 ml 1% NaOH and stored. 1 ml cooled sample (extract) were

taken in test tube with 2 ml DNS reagent and placed in boiling water bath for about 5 minutes. Then the sample was cooled in cold water bath for about 3 minutes. Then 1:10 dilution of the coloured sample and was made absorbance was read at 530 nm on spectrophotometer.

Using calibration curve of standard glucose, the glucose equivalent percentage were calculated. Values expressed as g 100^{-1} g fresh tissue.

9. **Total Polyphenols**

Total polyphenol content in leaf tissue of three *Amaranthus* species were determined according to 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 final volume was made 50 ml with 80% acetone. 1 ml extract along with a series of standards (std. tannic acid, 0.1 mg/ml) were taken in separate Nessler's tubes and to each tube 10 ml of 20% Na_2CO_3 and Folin Denis reagent (100 g Na tungstate mixed with 20 g phosphomolybdic acid in about 800 ml distilled water, to this 200 ml 25%. Phosphoric acid was added and the mixture was reflexed for 2.5 h to room temperature and volume was made 1000 ml with distilled water) were added. The final volume of reaction mixture was made 50 ml with distilled water. After 20 minutes, absorbance was read at 660 nm using reagent blank.

Total polyphenols were calculated with the help of standard curve of tannic acid and expressed as g 100 g^{-1} fresh tissue.

10. Free Proline

Method described by Bates et al. (1973) was used for determination of free proline content from leaves of three *Amaranthus* species. For this 0.5 g fresh leaf material was homogenized in mortar with 10 3% sulfosalicylic acid thoroughly and filtered through Buchner funnel using Whatman No.1 filter paper. The volume of filtrate was adjusted to 20 ml with 3% sulposalicylic acid. Then 0.5 ml filtrate was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin reagent (prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid with agitation, cooled and stored at 4°C) in 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 solutions (0.1 mg proline/ml). After 1 hour, the reaction was stopped by transferring the test tubes immediately to ice bath. Then 4 ml of toluene was added in each test-tube and mixed vigorously for 15-20 sec. Reaction mixtures were brought to room temperature and then absorbance of toluene chromophore was measured at 520 nm on a Shimadzu UV-190-Double beam spectrophotometer against toluene blank. Proline

concentration was calculated from calibration curve and final values were expressed as µ moles g^{-1} fresh tissue.

11. Glycinebetaine

The method described by Ishitani et al. (1993) was employed for determination of glycinebetaine content in leaf tissue of three *Amaranthus* species. 100 mg oven dried plant material was incubated in 20 ml of 1 N H_2SO_4 for 18 h. at 25°C. Each suspension was centrifuged at 200°g for 10 min and supernatent used as extract. 0.25 ml extract and 0.75 ml 1 N H_2SO_4 were reacted with cold KII_2 reagent. The contents of the tubes were mixed and cooled to 0°C for 2 h in an ice bath with stirring of the reaction solution at frequent intervals. The tubes were centrifuged and 10 ml of ethylene dichloride was added to each precipitate and absorbance was measured at 365 nm on Shimadzu UV-190 double beam spectrophotometer. Glycinebetaine concentration was calculated from calibration curve of 0.1 mg/ml betaine (sigma) prepared following similar procedure as described above and final values were expressed as µ moles g^{-1} dry tissue.

12. Total Nitrogen

Total nitrogen contents from leaves and roots of three *Amaranthus* species was determined using the method of Hawk et al. (1948). 0.2 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. To avoid bumping few glass beads were added 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.

One ml plant extract and different concentrations of standard ammonium sulphate solution (0.236 g of oven dried Ammonium sulphate dissolved in distilled water and few drops of H_2SO_4 were added. The volume was made 1000 ml with distilled water. This solution contains 0.05 mg of nitrogen per ml) were taken in Nessler's tubes. In control tube 1 ml distilled water was taken. To this 1 drop 8% $KHSO_4$ was added and volume was made 35 ml with distilled water. To this 15 ml Nessler's reagent was added (Reagent A : 7 g KI + 10 g $HgCl_2$ 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). The reaction between sample and the reagent gives the product $NH_4Hg_2I_3$ which has orange brown colour. This colour was measured after 15 minutes at 520 nm on double beam spectrophotometer (Shimadzu UV-190).

13. Soluble Proteins

The proteins from the enzyme extract were estimated by using 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 aqueous 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 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.

The values recorded in chapter Result and Discussion represent the average of three determinations.

14. **Enzymes**

a. Nitrate Reductase (EC. 1.6.6.1)

The method described by Jaworski (1971) was employed for the determination of activity of enzyme nitrate reductase. The leaf tissue was incubated in the medium containing 1 ml, 1 M KNO_3; 2 ml, 5% n-Propanol; 5 ml; 0.2 M Phosphate buffer pH 7.5 and 2 ml 0.5% Triton-x-100 for 1 h in dark under anaerobic conditions. After one hour, 1 ml of reaction mixture was taken out and mixed with 1 ml, 1% sulfanilamide in 1 M HCl and 1 ml 0.02% NEEDA (N-(1-Naphthy)

Ethylene Diamide, Dihydrochloride], while mixture of 1 ml incubation medium, 1 ml sulfanilamide and 1 ml NEEDA served as a blank. The absorbance was read at 540 nm on Shimadzu UV-190 double beam spectrophotometer. Standard curve was prepared with the help of different concentrations of KNO_2 and enzyme activity is expressed as μ moles of NO_2 liberated $h^{-1}g^{-1}$ fresh tissue.

b. Acid Phosphatase (EC. 3.1.3.2)

Enzyme acid phosphatase was studied by the method of McLachlan (1980). 0.5 grams of leaf segments were homogenized 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 rpm 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 1 ml 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. $h^{-1}mg^{-1}$ proteins.

c. Catalase (EC. 1.11.1.6)

The enzyme catalase was extracted from 1 g fresh leaves in 10 ml cold distilled water. It was then filtered through moist double layered cheese cloth and filtrate so obtained was centrifuged at 10,000 rpm for 10 minutes at 0°C to 4°C and the supernatant was used as an enzyme source. Assay of enzyme was done following the method described by Herbert (1955). Assay mixture contained 1 ml 0.01 N H_2O_2 in 0.02 M phosphate buffer pH 6.8 and 0.5 ml enzyme. Reaction was stopped by adding 5 ml 1 N H_2SO_4 after 1 minute of reaction. To this 1 ml 10% KI and 3 drops of 1% ammonium molybdate were added and liberated iodine was titrated against 0.01 N sodium thiosulphate using starch as an indicator. The decolourization of blue colour was taken as an end point. Blank or zero minute reading was taken by mixing all the ingredients except enzyme. The proteins in enzyme extract were determined by following method of Lowry et al. (1951). Activity of the enzyme is expressed as mg of H_2O_2 liberated $min^{-1}mg^{-1}$ protein.

d. Peroxidase (EC. 1.11.1.7)

Peroxidase activity was assayed according to method of Kondo and Morita (1951) as described by Horiguchi (1988).

The enzyme peroxidase was extracted from 2 g fresh

leaves in 15 ml (1/5 M) phosphate buffer (pH 6.8) and filtered through four layers muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 min and supernatant was taken as a crude extract of enzyme.

5 ml of 1/5 M acetate buffer (pH 5.0), 0.5 ml of 0.1% guiacol 2 ml of extract, 6 ml of water, 0.5 ml of 0.08% H_2O_2 were mixed and incubated at 30°C. After 30 min. of incubation 1 ml of 1 N H_2SO_4 was added to stop the reaction. It is followed by the measurement of absorbance at 470 nm. Enzyme activity is expressed as Shimadzu UV-190 double beam spectrophotometer. Δ O.D. $hr^{-1}mg^{-1}$ protein.