CHAPTER-II

Material and Methods...

The seeds of different cultivars of Setaria italica (L.) Beauv. have been collected from various Research Stations in India. For screening the cultivars for their salt tolerance capacity at germination and early seedling stage, improved method of Ponnamperuma (1977) was followed using sand culture. Discriminating level of salt was considered to be 0.5% NaCl (W/V). The various cultivars were classified as sensitive, moderately tolerant and tolerant based on their performance (biomass production) under saline conditions. From these studies, two cultivars namely SIC-1 (sensitive) and CO-5 (medium tolerant) differing in their salt tolerance were selected for further studies.

1. GERMINATION STUDIES :

25 seeds of the selected cultivars of Setaria were kept in petridishes for germination on Whatman No.1 filter paper. The filter paper was moistened with 15 ml distilled water (control) or salt solutions of the strength of 5, 10, 25, 50, 100, 200 & 300 mM NaCl. The number of seeds germinated in each treatment at 24, 48, 72, 96 & 120 hours were recorded. Radicle emergence was considered as a sign of germination. The experiment was carried out in triplicate at normal temperature and light conditions.

In order to study the changes in biomass during germination under saline conditions, 50 seeds of each cultivar were allowed to germinate in petridishes on Whatman No.1 filter paper. The controls received 20 ml distilled water only, while the others were treated with 25 ml salt solution in increasing order like 5, 10, 25, 50 & 100 mM NaCl. After 120 h of germination 20 seedlings at random were taken out, washed with distilled water and blotted to dry. The fresh weight of each seedling from every treatment was recorded. The observations were statistically analysed.

A. NITRATE REDUCTASE (EC 1.6.6.2) :

Similar set was prepared in order to study the activity of enzyme nitrate reductase (NR). At 120 h the seedlings were taken out (at random), washed, blotted to dry and cut into small pieces of about 0.5 cm^2 and then were used to determine NR activity. Activity of this enzyme (*in vivo*) was determined following the method described by Jaworski (1971). The material 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. After 1 h, 1 ml of reaction medium was taken out and mixed with 1 ml, 1% sulfanilamide in 1 M HCl & 1 ml 0.02% NEEDA (N-1 Naphthylenthylene diamine di hydrochloride). The absorbance was read at 540 nm on UV - VIS double beam spectrophotometer (Shimadzu-190).

Standard curve was prepared with 0.03 mM KNO_2 (0.0026 mg KNO_2/ ml distilled water), while mixture of 1 ml incubation medium, 1 ml sulfanilamide and 1 ml NEEDA served as a blank.

After taking out 1 ml of reaction medium, the material was filtered through Whatman No.1 filter paper, washed thoroughly with distilled water first and then with 0.2 M phosphate buffer (pH 7.5). Then it was homogenized in 10 ml 0.2 M phosphate buffer (pH 7.5). The extract was filtered through Whatman No.1 filter paper and from the filtrate, nitrate (NO_3^-), nitrite (NO_2^-) and proteins were estimated.

For estimation of NO_3^-, the method has been standardized following the principle that diphenylamine -H_2SO_4 reagent gives blue colour with NO_3^- (Kolhoff & Noponen, 1933). The method gives good results with varying concentrations of KNO_3. The blue colour obtained showed absorption maxima at 590 nm. The assay mixture contained 0.5 ml extract, 1.5 ml distilled water and 1.8 ml diphenylamine - H_2SO_4 reagent (1 g diphenylamine/100 ml concentrated H_2SO_4). After vigorous shaking the colour was allowed to develop for 10 min. Standard curve was prepared by mixing 0.02, 0.04, 0.06 and 0.08 ml 1 M KNO_3, diluted to 2 ml with distilled water and mixed with 1.8 ml diphenylamine indicator. Instead of extract, distilled water was used to prepare blank. Absorbance was recorded at 590 nm. From standard curve, nitrate content was calculated.

Nitrite was estimated following Jaworski's (1971) method. 0.5 ml extract was diluted to 2 ml with distilled water. To this 1 ml sulfanilamide (1% in 1 M HCl) and 1 ml NEEDA (0.02%) were added. After 15 min the optical density was read at 540 nm. Standard curve was prepared using 0.03mM KNO_2. Blank was prepared with distilled water in place of any nitrite.

Proteins were estimated following the method of Lowry (1951). For assay, 0.05 ml extract was diluted to 1 ml with distilled water. To this were added 5 ml of freshly prepared reagent C (50 ml 2% Na_2CO_3 in 0.1 N aqueous NaOH mixed with 1 ml 0.5% $CuSO_4 \cdot 5 H_2O$ in Na-K-tartarate. After 15 min 0.5 ml Folin Phenol reagent (100 g sodium tungstate mixed with 25 g sodium molybdate, 700 ml distilled water, 50 ml 85% phosphoric acid and 100 ml concentrated HCl was refluxed gently for 10 h using water condenser. To this 150 g lithium sulphate, 50 ml distilled water and few drops of bromine water were added. The mixture was boiled for 15 min without water condenser to remove excess bromine. The mixture was cooled and then adjusted to 1 N by titrating it against 1 N NaOH) was added to each test tube. The colour was allowed to develop for 30 min and then absorbance was read at 660 nM. Albumin (0.1 mg/ml) was used to prepare standard curve of protein. Blank contained distilled water, reagent C and Folin Phenol reagent.

B. NITRITE REDUCTASE (EC 1.6.6.4) :

The activity of enzyme nitrite reductase (NiR) was determined following the method described already for NR except that KNO_3 was replaced by 0.3 mM KNO_2 in the incubation medium and the incubation was done in light. The KNO_2 present in the incubation medium was determined by reading the optical density of the reaction mixture containing 1 ml incubation medium, 1 ml sulfanilamide and 1 ml NEEDA. The difference between the two readings, one at 0 min and the other after the enzymatic reaction gives an amount of KNO_2 utilised (reduced) by the enzyme. Blank was prepared by mixing 2 ml propanol, 6 ml phosphate buffer and 2 ml triton-X-100 and by mixing 1 ml of this medium with 1 ml sulfanilamide and 1 ml NEEDA. Standard curve of KNO_2 was prepared as described for NR.

NO_3^-, NO_2^- and proteins in the plant tissues were estimated after removing 1 ml enzyme preparation as described previously.

2. FIELD STUDIES :

The selected cultivars were raised in soil from their seeds. After stabilising the plants under natural conditions for one month, salt treatments were commenced. The plants were treated with increasing levels of NaCl like 50, 100 and 200 mM twice a week, alternating with watering, with equal amount of water to avoid salt accumulation and check the loss of

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water due to transpiration and evaporation. Control plants received only water. After about one month treatment i.e. when plants were two months old, they were used for experimental purpose.

A. GROWTH :

20 plants from each treatment were carefully uprooted and analysed for biomass (fresh weight and dry weight) and moisture content.

B. PHOTOSYNTHETIC PIGMENTS :

1) Chlorophylls : For estimation of chlorophylls and carotenoids, young and mature leaves were homogenized in 80% acetone at 0-4°C in dark. The extract was filtered through Whatman No.1 filter paper under suction. The residue was washed thoroughly, 2-3 times, with 10 ml aliquotes of 80% acetone, collecting all the washings in the same filtrate. Final volume was made to 100 ml with 80% acetone. Absorbance was read at 663 and 645 nm for chlorophyll a and chlorophyll b respectively. Chlorophyll a, chlorophyll b and total chlorophylls were calculated following the formulae by Arnon (1949).

$$\text{Chlorophyll a} = 12.7 \times A\ 663 - 2.69 \times A\ 645 = X$$

$$\text{Chlorophyll b} = 22.9 \times A\ 645 - 4.68 \times A\ 663 = Y$$

$$\text{Total Chlrophylls} = 8.02 \times A\ 663 + 20.2 \times A\ 645 = Z$$

$$\underset{(mg/100\ g)}{\text{Chlorophyll a/b/total}} = \frac{X/Y/Z \times \text{volume of extract} \times 100}{1000 \times \text{weight of plant material in g}}$$

ii) Carotenoids : Carotenoids were estimated according to the method of Kirk and Allen (1965). Carotenoids were calculated with the formula -

$$\text{Total carotenoids (mg/100 g)} = \frac{A\ 480 \times \text{volume of extract} \times 10 \times 100}{2500 \times \text{weight of material in g}}$$

C. POLYPHENOLS :

Polyphenols were estimated from the same acetone extract of chlorophylls following the method of Folin and Denis (1915). For assay 0.5 ml filtrate was mixed with 10 ml 20% Na_2CO_3 and 2 ml Folin Denis reagent (100 g Na-tungstate mixed with 20 g phosphomolybdic acid in about 800 ml distilled water, to this 50 ml 85% phosphoric acid was added and the mixture was refluxed for 2.5 h). This mixture was diluted to 50 ml with distilled water. With the help of tannic acid solution (0,1 mg/ml) standard polyphenol curve was prepared. A blank was prepared without polyphenolics. After development of colour the optical density was read at 660 nm.

D. NITROGEN FRACTIONS ANALYSIS :

For estimating various fractions of nitrogen viz. NO_3-N, NO_2-N, protein-N and insoluble-N from different plant parts like young leaves, mature leaves, roots and stems, oven-dried, powdered material was used. The material was homogenized in 20 ml 80% ethanol and filtered through Whatman No.1 filter

paper using Buchner's funnel. Filtrate was used to estimate the soluble fractions while from the residue insoluble nitrogen was estimated.

The filtrate was condensed on boiling water bath upto 1-2 ml. 10 ml distilled water was then added to it and mixed well. It was filtered through Whatman No.1 filter paper, with frequent washing. All the washings and the filtrate were collected together and diluted to 100 ml with distilled water. From this filtrate NO_3-N, NO_2- N (Jaworski, 1971) and protein-N (Lowry et al., 1951) were estimated as described earlier.

From the residue insoluble-nitrogen (insoluble in alcohol and water) was estimated. For this purpose residue along with filter paper was digested with 7 ml 1:1 H_2SO_4 in Kjeldahl's flask. A pinch of microsalt (200 g K_2SO_4 + 5 g dehydrated $CuSO_4$) was added to accelerate the digestion. Digestion was carried out till a clear solution was remained. After cooling to room temperature it was transfered quantitatively to 100 ml capacity volumetric flask. Volume was made and was stored overnight at room temperature. On next day it was filtered through Whatman No.1 filter paper. From this filtrate nitrogen was estimated following the method by Hawk et al., (1948). 2 ml of extract was mixed with a drop of 8% $KHSO_4$, 15 ml Nessler's reagent (reagent A - 7 g KI + 10 g HgI_2 in 40 ml distilled water, reagent B - 10 g NaOH in 50 ml distilled water. A & B were mixed in the proportion of 4:5) and diluted to 50 ml with

distilled water. The absorbance was read at 520 nm against reagent blank. A standard curve of ammonium sulphate (0.05 mg N/ml) was prepared and the nitrogen content was calculated.

E. PROLINE :

Free proline content in different parts viz. young and mature leaves and roots was estimated following the method of Bates et al. (1973). The material was homogenized in 10 ml, 3% sulposalicylic acid and then filtered through Whatman No.1 filter paper. 2 ml of the filtrate was mixed with 2 ml acid-ninhydrin reagent (mixture of 2.5 g ninhydrin, 60 ml glacial acetic acid and 40 ml, 6 M orthophosphoric acid heated for 5-10 min) and 2 ml acetic acid. The contents were boiled on waterbath for 1 h and cooled rapidly in ice-bath. 4 ml tolune was then added and absorbance of tolune chromophore at room temperature was recorded at 520 nm against tolune blank. Standard curve of proline (0.1 mg/ml) was prepared taking different concentrations as 0.2, 0.4, 0.6 and 0.8 ml of L-proline.

F. ENZYMES :

i) NITRATE REDUCTASE (NR EC 1.6.6.2) :

Activity of NR (in vitro) in young leaves, mature leaves and roots was determined. One g plant material was homogenized in 20 ml extraction medium containing 3.3 mM DTT (Dithiothretol) and 0.1 mM Na_2 - EDTA (Sodium salt of ethylenediaminetet -

racetate) in 3.3 mM tris buffer pH 7.2. The extract was then filtered through double layered cheese cloth. For estimating chlorophylls 1 ml of this crude preparation was taken out and remaining filtrate was centrifuged at 6000 g for 40 min. The supernatent served as an enzyme source.

2.1 ml of assay mixture for NR (Kaufman et al., 1971) contained 0.5 ml enzyme; 0.3 ml, 0.7 M KNO_3; 0.8 ml, 0.2 M phosphate buffer pH 7 and 0.5 ml 1.36 mM NADH (nicotinamide adenine dinucleotide reduced). The enzyme assay was done in dark. The enzymatic reaction was terminated by adding 1 ml sulfanilamide (1% in 1 M HCl) and 1 ml 0.02% NEEDA after 30 min in case of leaves and after 60 min in case of roots. Absorbance was read at 540 nm. Standard curve of KNO_2 was prepared as described elsewhere. NO_3-N and NO_2-N were also determined using the same extracts, following the methods already described.

ii) NITRITE REDUCTASE (NiR - EC 1.6.6.4) :

Activity of NiR was determined from the same extract used for NR. 2.1. ml of NiR assay mixture contained 0.5 ml enzyme; 0.3 ml, 0.3 mM KNO_2; 0.8 ml, 0.2 M phosphate buffer pH 7 and 0.5 ml, 1.36 mM NADH. The enzyme assay was done in light. The enzymatic reaction was terminated by adding 1 ml sufanilamide and 1 ml NEEDA after 30 min in case of leaves and after 60 min in case of roots. Standard curve of KNO_2 was prepared as described earlier. The difference between 0 min

reading and that after enzymatic reduction corresponds to the amount of KNO_2 utilised.

iii) GLUTAMATE OXALOACETATE TRANSAMINASE (GOT - EC 2.6.1.1) :

From the same supernatent of NR, activity of GOT was determined following the method of Harper and Paulsen (1969). For assay 0.5 ml enzyme was mixed with 1 ml assay mixture containing 0.02 M L-aspartate and 0.02 M α - ketoglutarate in 0.2 M potassium phosphate buffer, pH 7.5 (pH was adjusted with 0.2 N HCl). Two sets were prepared. In the first set the reaction was terminated at the beginning only (0.0 min), while in the second set the reaction was terminated after 60 min by adding 1 ml DNPH (Dinitrophenyl hydrazine, 0.1 g/100 ml 2 N HCl). After 15 min 5 ml NaOH (0.75 N) was added to each DNPH added test tubes followed by 5 ml distilled water. The absorbance was read at 504 against blank which was prepared with distilled water.

Standard curve of keto acid (oxaloacetic acid) was prepared (0.1 mg/ml distilled water). The difference between 60 min and 0 min readings was proportional to the amount of keto acid formed.

iv) ALANINE AMINOTRANSFERASE (AAT, EC 2.6.1.2) :

The activity of AAT was determined following the method described hy Harper & Paulsen (1969) from the same extract prepared for NR. The method was similar to GOT assay, except with the difference that the assay mixture for AAT contained 0.02 M

L-alanine instead of L-aspartate. Same standard curve prepared for GOT was used.

v) GLUTAMINE SYNTHETASE (GS, EC 6.3.1.2) :

GS activity was determined from young and mature leaves according to the method of Lea (1982). The material was homogenized in 10 ml extraction medium (0.5 mM EDTA, 1 mM DTT, 2 mM $MnCl_2$, 20% glycerol in 50 mM imidazole acetate buffer, pH 7.8). The extract was filtered through double layered muslin cloth. 1 ml of the filtrate i.e. crude enzyme preparation was used for chlorophyll estimation. Remaining filtrate was centrifuged at 10,000 g for 15 min, at 0.4°C. 0.25 ml of the enzyme preparation (supernatant) was incubated in 1 ml of assay mixture (12.5 mM glutamate, 5 mM ATP, 10 mM $MgCl_2$, 6 mM hydroxylamine hydrochloride and 2 mM EDTA in 100 mM imidazole acetate buffer pH 7.8). Two sets were prepared - first for 0.0 min in which the reaction was terminated by adding 1 ml $FeCl_3$ reagent (mixture of 6 g $FeCl_3$, 2.11 ml concentrated HCl and 3.27 g TCA in 100 ml distilled water) at 0.0 min and the second for 15 min in which the reaction was terminated after 15 min. Change in optical density was recorded at 540 nM. Blank was prepared using distilled water in place of enzyme.

vi) GLUTAMATE DEHYDROGENASE (GDH, EC 1.4.1.3) :

GDH was studied following the method described by Jain and Srivastava (1981) with slight modifications. The material

(young and mature leaves) was homogenized in 10 ml extraction medium containing 120 mM sucrose, 0.6 mM EDTA, 0.3 mM cysteine in 15 mM phosphate buffer, pH 7.5. The extract was filtered through double layered muslin cloth and the filtrate was centrifuged at 5000 g for 15 min at 0-4°C. 0.2 ml supernatent i.e. enzyme preparation was mixed with 2.8 ml assay mixture containing 4 mM α- ketoglutarate, 30 mM ammonium sulphate and 0.15 mM NADH in 400 mM phosphate buffer, pH 8.1. The reaction was started by adding enzyme and decrease in absorbance at 340 nm following the oxidation of NADH was recorded continuously for every 30 seconds till 5 min on double beam UV-viz spectrophotometer (Shimadzu-190). 0.2 ml distilled water in place of enzyme served as a blank.

All the operations during enzyme studies were carried out at 0 to 4°C. The enzyme chlorophyll was estimated following the method of Bradbeer (1969). 1 ml of the filtrate i.e. crude enzyme preparation (before centrifugation) was mixed with 4 ml acetone. After vigorous shaking, the contents were centrifuged at 4000 g for 10 min and the absorbance of supernatant was read at 663 and 645 nm using acetone blank.

Enzyme protein was estimated according to the method of Lowry _et al_. (1951).