Chapter II

MATERIALS AND METHODS

A. Materials

1. Soil culture :

Dodonaea viscosa L. plants were raised from the seeds in earthenware pots. After 30 days of growth of seedlings in normal conditions, the plants were exposed to salinity stress and water stress separately in different sets.

Salt treatment was given with NaCl salt solution at different concentrations such as 25, 50, 100 and 200 mM. The treatments were given twice a week, alternating with watering the plants, with equal amount of water to check the loss of water from pots and to avoid excess accumulation of salt in the pots. Drainage of the water or salt solution was well maintained. plants grown under non-saline conditions were taken as control.

In other set, plants were exposed to water stress by withholding water from the pots for different periods, viz. 2, 4, 8, 12 and 16 days. Plants were irrigated equally at the beginning. At the end of treatments (two months age), the plants were analysed for different growth parameters, such as shoot length, root length, leaf area and biomass production. The plants were also analysed for organic constituents, like chlorophylls, carbohydrates, polyphenols and proline. The acidity status and electrical conductivity of plant cell sap were also determined. The inorganic constituents with respect to macronutrients as well as micronutrients in different plant parts were also determined.

B. Methods:

1. Growth Analysis :

For the growth analysis plants from respective pots were carefully uprooted without disturbing the root system and washed thoroughly with water. At a time, 10 plants from each pot were collected. Thus, altogether 20 plants were used for growth analysis for each treatment. From this fresh plant material, growth parameters like average shoot length, average root length, average plant height, root to shoot ratio, number of leaves plant^{-1} and fresh weight plant^{-1} were determined. plants were then separated in individual parts like leaves, stem and roots. Some of this plant material was used for determination of some organic constituents and enzyme studies. The remaining material was oven dried and used for determination of inorganic constituents.

2. Organic constituents :

a) Moisture Content : The difference in fresh weight of individual plant parts and their oven dry wt was used to calculate the moisture percentage in leaves, stem and roots. From this the moisture content of a whole plant was also determined.

b) Titratable Acid Number (TAN) : The method described by Thomas and Beevers (1949) was used to determine TAN. Fresh leaf material was washed thcroughly and rinsed with distilled water and blotted to dryness. It was accurately weighed (2g) and boiled for half an hour in 100 ml distilled water with occasional addition of hot water to prevent drying. After cooling, it was filtered through double layered cheese cloth and volume was made to 50 ml with distilled water. The filtrate was then titrated against standardized NaOH (approx. N/40) using phenolphthalein as an indicator. NaOH was

standardized against N/40 Oxalic acid using the same indicator. TAN represents the number of ml of decinormal NaOH required to neutralize the acids in 100 g fresh tissue.

c) pH : To determine the acidity status of the cell sap, in addition to TAN, the pH of leaf extract in distilled water was determined on pH meter (Elico Pvt.Ltd.).

d) Chlorophylls : Chlorophylls were estimated from the leaves following the method of Arnon (1949). Chlorophylls were extracted in 80% acetone from 0.5 g of fresh plant material. The extract was filtered through Buchner's Funnel using Whatman No.1 filter paper. Residue on filter paper was washed repeatedly with 80% acetone, collecting the washings in the same filtrate. Volume of the filtrate was made to 100 ml with 80% acetone. All these operations were done in very dim or diffused light and the extract was stored in dark in a conical flask covered with black paper. The absorbance of this extract was read at 663 and 645 nm for chlorophylls "a" and "b" respectively on UV-VIS-190 double beam spectrophotometer (Shimadzu, Japan). Chlorophylls (mg 100^{-1} g fresh tissue) were calculated using the following formulae:

Chloro."a" = 12.7 x A 663 - 2.69 x A 645 = 'X'

Chloro."b" = 22.9 x A 645 - 4.68 x A 663 = 'Y'

Chloro.(a+b)= 8.02 x A 663 + 20.20 x A 645 = 'Z'

$$\text{Chloro 'a' or 'b' or (a+b) (mg/100 g fresh tissue)} = \frac{\text{X or Y or Z x Volume extract x 100}}{\text{1000 x weight of plant material (g)}}$$

e) Carbohydrates : Carbohydrates (Total sugars and starch) were estimated spectrophotometrically by the method of Nelson (1944). Fresh plant material (2g) was homogenized in 80% ethanol. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was used to estimate soluble

sugars while the residue on filter paper was used for starch estimation.

The filtrate thus obtained was condensed on a boiling water bath to about 3-5 ml. This extract was then treated with lead acetate and potassium oxalate (0.5 g each) with about 40 ml. distilled water to decolorize it. After decolorization the aliquats were filtered through Whatman No.1 filter paper and the volume was measured. The extract was used for estimation of reducing sugars. A known volume (20 ml.) was hydrolysed with 2 ml. conc. HC1 for 30 min under 15 lbs pressure in a pressure cooker. The contents were cooled, neutralized with Na_2CO_3 and filtered. The filtrate was used for estimation of total soluble sugars.

The residue of alcoholic extract obtained at the beginning was transferred to a conical flask. To it, 50 ml of distilled water and about 3 ml of concentrated HC1 were added and hydrolysed at 15 lbs pressure, for 30 minutes and cooled to room temperature. It was then neutralized by Na_2CO_3 and filtered through Whatman No.1 filter paper. The volume of the filtrate was noted. This filtrate containing reducing sugars produced as a result of hydrolysis of starch served as a source for starch estimation. The sugars so obtained were estimated to determine the starch present in the material.

The requisite quantity of the above filtrates (extracts) were taken separately in 10 ml marked test tubes. In such other test tubes, different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 ml) of standard glucose solution (0.1 mg ml^{-1}) were taken 1 ml. of Somagyi's alkaline copper tartarate reagent (4 g $CuSO_4,5H_2O$, 24 g anhydrous Na_2CO_3; 16 g Na-K-tartarate, Rochelle salt; and 180 g anhydrous Na_2SO_4 dissolved in 1000 ml distilled water) was added to each test tube. All the reaction mixtures were then

subjected to boiling water bath for about 10 min. After cooling to room temperature 1 ml of arsenomolybdate reagent (25 g ammonium molybdate in 450 ml distilled water, to which were added 21 ml concentrated H_2SO_4. To this was then added 3 g sodium arsenate, $Na_2HAsO_4,7H_2O$, dissolved in 25 ml distilled water. All ingredients were mixed well and the solution was placed in an incubator at 37°C for 48 h before use) was added to each reaction mixture. The contents of each tube were then diluted to a volume (10 ml). A blank was prepared by the same way but without sugar solution. After 10 min the absorbance of each reaction mixture was read at 560 nm on UV-VIS-190 double beam spectrophotometer, (Shimadzu).

By the interpolation from the glucose standard curve, the sugar percentage in the above fractions was determined.

f. Polyphenols : Polyphenols were estimated by the method of Folin and Denis (1915). 0.5 g fresh plant material was homogenized in mortar with pestle and extracted with 80% acetone (30 ml). Extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. Polyphenols were extracted repeatedly from the residue. The volume of the filtrate was made to 50 ml. This filtrate was used for the estimation of polyphenols.

2 ml of the filtrate was taken in a 50 ml. marked Nesseler's tube. In other such tubes different concentrations (0.5, 1, 2 and 4 ml) of standard polyphenol solution (tannic acid, 0.1 mg ml^{-1}) were taken. 10 ml 20% Na_2CO_3 were then added to each tube to make the medium alkaline 2 ml of Folin Denis reagent (100 g of Sodium tungstate and 20 g of phosphomolybdic acid dissolved in 200 ml distilled water were mixed with phosphoric acid, 25%. It was refluxed for 2.5 hours, cooled to room temperature and diluted to one litre with distilled water) were then added to each

test tube and finally the volume was made to 50 ml with water. A blank was prepared without polyphenolic, which helped to determine calibration curve. After some time the optical density of each mixture was read at 660 nm on UV-VIS-190 double bean spectrophotometer (Schimadzu). Polyphenols were calculated from the calibration curve of standard tannic acid.

g. Proline : Proline was estimated by the method of Bates et al (1973). The known quantity (1g) of fresh leaves was homogenized in 10 ml of 3% Sulfosalicylic acid. After homogenization, it was filtered. The volume of filtrate was recorded. 2 ml of this filtrate was taken in a test tube to which were added 2 ml of acid ninhydrin reagent (prepared by adding 2.5 g ninhydrin in 60 ml of glacial acetic acid, warmed to dissolve and after adding 40 ml of 6 M Orthophosphoric acid, cooled and used fresh) and 2 ml of glacial acetic acid. The reaction was allowed to continue for 1 hour at 100^{o}C on boiling water bath.

The reaction was quickly terminated in ice bath and 4 ml of toluene were added in each reaction mixture. The reaction mixture was shaken vigorously for 15-20 seconds. The reaction mixture was then brought to room temperature and the absorbance of toluene chromophore (upper layer) was measured at 520 nm on UV-VIS-190 double beam spectrophotometer (Shimadzu) using reagent blank. To take toluene chromophore vaccupipette was used.

At the same time, reaction mixtures for standard curve of proline (0.1 mg ml^{-1}) were prepared by taking different concentrations of proline solution. The calibration curve for standard proline was drawn and the amount of free proline in the leaves was determined and expressed in terms of ug g^{-1} fresh tissue.

h. Electrical conductivity : The electrical conductivity (mmhos cm^{-1}) of leaf cell sap was determined from the

water extract of fresh leaf material using MHOS pH meter Model PE-133 (Elico Private Limited, Hyderabad).

3. Enzyme studies

Enzymes hydroxyperoxidases (Peroxidase, E.C. 1.11.1.7 and Catalase, E.C. 1.11.1.6), acid phosphatase (E.C. 3.1.3.2) and nitrate reductase (E.C. 1.6.6.1) were isolated from fresh plant material. Isolation and assay of enzymes were done at lower temperatures (0-4^{o}C).

a. Hydroxyperoxidases :

i. Peroxidase : Peroxidase from leaf, stem and roots of fresh plants was determined following the method described by Maehly (1954). Enzyme was extracted by homogenizing the plant material (0.5 g) in cold distilled water. It was then filtered through moist cheese cloth and the filtrate so obtained was centrifuged at 10,000 x g for 10 min at 0 to 4^{o}C and the supernatent was used as an enzyme source. Enzyme assay mixture contained 2 ml phosphate buffer (pH 7, 0.1 M), 1 ml Guiacol (20 mM) and 0.5 ml enzyme preparation. The reaction was started by the addition of 0.04 ml H_2O_2 (10 mM). Change in optical density due to oxidation of guiacol was recorded per minute at 470 nm on UV-VIS-190 double beam spectrophotometer (Shimadzu) with frequent stirring of the reaction mixture with a fine glass rod. Enzyme activity is expressed as O.D. $min^{-1}g^{-1}$ fresh tissue as well as O.D. $min^{-1}mg^{-1}$ protein.

ii. Catalase : The enzyme preparation made for the assay of peroxidase was also used for the assay of catalase. Assay of the enzyme was done following the method described by Herbert (1955). The assay mixture contained 1 ml 0.45 M H_2O_2. in phosphate buffer (pH 6.8, 0.1 M) and 0.5 ml enzyme. The reaction was terminated by adding 5 ml 1N H_2SO_4 after 4 minutes of the reaction. To this 1 ml 10% KI and a drop of 1% ammonium molybdate were added and liberated iodine was

titrated against 0.01 N sodium thiosulfate using starch as an indicator. Blue to colourless was taken as an end point. Blank or 0 min reading was taken by mixing all the ingredients except enzyme as given above. Activity of the enzyme is expressed as mg H_2O_2 broken $min^{-1}g^{-1}$ fresh tissue. The specific activity of the enzyme was also determined.

b. Acid phosphatase :

The enzyme preparation in cold distilled water was also used for the assay of acid phosphatase. Assay of the enzyme was done following the method of De Leo and Sacher (1970). The assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg ml^{-1}), 2 ml of acetate buffer (pH 5, 0.1 M) and 1 ml enzyme preparation. Enzymatic reaction was terminated after 1 h by adding 1.5 ml of NaOH (1.68N). The intensity of yellow coloured complex formed as a result of reaction between p-nitrophenol formed in the enzymatic breakdown of p-nitrophenyl phosphate and NaOH was read at 400 nm on UV-VIS-190 double beam spectrophotometer (Shimadzu). Enzyme activity is expressed as O.D. h^{-1} g^{-1} fresh tissue. The activity is also expressed on protein basis.

c. Nitrate Reductase :

Nitrate reductase (E.C. 1.6.6.1) was determined (in vivo) by employing the leaf disc method (Jaworski, 1971). 0.5 g fresh leaf discs, stem and roots were suspended in a medium containing 1 ml, 1M KNO_3; 2 ml 5% n-propanol; 2 ml, 0.5% Triton X-100 and 5 ml phosphate buffer, pH 7.5,0.2 M. The test tubes with reaction mixture containing leaf discs or small cut pieces of root or stem were then sealed and kept in darkness for incubation for 1 hour. The enzyme activity was measured by treating 1 ml of incubated reaction mixture with 1 ml, 1% sulfanyl-amide in 1 M HC1 and 1 ml, 0.02% NEEDA (N-1-napthyl ethylene diamine dihydrochloride). The absorbance of color developed was

read at 540 nm on UV-VIS-190 double beam spectrophotometer (Shimadzu). Standard curve was prepared with 0.03 mM KNO_2 (0.0026 mg KNO_2 ml^{-1} distilled water) while , mixture of 1 ml incubation medium, 1 ml sulfanylamide and 1 ml NEEDA served as a blank. Activity of the enzyme is expressed as mg of NO_2 liberated $h^{-1}g^{-1}$ fresh tissue.

d. Determination of enzyme proteins :

Proteins were estimated according to the method described by Lowry et al.(1950). 0.05 ml of the enzyme preparation was taken in a test tube. In other such test tubes different concentrations (0.1, 0.2, 0.3, and 0.4 ml) of standard protein solution (bovine serum albumen, 0.1 mg ml^{-1}) were taken. 5 ml reagent `c' (50 ml of reagent `a' was mixed with 1 ml of reagent `b'. This reagent is stable for one day. Reagent `a' = 2 % Na_2CO_3 in 0.1 N aqueous NaOH, reagent `b' = 0.5 g $CuSO_4$, $5H_2O$ in 1% sodium tartarate) was added to each reaction mixture. it was kept for 15 min at room temperature. Then 0.5 ml of Folin phenol reagent (100 g sodium tungstate, Na_2WO_4,H_2O and 25 g sodium molybdate, $Na_2MoO_4,2H_2O$ were added to 700 ml distilled water).

After 30 min the absorbance was read at 660 nm on US-VIS-190 double beam spectrophotometer (Schimadzu).

A reagent blank was prepared by mixing 0.5 ml reagent C and 0.5 ml Folin phenol reagent.

4. Inorganic Constituents :

The inorganic constituents were determined from oven dried plant material.

Preparation of plant extract :

For estimation of inorganic constituents an acid digest from the oven dried plant material was used. The plant material was digested following the method of Toth et al. (1948). 0.5 g of the oven dried powdered plant material was taken in a 150 ml capacity

beaker. 20 ml concentrated HNO_3 were added to it and allowed to stand for sometime till the initial reactions subsided. It was then subjected to heating till the particles of plant material were completely dissolved. Then, 10 ml of concentrated perchloric acid (60%) were added to it. It was then heated strongly until the volume of the plant extract was reduced to approximately 3-5 ml It was cooled to room temperature, transferred quantitatively to 100 ml capacity volumetric flask and diluted to 100 ml with distilled water. It was kept over night and next day filtered through a dry Whatman No.44 filter paper . The filtrate was used for estimation of inorganic constituents.

a. $\underline{Na^{+}, K^{+}}$ and $\underline{Ca^{2+}}$:

Na^{+}, K^{+} and Ca^{2+} were estimated flame photometrically following the procedure standardized in our laboratory. Stock solutions of known concentration in parts per million (ppm) of K^{+} in KCl and Na^{+} in NaCl and Ca^{2+} in $CaCl_2$ were prepared. For the calibration curve, various concentrations of these elements were prepared as follows,

Various concentrations of Na^{+} in NaCl ranging from 1 to 10 ppm were prepared by diluting the stock solution of NaCl (10 ppm) accordingly. Similar concentrations were prepared for K^{+} in KCl (10 ppm) also. However, various concentrations of Ca^{2+} in $CaCl_2$ ranging from 20 to 200 ppm were prepared by diluting the stock solution of $CaCl_2$. (200 ppm) accodingly. The deflections of galvanometer for these different concentrations of Na^{+} K^{+} and Ca^{2+} using Na K and Ca specific filters respectively on flame photometer (Toshniwal, India.) were noted and calibration curves for these three elements were prepared. From these calibration curves and the galvanometer readings for plant extracts the concentrations of Na^{+}, K^{+} and Ca^{2+} in the plant

material were calculated.

b. Phosphorus :

Phosphorus was estimated following the method given by Sekine et al. (1965). Phosphorus gives yellow colour raction with molybdate vanadate reagent by estimating colourimetrically the intensity of the developed colour and by comparing it with that of known standards, P^{5+} can be estimated.

2 ml of acid digest was pipetted in a test tube to which 2 ml of 2 N HNO_3 were added followed by 1 ml of Molybdate vandate reagent (A -1.25 g ammonium vandate dissolved in 500 ml of 1 N HNO_3. B-25 g of ammonium molybdate in 500 ml distilled water. Then A and B were mixed in equal volumes). Volume was made to 10 ml with distilled water . The reaction mixture was shaken well and kept for 20 minutes to allow the colour to develop. Absorbance was measured at 420 nm on UV-VIS-190 spectrophotometer (Shimadzu,Japan) using reagent blank containing no phosphorus.

A curve of standard P^{5+} was prepared for different concentrations of phosphorus (0.5, 1, 2 and 4 ml.) using standard KH_2PO_4 solution of the strength of 0.025 mg P ml^{-1}. By comparing the absorbance of the extract with that for standards, amount of phosphorus in the plant material was calculated.

c. Iron, Manganese, Magnesium, Cobalt, Copper, Cadmium, Nickel, Lead and Zinc.

These inorganic elements were estimated from the acid digest using atomic absorption spectrophotometer (Perkin-Elmer, 3030).