CHAPTER - II

MATERIAL AND METHODS

2.1 MATERIAL :

Eichhornia crassipes solms. a members of family Pontederiaceae is worked out for its eco-physiology during present investigation. It is commonly found as an aquatic weed in fresh water, ponds, pools, tanks, lakes, reservoirs, streams, rivers and in irrigation chanals throughout the year [Fig.2].

For general study the young ramets of E. crassipes were maintained in the tank of botanical garden of Yashwantrao Chavan College of Science, Karad. At the time of experiment the tank water was analysed for its DO.

So as to understand the behaviour of water hyacinth in normal fresh water and polluted pond fresh water, the plants were collected at two different localities as running fresh water and pond. Surrounding water sample was collected simultaneously and analysed for its Dissolved Oxygen condition.

Stomatal density was measured in a plant which thrived well, multiplied vegetatively continuously in the tank of the college botanical garden.

Effect of light intensity on morphology of the plant was studied in the plants growing in tank under full light and shade condition.

Inorganic and organic constituents were determined from the fully grown plants which were simultaneously used for general studies.

Every time at least two replicates were maintained for each analytical experiment. Morphological peculiarities, habitat details etc. were traped in form of photographs.

2.2 Methods :

2.2.1 Growth performance :

Growth performance of Eichhornia crassipes under different light conditions - full light and shade was studied with reference to the remark of Boresch (1972). Natural condition of the tank in botanical garden was so that one tank was exposed to full light during entire day length while the tank in shade was in constant shade. Morphological parameters like length and diameter were measured.

2.2.2 Stomatal density :

Stomatal density was measured by method of Stoddard (1965) as described by Waghmode (1982), with help of natural colour nail paint.

2.2.3 Organic constituents :

For estimation of organic constituents like moisture content and chlorophylls; different parts of mature and fully grown plants were used.

Moisture content :

Percent moisture content of the plant/plant parts was measured according to the method described by Patil (1988). The plant material was air dried and the formula used was,

$$\% \text{ moisture} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{fresh weight}} \times 100$$

From moisture % the amount of total solids was determined.

Chlorophylls :

The chlorophylls were estimated by the method of Arnon (1949). The leaves were washed thoroughly with distilled water and blotted dry. 0.5 g of the leaves were accurately weighed and homogenized in mortor with pestle. Chlorophylls were extracted in 80% acetone (4 ml of liquer ammonia/liter of 80% acetone) A pinch of Mg CO_3 was added while crushing. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The residue was washed thoroughly with 80% acetone collecting the washings in the same container. Finally the volume was made to 100 ml. with 80% acetone. All the operations were done in dim

light, and at the lowest possible temperature. Optical density of the filtrate was read at 645 and 663 nm.

Chlorophylls (mg/100 g fresh tissue) were calculated by using the following formulae,

Chlorophyll 'a' = (12.7 x A.663) - (2.69 x A.645) = X

Chlorophyll 'b' = (22.9 x A.645) - (4.68 x A.663) = Y

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

$$\text{Chlorophyll a/b/a+b mg/100 g fresh tissue} = \frac{x/y/z \times \text{vol. of extract} \times 100}{100 \times \text{wt.of plant material (g)}}$$

$$\text{Chlorophyll a:b ratio} = \frac{\text{mg of chlorophyll 'a' / 100 g}}{\text{mg of chlorophyll 'b' / 100 g}}$$

2.2.4 Nitrogen metabolism :

To study nitrogen metabolism with respect to total nitrogen, total proteins and enzymes, fresh and oven dry material of Eichhornia crassipes was used. From oven dried root, stem (off set) and leaves total nitrogen and total proteins were estimated. While fresh root, stem (offset) and leaves were used to study activity of enzyme nitrate reductase.

i) Total nitrogen :

The total nitrogen content in the oven dried root, stem and leaves were estimated, according to the method of Hawk et

al., (1948). 0.5 g of oven dried material was digested in Kjeldahl flask with sulphuric acid (1:1 dilution) and a pinch of microsalts (mixture of anhydrous copper sulphate and potassium sulphate in the proportion of 1:40) till a colourless liquid was obtained at the bottom of the flask. It was then cooled to room temperature and transferred quantitatively to the volumetric flask and volume was adjusted to 100 ml with distilled water. Then it was filtered on next day through dry filter paper. The filtrate was used for estimation of nitrogen.

Two ml.of this filtrate was taken in Nesslor's tube (35 and 50 ml marked). In other tubes different concentrations of standard ammonium sulphate (0.05 mg nitrogen/ml.) were taken. One tube was kept as a blank without ammonium sulphate. To each of these tubes was added a drop of 8% potassium bisulphate. The volume of all these tubes was adjusted to 35 ml. with distilled water. 15 ml of Nessler's reagent was then added in each tube. Nessler's reagent is a mixture of reagent A (7 g KI and 10 g HgI_2 dissolved in 40 ml distilled water) and B (10 g NaOH dissolved in 50 ml. distilled water) in the proportion of 4:5. The colour intensity of the orange brown product ($NH_4Hg_2I_3$) produced by the reaction between NH_3 liberated from the sample and reagent, was measured at 520 nm on Toshniwal's VIS - Spectro-

photometer. The amount of nitrogen in the sample was calculated from the standard curve of ammonium sulphate.

ii) Total proteins :

To estimate total proteins in the sample, similar method for estimation of total nitrogen was used and total proteins were determined by using the formula, as described by Patil (1988).

Total proteins = Total nitrogen x 5.7 (factor)

Enzyme of Nitrogen Metabolism :

Nitrate reductase (E.C. 1.6.6.2) :

To determine activity of enzyme nitrate reductase (NR) in fresh root, stem (offset), petiole and leaf lamina of E. crassipes solms, in vivo method of Jaworski (1971) as described by Knyl (1974) was followed with slight modifications.

Assay of the Enzyme :

Leaf discs of 1 cm. diameter were cut with the help of leaf punch, while stem and root were cut into small pieces of uniform size. 500 mg of plant tissue was suspeneded in 10 ml of a standard incubation medium containing phosphate buffer (pH 6.2), 20 mM KNO_3, 5% (v/v) n-propanol and 1.25% Triton x - 100 in sealed jars and incubated in the dark for 30 minutes in case of

leaves, while root and stem (offset) samples were incubated in the dark for 60 minutes. Nitrate reductase activity was measured by NO_2^- production which was detected by treating 0.4 ml of reaction mixture with 0.3 ml of 1% sulfanilamide in 2 N. HCl and 0.3 ml of 0.02% NEEDA (N% 1 Naphthylethylene diamide hydrochloride). After 20 minutes the solution was diluted to 4 ml with distilled water and absorbance was read at 540 nm on Toshniwal's VIS-Spectrophotometer. The amount of nitrite was estimated from a standard curve of sodium nitrite prepared in a similar manner.

Study of various factors influencing nitrate reductase activity :

Buffer pH

For the study of effect of buffer pH on the enzyme activity, the 0.2 M phosphate buffer of different ph values was prepared. The other components of the medium and the experimental conditions were essentially the same as employed in standard assay.

Tissue weight :

For the study of effect of tissue weight on enzyme activity, the samples of different weights (100 mg, 200 mg, 300 mg, 400 mg, 500 mg) of leaf tissue were suspended in the standard

incubation medium in darkness for one hour. After this nitrite released in each case was estimated.

Triton X-100 :

For the study of effect of Triton - x-100 on the enzyme activity, plant tissue was suspended in standard incubation medium containing Triton x-100 and tissue was also suspended in incubation medium which was not containing Triton x-100. After this nitrite released in each case was estimated and further procedure was adopted as employed in standard assay.

Substrate concentration :

To study the effect of substrate concentration on enzyme activity, different concentrations of KNO_3 (10 mM, 20 mM, 30 mM, 40 mM and 50 mM) were employed during the preparation of incubation medium, keeping the other components of the incubation medium same and the experimental conditions essentially similar to standard assay.

Temperature effect :

To study the thermolability of the NR the assay was carried out at various temp. (little below and up of the room temp.) Enzyme assay was performed by maintaining the temp. of reaction mixture at 22°C, 27°C, 32°C, 37°C and 42°C, keeping all other experimental conditions similar.

2.2.5 Inorganic constituents :

To estimate inorganic constituents, plant samples were digested by wet digestion method of Toth et al. (1948). 0.5 g oven dried powdered sample was taken in 100 ml clean corning beaker and to it 15 ml of concentrated nitric acid was added. The beaker was covered with watch glass and kept at room temperature till the initial reaction subsided. Then it was heated on a gas flame till all the particles of plant material were dissolved. The digest was then cooled to room temperature and to it 10 ml of 60% perchloric acid was added. It was then heated strongly untill a clean and colourless solution resulted. Heating was stopped when the volume of the extract was reduced to 2 to 3 ml. It was then cooled and transferred quantitatively to a 100 ml volumetric flask. Finally volume was adjusted to 100 ml with distilled water. On next day the digest was filtered through Whatman No.1 filter paper. The filtrate was used for estimation of various inorganic elements.

Inorganic elements Na^+, K^+, Ca^+ were estimated with help of Atomic Absorption Spectrophotometer (3030/PERKIN-ELMER).

2.2.6 Dissolved oxygen [DO]

Dissolved oxygen in water (DO) is one of the parameters of monitoring the degree of pollution. 'DO' of the water samples

from different localities was measured by the Winkler's method as described by Nagbhushanam et al (1981).

Plant material and water samples were collected at two localities (Fig.3). Water sample was taken in sample bottles which were stoppered immediately. The care was taken that, no air bubble is inside. One ml of Winkler's 'A' solution was taken in reagent bottle with help of pipette. After shaking well exactly 1 ml of Winkler's 'B' solution was added. After thorough mixing the brown ppt was allowed to settle down. After this concentrated HCl was added dropwise in the reaction mixture till the ppt. is dissolved completely. Then, 100 ml of this; were taken in conical flask. To it few drops of 1% starch solution were added as an indicator. After addition of starch, the colour of the solution turns blue. This solution was titrated against N/80 sodium thiosulphate till the blue colour disappears. Burrette reading was recorded accurately.

Volume of the dissolved oxygen in water samples was calculated with the formula -

$$DO = \frac{V \times 0.0001 \times 22400 \times 1000}{32 \times \mu}$$

where,

v = volume of sodium thiosulphate used for titration.

μ = volume of water sample taken.