MATERIALS AND METHODS

Plant species Euphorbia hirta, L., a C_4 plant belonging to the family Euphorbiaceae is seen growing on soils with different soil moisture levels in the University Botanical garden. The leaves of this plant are showing either green (normal) or varying intensities of purple pigmentation. The purple pigmented plants are seen growing on soils with low moisture levels. The purple pigmentation was very intense during summer months, where the plants were exposed to continuous long dry spell. The purple pigmentation was more intense in the mature and fully expanded leaves than in young leaves of the plant. These plants of Euphorbia hirta growing on soils with different soil moisture levels in the university botanical garden were taken as experimental material in the present investigation. A negative correlation was observed between the soil moisture content and the intensity of purple pigmentation. Accordingly the purple pigmented plants were classified as light, moderate and heavy pigmented plants. The observations recorded in the present study were made during March to June 1984 and 1985.

Soil moisture content :

Soil samples were collected at a depth of 6" and 10g of soil was weighed immediately in a petriplate and dried at 120°C in an hot air oven for 48h. The dry weights were taken and moisture content was expressed on percentage basis.

Leaf area and moisture content :

Fully expanded and mature leaves (3rd to 6th) from the apex were collected, washed in distilled water and blotted dry immediately. The area was noted on a graph paper and fresh weight was noted. Then the leaf samples were dried at 80°C in an hot air oven for 48 h and the dry weights were recorded.

Estimation of pigments :

Anthocyanins: Anthocyanins were estimated according to Mancinelli et al., (1975). Leaf material was collected from the plants, washed in distilled water and blotted to dry. One gram of leaf tissue was extracted with 15 ml of 1% (V/V) HCl in methanol for 24 h. The extraction was carried at 3°-5° C with continuous shaking in a water bath. The extracts were cleared by

filtration and made up to 25 ml. The absorbance were recorded at 530 nm and 657 nm in a spectrophotometer. The absorbance readings at 530 nm were corrected using the formula (A_{530} - 0.33 A_{657}).

Chlorophylls : Total chlorophyll content was estimated according to Arnon (1949). 500 mg of leaf tissue was ground in cold 80% acetone in a prechilled mortar and pestle. The homogenate was centrifuged and the supernatnat was collected. The sediment was reextracted with 80% acetone, and centrifuged. The supernatants were pooled and made to known volume with 80% acetone. Absorbance of the solution was recorded at 645 and 663 nm in a spectrophotometer. Chlorophyll content was calculated using the following formula.

$$20.2 \times \text{O.D. at 645 nm} + 8.02 \times \text{O.D. at 663 nm.} \times \text{volume of the acetone extract} \times \frac{1000}{\text{weight of the tissue}} \times 1/1000 =$$

mg of chlorophyll/g. fresh weight

Stomatal frequency :

Green and purple pigmented leaves (3rd to 5th from apex) were collected and washed in distilled water.

Epidermal peelings (1.5 x 2.0 mm) from adaxial and abaxial surfaces were peeled with the help of a sharp blade, mounted on a slide and observed under a research microscope. Number of stomata in the microscopic field area was noted. Microscopic field area was calculated with the help of a staye micrometer. Stomatal frequency was expressed as number of stomata per mm^2 area of leaf.

Further, stomata with two or three subsidiary cells were observed. Purple pigmentation was observed in the subsidiary cells and was not observed in guard cells. Frequency of stomata with one or more pigmented subsidiary cells was also calculated and expressed as number per mm^2 area.

Frequency of defunct guard cells (one of the two guard cells loosing crescent shape and remaining straight, causing partial closure of stoma) in green, and light, moderate and heavy pigmented leaves was calculated and expressed as number per mm^2 area.

The observations were made in five different regions of 10 epidermal preelings of five different plants.

Transpiration rate :

The transpiration rate of green and purple pigmented plants was determined by using a simplified potometer as described by Rama Das et al., (1979). The rate of transpirational water loss of the twigs cut under water was determined indirectly by measuring the rate of absorption with an assumption that the absorption balances the water lost in transpiration. The apparatus consisted essentially of a water reservoir (U-tube) in which the twigs cut under water was sealed on to one side, and the other side was closed with oil layer. The U-tube was graduated on both arms. The whole apparatus was kept in the open atmosphere for 1 hour and the volume of water loss (ml) was calculated per unit leaf area. The transpiration rate was expressed as $ml/h/dm^2$.

Stomatal movements in detached leaves :

Green and purple pigmented leaves (3rd to 5th from apex) were collected from the plants in the early hours, washed in distilled water and kept in 50 mM phosphate buffer pH 7.0. The leaves were made into two sets, each set containing five petriplates, with ten

leaves in each petridish. One set was placed in darkness and other set was placed under light provided by bank of 8 x 100 W incandescent lamps. To avoid temperature affect the light source was filtered through a 3" column of continuous flowing water. The adaxial surface of the leaf was exposed to light source. Stomatal pore size was measured in both light and dark exposed leaves at required intervals, by taking the epidermal peelings 1.0 mm x 1.5 mm under a research microscope using a calibrated occular micrometer.

Diurnal variations in stomatal movements :

Stomatal movements in intact plants of green and purple pigmented leaves of Euphorbia hirta were studied by following the nail polish imprint method according to Sampson (1961). The pore size was measured on the adaxial surface of the leaf. Thin film of nail polish was applied on the surface of the leaf of intact plant and allowed to dry. Then the leaf was cut and brought to the laboratory. Immediately the thin film was separated from the leaf, mounted on a slide and observed under a research microscope. The pore size was measured using calibrated occular micrometer.

Estimation of free acids :

2 grams of leaf material was cut into small bits, and boiled with 100 ml of distilled water on a water bath for 30 minutes. It was filtered through a whatman No.1 filter paper and made upto 100 ml with distilled water. 20 ml of this leaf extract was taken into a conical flask, and titrated against 0.01N sodium hydroxide using phenolphthalein as an indicator. Appearance of pink colour was taken as the end point of titration. The acid levels were expressed as u equivalents of NaOH/g leaf fresh weight/hour.

Determination of pH :

25 ml of the above leaf extract was taken into a beaker, and pH was measured in an Elico model LI 122 pH meter.

Estimation of malic acid :

Malic acid was estimated according to Gutmann and Wahlefeld (1963).

2 grams of leaf tissue was homogenized in about 10 ml of 0.6N perchloric acid, in a cold mortar with pestle. The homogenate was centrifuged for 10 minutes at

3000 g. The supernatnat was decanted and the sediment was reextracted with 0.6N perchloric acid and centrifuged. The supernatants were pooled and made upto known volume with water to get 8 ml/g of tissue.

The extract was neutralized with 5M K_2CO_3 solution using methyl orange indicator. The K_2CO_3 solution was added to the extract drop wise with constant stirring till the mixture changes to salmonpink to give a pH of 3.5 approximately. The neutralization process was done under cold conditions. The extract was allowed to stand for 10 minutes in ice water and the supernatant was decanted from the sediment perchlorate. Malic acid was estimated by following the NAD reduction at 340 nm in a Bausch and Lomb Spectromic 88 spectrophotometer. The reaction mixture in 3.0 ml contained 0.43M glycine/ 0.34 M hydrazine hydrate buffer pH 9.0, 2.75 mM NAD, 34 units/mg of malate dehydrogenase and 0.2 ml sample. Reaction mixture was incubated for 10 minutes at 37°C. NAD reduction was measured at 340 nm and the results were tabulated.

PREPARATION OF SOLUTIONS :

1. 5M K_2CO_3 solution: 69g K_2CO_3 was dissolved in water and made upto 100 ml.

2. Methyl Orange indicator : 50 mg Methyl orange was dissolved in water and made upto 100 ml.

3. 0.6N perchloric Acid : 7.7 ml of $HClO_4$ was diluted to 150 ml with water.

4. Hydrazine glycine buffer (0.4 M hydrazine; 0.5 M glycine; pH 9.0) :

11.4 g glycine and 25 ml hydrazine hydrate were dissolved in 20 ml water, and made upto 300 ml with water.

5. 40 mM NAD : 150 mg NAD was dissolved in water and made upto 5 ml.

Stomatal movements in isolated epidermal strips :

Green and purple pigmented leaves were collected from the plants at 8.00 AM and washed in distilled water. Epidermal peelings were peeled from the leaves in 50 mM phosphate buffer pH 7.0. Uniform peelings were selected and placed in petridishes under a bank of incandescent lamps (100 W x 8). Temperature effect was avoided using water filter. Stomatal response to ABA, proline, KCl, ADP, ATP

and HCO_3 was studied by preparing solution in phosphate buffer pH 7.0. Observations were made at 3 h interval, at 8, 11, 14 and 17 hours. Observations in phosphate buffer were taken as control.

Temperature effect on stomatal movements was studied by maintaining the buffer temperature at 25, 32 and 40°C.