# Chapter II

## * Material and Methods *

## MATERIALS AND METHODS

(A) Material :

For the present investigation, three plant species (i) Acacia auriculiformis, Cunn, (ii) Anong squamosa, Linn. (iii) Eucalyptus globulus, Labill., were selected, from the afforested area in University Campus, with permission of Director of Forest Division, Kolhapur. In this locality, the plantation was only one year old. The plant species growing in identical conditions in this area were selected. The experiments were commenced in the month of November, 1987 and were continued upto August 1988. From these plants, leaf material was collected for monthly experiments. The monthly data about environmental parameters like rainfall, humidity, air temperature, soil temperature at various depths (5 cms, 10 cms and 20 cms) were collected at the experimental site with the help of Meteorology Department of Agriculture College, Kolhapur. The soil samples in triplicate were collected from the root environment for the determination of moisture percentage in it, at the time of experiments.

Soil moisture percentage : For determination of soil moisture percentage, the soil samples from each plants root environment (1 to 1.5 feet depth) were collected in polyethylene bags. From these soil samples, exactly 10 gms in triplicate were weighed and kept in oven at $60^{o}C$ temperature for 15 days.

After 15 days, these soil samples were again weighed and by considering difference between two weights, soil moisture percentage was determined. This was followed for every month and at the exact date of experiments.

The analysis of leaf moisture status and free proline was done from the fresh leaf samples every month on a fixed date. The stomatal studies were also carried out on the same day of sampling. The sugars mineral elements and total nitrogen were determined from the oven dried plant material which was collected on the same date.

(B) Methods :

(1) Determination of leaf moisture percentage :

Fresh leaves of each plants in triplicate were weighed exactly 10 gms and kept in oven at 60°C temperature for 15 days. The oven dried leaves were taken out and again weighed. Their weights were recorded accordingly and by considering difference in weights, the leaf moisture percentage was determined. This practice was followed for every month in a year.

(2) Free proline estimation :

The leaves of various stages of growth were collected randomly from the three plant species. These leaves were brought to laboratory within short duration. The leaves were

first washed with tap water and then by distilled water. They were made dry by using blotting paper.

With the help of one pan balance, 0.5 gms. each leaf pieces were taken for quantification of free proline, by the Standard method described by Bates et al. (1973). In this method 3% sulfosalicylic acid was used as an extraction medium. The acid ninhydrine reagent was prepared by dissolving 1.25 gms ninhydrine powder in 30 ml. glacial, acetic acid and 20 ml. 6 M. phosphoric acid. The content was slightly warmed and stirred until crystals of ninhydrine were dissolved. The reagent was stored in freeze. Each time fresh reagent was prepared.

(a) 0.5 gms of leaf pieces of each plant in triplicate were homogenized in 10 ml of 3% sulfosalicylic acid and homogenate was then filtered through Whatman No.2 filter paper.

(b) 2 ml. of the filtrate was mixed with 2 ml. of acid ninhydrine and 2 ml. of glacial acetic acid in a test tube. These test tubes were heated on water bath (100$^{o}$C) for one hour and then the reaction was terminated by dipping these test tubes in an ice bath.

(c) In the reaction mixture 4 ml. of toluene was added and test tubes were vigorously stirred for 15-20 seconds.

(d) The chromophore containing toluene was brought to room temperature and the absorbance was read at 520 nm using toluene as a blank.

(e) The proline concentration was determined from a standard curve and calculated on dry weight basis.

3. Total sugar estimation :

The total sugars were estimated by the method of Nelson (1944). 0.5 gms of dry plant material (leaves) was crushed in morter with pestle and extracted with 20-25 ml. of 80 % alcohol. It was filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was used for estimation of soluble sugar. The filtrate obtained, was condensed on a waterbath till the volume was about 2-3 ml. This was mixed with lead acetate and potassium oxalate in the ratio of 1:1 to decolorise it. To this distilled water was added and filtered through Whatman paper No.1. It was again washed with distilled water for two times and the washings were collected in the same filtrate. The total quantity of filtrate was noted down and labelled as (A) and used for estimation of soluble sugars.

The known volume from (A) stock solution was taken in conical flask for hydrolysing it with concentrated HCl in pressure cooker at 15 lbs. pressure for half an hour. The content was cooled to room temperature and neutralised with $Na_2CO_3$ and then filtered through Whatman No.1 paper. The filtrate was used for the estimation of soluble sugars (B).

The requisite quantity (0.1 ml.) of the above filtrates

(B) and (C) was taken separately in 10 ml. marked test tubes. In other set of test tubes, different concentrations (0.1, 0.2, 0.3 and 0.4 ml) of standard glucose solution (0.1 mg.ml) were taken. One ml. of Somogyi's alkaline copper tartarate solution (4 gms. of $CuSO_4$, $5H_2O$; 24 gms. of unhydrous $Na_2CO_3$; 16 gms. of Na - K - tartarate (Rochella Salt); and 180 gms anhydrous $Na_2SO_4$. All were dissolved in 1000 ml. of ~~distilled~~ water), was added to each test tube. All the test tubes containing reaction mixtures were then subjected to boiling water bath for 10 minutes.

After cooling to room temperature, 1 ml. of arsenomolybalate reagent (25 gms of ammonium molybdate dissolved in 450 ml of distilled water and to it, 21 ml concentrated $H_2SO_4$ is added. To this, 3 gms of sodium arsenate ($Na_2HASO_4 \cdot 7H_2O$) was added by dissolving it in 25 ml distilled water. This mixture of chemicals was placed in an incubator at 37°C. for 24 hours before use )was added to each test tubes containing reaction mixture. This content of each tube was diluted with distilled water to a volume of 10 ml. A blank was prepared by the same way but without sugar solution. After 10 minutes the absorbance of each reaction mixture was read at 560 nm on double beam spectrophotometer (Shimadzu). With the help of glucose standard curve, the amounts of total sugars were determined.

(4) Estimation of mineral elements ($Ca^{++}$ and $K^{+}$) :

$Ca^{++}$ and $K^{+}$ were estimated flame photometrically following the procedure standardised in our laboratory. Stock solutions of known concentrations in parts per million (PPM) of $Ca^{++}$ in $CaCl_2$ (10 ppm..... 100 ppm) and $K^{+}$ in KCl (10 ppm..... 100 ppm) were prepared. The readings of these standard solutions were used for determining the amounts of $Ca^{++}$ and $K^{+}$ in the acid digest of plant material.

For preparation of the acid digest, oven dried plant material was used and the method of Toth et al. (1948) was followed, 0.5 gms oven dried material of each plant species was taken in 150 ml. capacity beaker to which 20 ml. concentrated $HNO_3$ were added. The beaker was covered with watch-glass and was kept till the primary reaction subsided. It was then heated slowly on hot plate to dissolve solid material. After cooling to room temperature, 10 ml. of perchloric acid (60%) were added to it and mixed thoroughly. It was then heated strongly until a clear and colourless solution (2-3 ml.) was obtained. While heating the liquid was not allowed to dry. It was then cooled and transferred quantitatively to 100 ml. capacity volumetric flask, diluted to 100 ml with distilled water and kept overnight.

Next day it was filtered through dry Whatman No.44 (ashless) filter paper and the filtrate was used as the source of different inorganic elements like $Ca^{++}$ and $K^{+}$.

(5) Total nitrogen estimation :

The total nitrogen in the plant material was estimated colorimetrically by the method of Hawk et al. (1948).

Five hundred mgs of oven dried plant material was digested in a Kjeldahl flask with conc. sulphuric acid and water (1:1) and a pinch of microsalt (anhydrous Copper sulphate + Potassium sulphate in the ratio of 1:40). Glass beads were added to avoid bumping. The clear and colorless solution (about 2 ml) was obtained at the bottom of the flask. It was then cooled to room temperature and transferred quantitatively to the volumetric flask and the volume was made to 100 ml. with distilled water. It was kept overnight and was filtered through Whatman filter paper. The filtrate was used for the estimation of total nitrogen.

2 ml. of this filtrate was taken in a Nesseler's tube. In other tubes different concentrations of standard ammonium sulphate (0.05 mgs. nitrogen $ml^{-1}$) were taken. One test tube was kept blank without ammonium sulphate. To these tubes, was added a drop of 8% of potassium bisulphate and 1 ml. $H_2SO_4$ (1:1 wherever needed). The volume of all the test tubes was adjusted to 35 ml. using distilled water. 15 ml. of Nesseler's reagent was then added to each tube. Nesseler's reagent is a mixture of A (7 gms KI and 10 gms $H_gI_2$ dissolved in 40 ml distilled water) and B (10 gms NaOH dissolved in 50 ml of distilled water), in the proportion of 4:5. The colour

intensity of orange brown product ($NH_4HgI_2$) produced by the reaction between $NH_3$ liberated from the sample and the reagent was measured at 520 nm on double beam spectrophotometer.

(6) Stomatal studies :

Stomatal studies were performed at 12 noon on the stipulated date in each month from November to August. The stomatal behaviour was investigated with the help of LiCoR autoporometer, from three leaves at different positions on the plant and average of the three readings was taken and mean transpiration rate was determined.

(7) Study of Influence of artificial irrigation :

In summer months like April and May a marked collapse of the plants of all the three species was noticed (Fig.5). At this stage we thought it worthwhile to artificially water few plants from each species and 5 litres tap water was given to each plant on every alternate day. The analysis of artificially watered plants and naturally water stressed plants with respect to leaf moisture and leaf proline was carried out according to methods described earlier. In order to understand whether such watering is beneficial for restoring normal metabolic activity in plants, further in the watered and stressed plants of these three species the activity of a key enzyme of nitrogen assimilation, nitrate

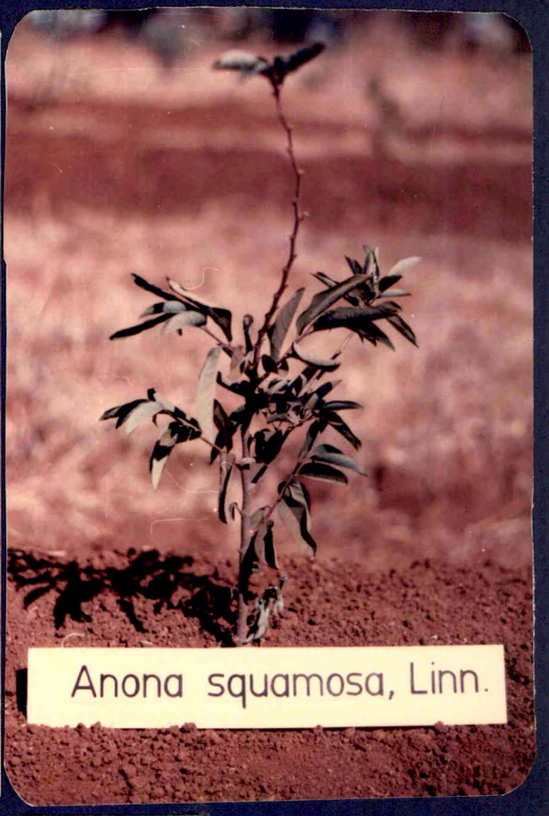


Fig. 5

**The Plant species suffering from water stress in field conditions.**

reductase was studied using in vivo method of Jaworski (1971).

For determination of nitrate reductase activity, the in vivo method of Jaworski (1971) as described by Knypl (1974) was followed.

Small leaf discs were cut with the help of leaf punch. Five hundred milligram of leaf material was suspended in 10 ml. of a standard incubation medium containing 0.1 M Phosphate buffer (pH 6.2), 20 ml. $KNO_3$, 5% (V/v), n-propanol and 1.25% Triton X-100) in test tubes. The test tubes were sealed and incubated in the dark for 60 minutes. Nitrate reductase activity was measured by estimating $NO_2$ production which was detected by treating 0.4 ml. of the incubation mixture with 0.3 ml of 1% sulfanilamide in 2N HCl and 0.3 ml of 0.02% N - (1-Naphyl) ethylene diamide hydrochloride. After 20 minutes the solution was diluted to 4 ml with distilled water and absorbance was read at 540 nm, on Shimadzu double beam spectrophotometer.

The amount of nitrite released due to enzyme was estimated from a standard curve of sodium nitrite prepared in a similar manner.

The values of various inorganic and organic constituents, environmental parameters, transpiration rate and enzyme activity depicted in the Chapter 'Results and Discussion' represent average of three independent determinations.