CHAPTER-II

MATERIAL AND METHODS

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A) Plant Material :

(i) Cultivation of Agave plants :

The plants of Agave cantala were raised in the pots as well as in garden soil by using bulbils for the growth studies. However the growth being very slow, only growth pattern was studied in these plants. For the well development Agave requires about 4 to 5 years of period.

To overcome this difficulty, during present investigations the Agave plants raised by the farmers as hedge plants in Kolhapur and Shivnagar were used. About 10 to 15 plants of same age (5 years maturity) were selected. These plants were provided with adequate amount of water in specific intervals. The required plant material i.e. leaves were taken from these selected plants and used oftenly during experimental studies.

(ii) Sampling Method :

From healthy and insectfree Agave plants young (developing, upper) leaves, mature (fully expanded, middle and green) leaves and senescent (old, yellowing and basal) leaves were carefully selected from identical positions, in three seasons namely summer, rainy and winter. The selected leaves were first washed and cleaned with tap water following with distilled water. Then they were blotted dry and subse-

quently cut into small pieces. These pieces were mixed randomly and this leaf material was taken for the different analytical experiments as follows.

The metrological readings such as soil moisture, maximum and minimum temperature, humidity, relative humidity, evaporation, soil temperature at various depths, light intensity were also recorded at the time of each harvest(Table No.1).

To determine soil moisture percentage, the soil was collected from the root zone of selected plants of Agave cantala. The collected soil was mixed thoroughly and known amount of soil (10g) was kept in oven at 70°C for 8-10 days for drying, till a constant weight is obtained. The loss in weight per 100 g was expressed as moisture percentage.

B) Methods

1. Inorganic Constituents :

(i) Preparation of acid digest :

The plant material mentioned above was kept in oven at 70°C for 8 days till a constant weight was obtained. The oven dried material was powdered.

For the estimation of different inorganic constituents an acid digest was prepared following the method of Toth, et al. (1948). Five hundred mgs of ovendried powdered material was

Table No.1

Environmental parameters during the experimentation period in three seasons of the year

Sr. No.	Environmental parameter		Summer season	Rainy season	Winter season
1.	Maximum Temperature ^{o}C		34.5	26.6	31.5
2.	Minimum Temperature ^{o}C		20.7	18.3	11.3
3.	Light Intensity $\mu E\ m^{-2}\ Sec^{-1}$		1859	226.4	98.3
4.	Humidity percentage		76.0	88.0	91.0
5.	Relative Humidity percentage		31.0	49.1	45.0
6.	Evaporation		10.0	2.7	4.2
7.	Soil Moisture percentage		6.8	19.76	16.8
8.	Soil Temperature At Various Depths ^{o}C	5 cm	28.5	23.5	18.7
		10 cm	29.8	23.4	22.6
		20 cm	32.6	28.8	26.3

transferred to 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 reactions subside. It was then heated slowly to dissolve solid particles. 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 (about 2-3 ml) was obtained. While heating, the liquid was not allowed to dry. It was then cooled and transferred 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 constituents.

(ii) Estimation of sodium, potassium, calcium, magnesium and iron :

Sodium and potassium were estimated flame photometrically following the procedure standardized in our laboratory. Stock solutions of known concentrations in parts per million (ppm) of Na in NaCl(1 to 10 ppm), K in KCl (10 to 50 ppm) were used for calibration curves. From these calibration curves the concentrations of Na and K in the acid digested samples were calculated.

Calcium, magnesium and iron were estimated with the help of computarised atomic absorption spectrophotometer in

the Common Facility Instrumentation Centre of Shivaji University, Kolhapur.

2. Organic Constituents :

(i) Moisture percentage of leaf :

The young, mature and senescent leaves were taken as described earlier from the selected plants. The leaves were washed, blotted dry and cut into small pieces. These pieces were thoroughly mixed and 10 g leaf material was kept in oven at 70°C for drying, till a constant weight was obtained. The loss in weight per 100 g was expressed as moisture percentage.

(ii) Organic acid status (Titratable Acid Number) :

Titratable Acid Number (TAN) was estimated by the method of Thomas and Beevers (1949). TAN was estimated from young, mature and senescent leaves in each season. The fresh leaf material was cut in to small pieces and weighed amount (2 g) was immersed in boiling water and boiled for half an hour. It was cooled and filtered through cheese cloth. The final volume of filtrate was made to 50 ml with distilled water. This served as plant extract for determination of TAN. It was titrated against N/40 NaOH using phenolphthalein as an indicator. NaOH was standardized against N/40 oxalic acid using the same indicator. Titratable Acid Number (TAN) represents the number of ml of decinormal NaOH required to neutralize the acid present in 100 g of fresh tissue. It was estimated

by applying following formula :

$$TAN = \frac{\text{Volume of oxalic acid taken for titration}}{\text{Oxalic acid titration reading}} \times \frac{\text{Total volume of extract}}{\text{Wt. of plant material in g}} \times \frac{\text{Extract titration reading}}{\text{Volume of extract taken for titration}} \times \frac{100}{4}$$

(iii) Carbohydrates :

Carbohydrates were estimated according to the method described by Nelson (1944). These were estimated from young, mature and senescent leaves in each season. Five hundred mgs fresh leaf material was homogenised in mortar with pestle and extracted with 80 % alcohol. It was filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was used for the estimation of soluble sugars while the residue was used for starch determination. The filtrate thus obtained was condensed on a water bath till the volume was about 3 ml and treated with lead acetate and potassium oxalate (1:1) to decolourize it. To this distilled water was added and filtered. It was again washed with distilled water for 2-3 times collecting the washing in the same filtrate. This filtrate was used for estimation of reducing sugars (A). A known volume of this extract was hydrolysed with concentrated HCl in pressure cooker at 15 lbs pressure for half an hour. The contents were cooled, neutralised with Na_2CO_3 and filtered. The filterate

was used for the estimation of total (reducing + non-reducing) sugars (B) .

The residue obtained in the first filtration (alcohol extract) was transferred to a conical flask with 50 ml water and 2.5 ml of concentrated HCl. This was hydrolysed, neutralized and filtered as stated earlier. This filtrate contains reducing sugars produced as a result of hydrolysis of starch. The sugars so available were estimated to determine the starch present in the tissue (C).

The requisite quantity (preferably 0.1 ml) of the above filtrates A, B and C was taken separately in 10 ml marked test tubes. In other such test tubes different concentrations (0.1, 0.2, 0.3, 0.4 ml) of standard glucose solution (0.1 mg ml^{-1}) were taken. One ml of Somogyi's alkaline copper tartarate solution [4g $CuSO_4$, $5H_2O$; 24 g unhydrous Na_2CO_3; 16 g Na, K, tartarate (Rochelle salt) and 180 g unhydrous Na_2SO_4 dissolved in 1000 ml distilled water] was added to each test tube. All the reaction mixtures were subjected to boiling water bath for about 10 minutes. 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 3g sodium arsenate, $NaHASO_4$, $7H_2O$; dissolved in 25 ml distilled water. All ingradients were mixed well and the solution was placed in an incubator at 37°C for 48 hours before use] was

added to each reaction mixture. The contents of each test tube were then diluted with distilled water to a volume (10 ml.). A blank was prepared by the same way but without sugar solution. After 10 minutes the absorbance of reaction mixture was read at 560 nm.

With the help of glucose standard curve, the amounts of these carbohydrate fractions were determined.

(iv) Total Polyphenols :

Polyphenols were estimated according to method of Folin and Denis (1915); from young, mature and senescent leaves, in each season. Polyphenols were extracted from fresh leaf material in 80% acetone (30 ml). Extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. The phenolics were extracted repeatedly from the residue with acetone. The volume of the filtrate was made to 50 ml with acetone. One ml filtrate was taken in a 50 ml marked nesselor's tube. In other such tubes different concentrations (0.5, 1, 2, 3, 4ml) of standard polyphenol solution (tannic acid, 0.1 mg ml^{-1}) were taken. Ten ml. of 20% Na_2CO_3 were then added to each tube to make the medium alkaline. Two 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 %). This was refluxed for $2\frac{1}{2}$ hrs, 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 distilled water. A blank was prepared without polyphenols. The ingradients were allowed to mix thoroughly. After some time the optical density of each mixture was read at 660 nm on Shimatzu double beam Spectrophotometer. Polyphenols were calculated from the calibration curve of standard tannic acid.

(v) Proline :

The free proline contents were estimated by the method of Bates et al. (1973). Proline contents were estimated from young, mature and senescent leaves in each season. In this method 3% sulfosalicylic acid was used as an extraction medium. The acid ninhydrin reagent was prepared by dissolving 1.25 g ninhydrin powder in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid, it was slightly warmed up and stirred until dissolved. This acid ninhydrin reagent was stored for 24 hours in the refrigerator. Each time fresh reagent was prepared.

(a) Five hundred mgs oven dried powdered plant material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid. This homogenate was then filtered through Whatman No.2 filter paper.

(b) Two ml of the filtrate was mixed with 2 ml of acid

ninhydrin and 2 ml of glacial acetic acid in a test tube. The test tubes were heated in water bath (100°C) for 1 hr. and then reaction was terminated by dipping it in an ice bath.

(c) The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 15-20 seconds.

(d) The chromophore containing toluene was aspired from the aqueous phase and 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.

(vi) Pigments

(a) Chlorophylls :

Chlorophylls were estimated following the method of Arnon (1949). Fresh plant material (young, mature and senescent leaves) was used for estimation of chlorophylls in each season. Chlorophylls were extracted in 80% chilled acetone from 0.5 g of fresh leaf material in dark. This extract was filtered through Whatman No.1 filter paper using Buchner's funnel. Residue was washed repeatedly with 80% acetone, collecting the washings in the same filtrate. The volume of the filtrate was made to 50 ml with 80% acetone. The absorbance was read at 663 and 645 nm for chlorophylls a and b respectively.

Chlorophylls (mg 100 g^{-1} fresh tissue) were calculated 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

Total Chlorophylls = (8.02 X A 663) + (20.2 X A 645)... Z

$$\text{Chlorophyll a or Chlorophyll b or Total Chlorophylls (mg 100g}^{-1}\text{ fresh tissue)} = \frac{\text{X/Y/Z X volume of extract X 100}}{\text{1000 X weight of material (g)}}$$

(b) Carotenoids :

Carotenoids were estimated by reading the absorbance of acetone extract of leaf samples at 480 nm (Kirk and Allen, 1965).

Total carotenoids were estimated using the formula of Liagen-Jensen and Jensen (1971) :

$$C = D \times V \, f \, \frac{10}{2500}$$

where C = Total carotenoids in mg 100 g^{-1} fresh tissue.

D = Optical density

V = Total volume of extract in ml.

f = dilution factor

2500 = Average extinction.

3. Study of stomatal behaviour in Agave leaves during different seasons :

The stomatal behaviour with respect to diffusion resistance for water vapour and transpiration rate from the abaxial and adaxial surfaces of young, mature and senescent leaves of Agave cantala was studied with the help of steady state autoporometer (LI-COR INC USA). These readings were recorded at 12-00 noon from leaves at identical positions during different seasons.

4. Crassutacean Acid Metabolism Studies :

1) Study of diurnal fluctuations in Titratable Acid Number (TAN) during different seasons :

Seasonal variations in diurnal fluctuations in titratable acidity in the mature leaves of Agave cantala were studied. For this purpose the leaf material from the same plant was sampled at 6-00 a.m., 12-00 noon, 6-00 p.m. and 12-00 night and TAN was estimated according to the method described earlier.

ii) Study of diurnal fluctuations in Carbohydrates during different seasons :

The diurnal fluctuations in carbohydrate contents during different seasons were studied in mature leaves of Agave cantala at different hours of day (6-00 a.m., 12-00 noon

6-00 p.m. and 12-00 night). The carbohydrates were estimated according to the method described by Nelson (1944) which has been described in details in earlier section.

The values of various organic and inorganic constituents and stomatal parameters, presented in Chapter "Results and Discussion" represent average of three independent determinations.