# CHAPTER - II

# MATERIAL AND METHODS

## *A. MATERIAL*

### 1. ABOUT VARIETY CO.C.671

Number of promising sugarcane varieties are evolved by Sugarcane Research Institute Coimbtoor at Cadloor Research Station, Tamil-Nadu in last 13 years. Among these varieties the highly promising variety at present is Co.C-671., which was evolved by this institute in 1989. This variety was obtained by making cross between varieties Q.63 and Co.775.

VSI (Vasantadada Sugar Institute, Pune) released this variety as a commercially most profitable variety in Maharashtra in October 1994 because of some promising features such as more stalk girth, grater stalk length, good germination percentage, better tillering ratio, high sugar percentage and yield.

Some of important economic characteristics of this variety are as follows :

Brix value - 21.77%, Sucrose - 19.98%,

Puriety - 91.77%, CCS - 13.3%,

Fibre - 12.70% and Yield - 106.7 tone/ha

Further this variety is also extensively employed for Gour production. Hence this variety has been choosen for the present investigation. The plant material was obtained through the courtsey of Gour and Cane Research Station Kolhapur.

Plate No.1 : Sugarcane variety CO.671 growing at natural conditions in field of Kekhale

*B. METHODS*

**1. PRESOWING SOAKING TREATMENT**

Influence of presowing soaking treatment with different chemicals on the seed germination and growth was studied as early as 1918 and 1919 by Kidd and West.

Since then number of chemicals have been employed for treatment of seeds of different crops. These include salt solutions, growth promotors as well as growth retardants. Although in most of the crop species, seed is means of propagation, in sugarcane the stem cuttings popularly known as 'setts' are employed for this purpose.

The sugarcane setts (with two buds) were obtained from the sugarcane field at Nigve Dumala, Taluka - Karveer, Dist. - Kolhapur. Every care was taken to select disease free healthy and vigorously growing plants for this purpose. The healthy setts were sorted out and surface sterilized with water and air dried. In the present experiment for the presowing soaking treatment of sugarcane setts, distilled water, chloro choline chloride (CCC) and ethephon were employed. For giving the treatment the setts of the sugarcane were soaked in solution of above growth regulators. The concentration used was 100 ppm. They were soaked for the period of 20 hours in big enamel trays (2'X 1

1/2' X 4"). The setts were completely submerged in the solution. Then they were air dried at the room temperature till they attained their original weight. The setts were directly planted in furrows of about 12' X 12' in diameter. The untreated setts served as control. Each row was equally watered and every care was taken to raise weed and disease free plants. The experiment was performed in triplicate.

**2. BUD ANALYSIS**

In order to assess the immediate effect of presowing soaking treatment on some metabolic aspects in the sprouting buds, the sugarcane setts from different treatments were uprooted after 8 days and the buds were carefully isolated. The hard cover on the buds was removed and the bud tissue was employed for further biochemical analysis.

**a. Enzymes**

i) Nitrate reductase (E.C. 1.6.6.2)

Activity of nitrate reductase was determined following the *in vitro* technique developed by Jaworski (1971). The buds were washed with distilled water, cut into small pieces and then immediately weighed and incubated in the medium containing 1 ml 1 M $KNO_3$, 2 ml 5% n-propanol, 5ml 0.2 M phosphate buffer (pH 7.5) and 2 ml 0.5% Triton X-100 in sealed tubes for one hour in dark. After incubation

period 1 ml reaction medium was taken out and mixed with 1 ml 1% sulphanilamide in 1 M HCl and 1 ml 0.02% NEEDA (N-1, Naphthylene diamide dihydrochloride). The absorbance was read at 540 nm on Shimadzu double beam spectrophotometer UV-190.

Standard curve of nitrite 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 sulphanilamide and 1 ml NEEDA was used as blank.

The activity of NR is expressed on fresh weight basis in terms of $NO_2$ liberated $g^{-1}$ bud tissue $h^{-1}$.

ii) Peroxidase (E.C. 1.11.1.7)

The method of Maehly (1954) was employed for assay of enzyme peroxidase. Enzyme was extracted by homogenizing 0.5 gm bud tissue in cold distilled water in chilled morter and pestle. The suspension was filtered through four layered cheese cloth and the filtrate was centrifuged at 10,000 g for 20 minutes in refregerated centrifuge. The supernatant was used as enzyme source.

Enzyme assay mixture was prepared by adding 2 ml of 0.1 M phosphate buffer (pH-7), 1 ml 20 mM guicol and 1 ml

enzyme. The reaction was initiated by adding 0.04 ml of 10 mM $H_2O_2$ and the changes in optical density due to oxidation of gulcol were recorded at every 30 second for 2 minutes at 470 nm with frequant stirring of reaction mixture described earlier. The soluble proteins in enzyme extract were estimated according to method of Lawry et al. (1951). The enzyme activity is expressed as OD/min/mg protein.

iii) Catalase (E.C. 1.11.1.6)

The method of Herbert (1955) was followed for the study of enzyme catalase. The enzyme is extracted from fresh bud tissues in cold double distilled water. It was then filtered through moist double layered cheese cloth and filtrate so obtained was centrifuged at 10,000 rpm for 10 minutes at 9° to 4°C and supernatant was used as enzyme source.

The assay mixture was prepared by mixing 1 ml of 0.045 M $H_2O_2$ in phosphate buffer (pH-6.8) and 1 ml of aliquat of enzyme. Assay mixture was incubated at room temperature for 1 minute and then the reaction was terminated by the addition of 5 ml of $H_2SO_4$ (5 N). For 0 minute the reaction was stopped before addition of enzyme by 5 N $H_2SO_4$. To this 1 ml of 10% KI solution and a drop of 2% ammonium molybdate was added. The liberated iodine was titrated against 0.01 M sodium thiosulphate using starch indicator. The difference in titraton reading between 0 and

1 minute reaction mixtures was taken as measure of enzyme activity. The soluble proteins in the enzyme extract were estimated according to the method of Lawry et al. (1951) described earlier. Activity of enzyme is expressed as mg $H_2O_2$ broken down/min/mg protein.

iv) Dehydrogenase (E.C. 1.1.1.4)

For the study of dehydrogenase from buds of sugarcane setts the tetrazolium method of Kittoch and Law (1957), was employed. The buds were seperated carefully from setts and cut into the uniform pieces. These pieces were incubated for 4 hours in 4 ml 0.2% 2-3-5 Triphenyl tetrazolium chloride (TTC) solution in dark. After this bud pieces were washed 2-3 times with distilled water, surface dried and were treated with 5 ml of 2-methoxyethanol for extraction of red coloured formazon, which is formed due to the activity of dehydrogenase. The optical density of coloured formazon was measured at 470 nm. The enzyme activity is expressed as OD/hr/g bud tissue.

v) Soluble Proteins

Soluble proteins were estimated from the bud tissue by the method described by Lowry et al.; (1951). Bud tissue was homogenized in 0.1 M phosphate buffer (pH-7) and filtered through moist musline cloth and centrifuged at 5000 rpm, 0.5 ml of supernatant was diluted to 1 ml with

distilled water and 5 ml of alkalike copper solution prepared by mixing 50 ml of reagent a (2% $Na_2CO_3$ in 0.1 N aqueous NaOH) with 1 ml of reagent b (0.5% $CuSO_4$ 5 $H_2O$ in 1% sodium tartarate) was added to it. It was kept for 15 minutes at room temperature. 0.5 ml of Folin phenol reagent were added to it and it was kept for 30 minutes. Absorbance was read at 660 nm. Blank was prepared with distilled water instead of standard protein solution. The soluble proteins were determined with the help of a standard curve prepared on the basis of different concentrations of bovine serum albumin.

**3. GERMINATION STUDIES**

For the study of germination percentage, the germination percentage was recorded after 30th day of sowing. The emergence of young shoot was considered as criteria for germination.

In order to study effect of presowing soaking treatment on root growth, the setts were uprooted after 8 days of sowing and the length of roots from the point of emergence at the two nodes and the fresh weight of root mass was recorded.

**4. GROWTH AND YIELD STUDIES**

The height of the sugarcane plants raised from

variously pretreated setts was recorded. The total leaf area and thickness of 4th leaf with the help of micrometer (Mitrtoya No.2046-08) was also recorded.

The sugarcane variety Co.671 is generally harvested after 10 months. Hence after completion of 10 months growth the canes from each treatments were harvested and studied various yield parameters like length of cane, number of internodes, length of internode, diameter of cane and weight of each cane. The sucrose percentage was also determined with the help of Hand Refractometer (ERMA OPTICAL WORKS, LTD TOKYO, JAPAN) as a brix value.

**Sucrose Percentage**

Sucrose percentage in the cane juice obtained from plant subjected to different treatments was determined with the help of ERMA hand refractometer.

ERMA hand refractometer was discovered by ERMA OPTICAL WORKS, LTD, TOKYO, JAPAN. It employs the principle of total reflection to measure the refractive index. The scale shows the percentage of sugar density instead of the refractive index enabling immediate reading of desired result. ERMA hand refractometer is a choise instrument for speedy and accurate results and its reliability has been proved in many fields.

Rotate eyepiece knob and adjust eyepiece to focus clear image on scale. Put a drop of distilled water on stage of refractometer and observe the reading. It must be adjusted to zero. Now put one or two drops of juice of each treatment on the stage seperately and close the cover lightly. Look into eyepiece with projection inlet facing the light and the boundary line appears. Read the measurement of the point where the boundary line coincide with the scale for desired percentage of sugar content.

**5. PLANT ANALYSIS**

**a. Inorganic constituents**

i) Preparation of acid digest

The leaves from identical positions in the plants subjected to different treatments were brought to laboratory, washed throughly with distilled water. They were blotted to dry and cut into small segments. The leaf material was oven dried at 60°C for 8 days. The oven dried leaf material was throughly ground so as to yield a homogenous powder. For the estimation of different inorganic constituents an acid digest was prepared following the method of Toth et al. (1948). 500 mg of ovan dried powdered material was transfered to 150 ml capacity beaker to which 20 ml concentrated $HNO_3$ were added. The beaker was covered with watchglass and was kept till the primary reactions

subsided. It was then heated slowly to dissolve solid particals. After cooling to room temperature, 10 ml of perchloric acid (60%) were added to it and mixed throughly. 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 transfored quatitatively 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) filterpaper and filtrate was used as the source of different inorganic constituents.

ii) Phosphorus

Phosphorus was estimated according to the method of Sekine et al. (1965). 1 ml of leaf extract was pipetted in a test tube to which 2 ml of 2 N $HNO_3$ were added followed by 1 ml of molybdate vanadate reagent (A = 1.25 g of ammonium vanadate were dissolved in 500 ml 1 N $HNO_3$, B = 25 g of ammonium molybdate in 500 ml distilled water. The A and B were mixed in equal volumes). The volume was made to 10 ml with distilled water. The reaction mixture was shaken well and kept for 20 minutes. The yellow colour developed with molybdate vanadate reagent was measured calometrically at 420 nm using reagent blank compared with the optical density of known standards of phosphorus (Standard 'P' solution was prepared by dissolving 0.11 g of monobasic potassium

phosphate in distilled water and by adjusting the volume to 1 liter. This solution contained 25 ppm phosphorus) and the amount of phosphorus in the plant material was calculated.

iii) Calcium, Magnesium, Iron Manganese, Zinc, Copper and Cobalt

The inorganic elements like Calcium, Magnesium, Iron, Manganese, Zinc, Copper and Cobalt were estimated using Atomic Absorption Spectrophotometer (Perkin Elmer 3030).

**b. Organic constituents**

i) Carbohydrates

The soluble carbohydrates were extracted from 0.5 g oven dried plant material with 80% neutral alcohol. The extract was filtered through Buchner funnel using Whatman No.1 filter paper. The insoluble residue was reserved for estimation of starch. The filtrate thus obtained was condensed on water bath to about 2-3 ml. To this one gram each of lead acetate and potassium oxalate were added for decolorization. After decolorization 20 ml distilled water was added and aliquat was filtered. Final volume of filtrate was made to 50 ml with distilled water and it served as an extract for determination of reducing sugars. A known volume of this extract (20 ml) was hydrolysed with 2 ml conc. HCl by autoclaving at 15 lb atm. pressure for half an hour. The

content was cooled, neutralized with anhydrous sodium carbonate and filtered. The volume of filtrate was measured and this filtrate was used for the estimation of total (reducing and non-reducing) sugars.

For estimation of starch, the insoluble residue along with the filter paper obtained at the beginning after filtering the alcoholic extract was transferred to a 100 ml conical flask with 50 ml distilled water and 5 ml concentra-tion HCl and hydrolyzed at 15 lbs atm. pressure for half an hour. These conical flasks were cooled to room temperature, neutralized by addition of unhydrous sodium carbonate and filtered. The volume of filtrate was measured as this contains reducing sugars (glucose) formed as a result of hydrolysis of starch. These sugars represents the starch content in the residue.

The reducing sugars (glucose equivalents) present in the three extracts were estimated with the help of dinitrosalicylic acid (DNS) reagent (Miller, 1972).

For preparation of DNS reagent 1g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite were dissolved with continuous stirring in 100 ml 1% NaOH and stored. 1 ml cooled sample (extract) were taken in test

tube with 2 ml DNS reagent and placed in boiling water bath for about 5 minutes. Then the sample was cooled in cold water bath for about 3 minutes. Then 1:10 dilution was made of the coloured sample and absorbance was read at 530 nm on spectrophotometer.

Using caliberation curve of standard glucose, the glucose equivalent percentage in above three fractions were calculated. Values expressed as g $100^{-1}$ g dry tissue.

ii) Total nitrogen

Total nitrogen from leaves of sugarcane subjected to different treatments was estimated according to method of Hawk et al. (1948). 1g of oven dried, powdered plant material was transfered to Kjeldahl flask with a pinch of microsalt (200 g $K_2SO_4$ + 5 g $CuSO_4$, dehydrated) and to it 5 ml of $H_2SO_4$ (1:1) were added. Few glass beads were added to avoid bumping and material was digested on blue flame till it become yellow in colour. The flask was then cooled to room temperature. 15 to 20 ml distilled water were added and with thorough shaking the content were filtered. This was reported two to three times and the volume of filtrate was made 100 ml.

In very clean and dry Nessler's tube 1 ml of plant extract and different concentrations of standard ammonium

sulphate solution (0.236 g of ammonium sulphate dissolved in water and few drops of $H_2SO_4$ were added. The volume is made 1000 ml. This solution contains 0.05 mg of nitrogen per ml). In blank tube 1 ml distilled water was taken. To this drop of 8% $KHSO_4$ was added and volume was made 35 ml with distilled water. To this 15 ml Nessler's reagent were added (Reagent A : 7 g KI + 10 g $HgI_2$ in 40 ml distilled water. Reagent B : 10 g NaOH in 50 ml distilled water. A and B were mixed in proportion of 4:5 at the time of estimation). After 15 to 20 minutes the absorbance was read at 520 nm. Amount of nitrogen was calculated from the standard curve.

iii) Titratable acid number (TAN)

Titratable acid number (the number of ml of decinormal NaOH required to neutralise the acid content in 100 g of fresh tissue) was estimated according to the method described by Thomas and Beevers (1949). Fresh leaves from identical positions of each treatment were taken, washed with distilled water and blotted to dry. The leaves were cut into small pieces of uniform size. 2 g leaf pieces were transferred to 40 ml distilled water in 150 ml beaker and boiled for half an hour on hot plate. It was then allowed to cool at room temperature and filtered through muslin cloth. The volume of filtrate was made 50 ml with distilled water.

10 ml of filtrate was taken in conical flask and

titrated against N/40 NaOH using 3-4 drops of phenolpthalein indicator NaOH solution was standerdised against N/40 oxalic acid in the similar manner.

Titratable acid number (TAN) was calculated by using following formula.

$$TAN = \frac{\text{ml of oxalic acid taken for titration}}{\text{Oxalic acid titration reading}} \times \frac{\text{Extract titration reading}}{\text{ml of extract taken for titration}} \times \frac{\text{Volume of extract}}{\text{weight of plant material (g)}} \times \frac{100}{4}$$

iv) Ascorbic acid content

Ascorbic acid content in plant leaves was estimated following the method of Aberg (1958). The material was washed and cleaned with distilled water and immediately blotted to dry. It was weighed accurately (3 g) and cut into small pieces. Then pieces were placed in morter and oxalic acid (0.4% w/v) was added at the rate of 4 ml/g tissue. The plant material was throughly crushed for about 5 minutes and the extract was filtered through two layered cheese cloth. Then it is centrifuged at 1000 g for 20 minutes. The volume of supernatant was made to 15 ml (1 g tissue in 5 ml oxalic acid) with oxalic acid reagent (0.4% w/v). Ascorbic acid in extract was estimated by visual titration method based on reduction of 2,6 dichlorophenol indophenol dye.

Standard ascorbic acid solution was prepared by dissolving 50 mg of ascorbic acid in 50 ml of 0.4% oxalic acid solution in 250 ml volumetric flask and finally volume was made to 250 ml with oxalic acid (1 ml of solution contains 0.2 mg of ascorbic acid).

Indophenol reagent was prepared as follows. 150 ml of glass distilled water was added to 200 ml volumetric flask. Then 50 mg of sodium 2,6-dichlorophenol indophenol were added to it. Then the flask was gently heated in a hot water bath to dissolve the dye. The 42 mg of $NaHCO_3$ were added. The flask was allowed to cool. After cooling volume of solution was made to 200 ml with glass distilled water and the reagent was stored in dark glass bottle at 2°C for standerdization of indophenol reagent. 5 ml ascorbic acid solution were taken in white porcelain dish and then titrated against the indophenol dye untill the solution become pink (the pink colour persisted for at least 15 seconds).

After standerdization of indophenol reagent 5 ml of plant extract was titrated against standerdized indo-phenol reagent in the similar manner.

The ascorbic acid content of the extract was calculated by using formula,

$$I \times S \times \frac{D}{A} \times \frac{100}{W}$$

mg of ascorbic acid $100^{-1}$ g fresh tissue

where,

I = ml of indophenol reagent used in titration.

S = mg of ascorbic acid reacting with 1 ml of the reagent.

D = Volume of extract in ml.

A = The aliquot titrated in ml.

W = The weight of sample in gms.

The value of ascorbic acid so obtained was further corrected on the basis of moisture content in the fresh tissue and expressed on dry weight basis.

v) Total polyphenols

Total polyphenols were estimated following the method of Folin and Denis (1915). They were extracted from fresh material in 80% acetone (30 ml). Extract was filtered through Whatman No.1 filter paper using Buchner's funnel. Polyphenols were extracted repeteadly from the residue. The volume of filtrate was made to 50 ml with 80% acetone. 0.5 ml of filtrate was taken in a 50 ml marked Nessler's tube. In other such tubes different concentrations (0.5, 1,2,3,4 ml) of standard polyphenol solution (tannic acid 0.1 mg

$ml^{-1}$/dry weight) were taken. 10 ml of freshly prepared 20% $Na_2CO_3$ were 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 double distilled water were mixed with phosphoric acid (25%). This was refluxed for $2\frac{1}{2}$hours, cooled to room temperature and diluted to 1 litre with double 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 polyphenolics. The ingredients were allowed to mix thoroughly by shaking the tubes. After some time the optical density of each mixture was read at 660 nm. Polyphenols were calculated with the help of the calibration curve of standard tannic acid.

**c. Photosynthetic Pigments**

i) Chlorophylls

Chlorophylls were estimated following the method of 'Arnon (1949). The leaves from identical positions in the plants subjected to different treatments were brought to laboratory, washed with distilled water and blotted to dry. They were cut into small segments with razor and 0.5 g leaf material was accurately weighed. Chlorophylls were extracted in 80% chilled acetone in dark. This extract was filtered through Whatman No.1 filter paper using Buchner's funnel. Residue was washed repeatadly with 80% acetone collecting

the washings in the same filtrate. The volume of filtrate was made 50 ml with 80% acetone. The absorbance was read at 663 and 645 nm for chlorophylls a and b respectively.

Chlorophylls (mg $g^{-1}$ 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

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

ii) Carotenoids

Carotenoids were estimated by reading the absorbance of acetone extract at 480 nm (Kirk and Allen, 1965). Total carotenoids were estimated using the formula of Liaasen-Jensen and Jensen (1971).

$$C = D. \text{ X } V.F \text{ X } \frac{10}{2500}$$

where,

C = Total carotenoids in mgs.

D = Optical density.

V = Total volume in ml.

F = dilution factor and

2500 = average extinction.

iii) Chlorophyll stability index (CSI)

Chlorophyll stability index (CSI) of sugarcane plant leaves which were subjected to different treatments was determined with the help of Murthy and Majumdar method (1962). In this method 0.5 g leaf material was taken. It was boiled for 30 minutes in distilled water. Chlorophyll from this boiled material is determined by using the method of Arnon (1949), and it is compared with chlorophyll content of fresh unboiled leaf material at each treatment. Chlorophyll stability index (CSI) is determined by using following formula,

$$CSI = \frac{\text{Chlorophyll content of boiled sample}}{\text{Chlorophyll content of unboiled (fresh) sample}}$$

The values of various inorganic and organic constituents depicted in the following chapter Results and Discussion represent average of three independent determinations.