CHAPTER II

MATERIALS AND METHODS

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(1) Plant Material

The present investigations were carried out using :

(a) Callus tissues derived from the floral buds of *Nicotiana tabacum* L. var. Anand-2; and

(b) callus tissues derived from the wall of the anthers of *Gossypium hirsutum* L., cv. Sankar-5.

Floral bud callus of tobacco as well as the anther callus of cotton was initiated on Murashige and Skoog's (MS) medium (1962) supplemented with 2 mg/l each of indole-3-acetic acid (IAA), naphthalene acetic acid (NAA) and kinetin (KN). Both the tissues were later on transferred and maintained on the same medium, subculturing after 30 day cultural cycles.

(2) Chemicals

The chemicals used for the investigation were of high purity grade and were obtained from

1) Sigma Chemical Company, U.S.A.
2) British Drug House, England (Analar grade)
3) E. Merck, Germany (Guaranteed reagents) and
4) Sarabhai Merck, India.

Agar agar powder was purchased from Centron Research Laboratories, India or Sisco Research Laboratories, India.

(3) Culture vessels

The glasswares made of corning or pyrex glass were used during the course of experimentation. Before use they were cleaned by the following procedure :

The glassware was treated with 40% chromic acid overnight, after which the acid was completely washed off by thorough rinsing in tap water. Later the glassware was cleaned with 5% solution of detergent Teepol and then washed with jets of tap water. The glassware was finally rinsed with double glass distilled water, drained and dried overnight in an oven at 60° before use in the experiments.

Erlenmeyer flasks of 100 or 150 ml capacity served as culture vessels for the experiments. The flasks used were cleaned by the above procedure.

(4) Culture Medium

(A) Composition and preparation :

The composition of the culture medium employed in the investigation is shown in Table 1. Separate stock solutions were prepared according to the composition of the nutrient medium in glass distilled water. Stock solutions were stored

Table : 1. Murashige and Skoog's (1962) Modified Medium.

Stock Soln.	Constituents	Conc. in stock soln. gm/l	Vol.of stock soln. in final medium ml/l	Final conc. in medium mg/l
A.	NH_4NO_3	82.50	20	1650.00
B.	KNO_3	95.00	20	1900.00
C.	H_3BO_3	1.24		6.20
	KH_2PO_4	34.00		170.00
	KI	0.166	5	0.83
	$Na_2MoO_4.2H_2O$	0.05		0.25
	$Cocl_2.2H_2O$	0.005		0.025
D.	$Cacl_2.2H_2O$	88.00	5	440.00
E.	$MgSO_4.7H_2O$	74.00		370.00
	$MnSO_4.4H_2O$	4.46	5	22.30
	$ZnSO_4.7H_2O$	1.72		8.60
	$CuSO_4.5H_2O$	0.005		0.025
F.	Na_2EDTA	7.40	5	37.35
	$FeSO_4.7H_2O$	5.57		27.85
G.	Thiamine HCl	0.02		0.10
	Nicotinic Acid	0.10	5	0.50
	Pyridoxin-HCl	0.10		0.50
	Glycine	0.40		2.00

Addendum : Sucrose 20 gms/l, myo-inositol 100 mg/l.
The stock solutions A-G were prepared and stored in a refrigerator (never more than 4 weeks) and mixed, while preparing the final medium.
pH of the medium adjusted to 5.6-5.8.

in a refrigerator at 5-10°. Whenever hydrates of any salts were used, appropriate corrections were made.

The constituents of the medium were added in the order shown in Table 1. All supplements to the basal medium were added prior to the final adjustment of volume. After making the volume, pH of the medium was measured and adjusted to 5.7 with 1.0 N sodium hydroxide or 1.0 N hydrochloric acid (as found necessary) using an Elico pH Meter (Hyderabad). The medium was solidified with 0.8% (w/v) Centron or 0.6% (w/v) Sisco agar.

(B) Sterilization of medium and culture vessels

After adding agar, the medium was heated gently with constant stirring, till the agar dissolved. Later 40 ml of the medium was transferred to each of the culture vessels. The mouth of the culture vessels was closed immediately with non-absorbant cotton wool plugs wrapped with a double layered gauze. Brown papers were tied around the cotton plugs to protect them from condensed water during autoclaving. The medium and glassware were sterilized by autoclaving at a pressure of 15 psi for 20 minutes.

(5) Aseptic techniques

(A) Inoculation chamber

All aseptic manipulations were carried out in sterile

inoculating cabinet. The working table was rubbed with 5% solution of detergent Teepol, washed with water, dried and finally cleaned with 4% formaldehyde. The interior of the chamber was saturated with an aerosol of 2% thymol and 2% glycerine in 90% ethyl alcohol. Finally the cabinet was exposed to UV radiation germicidal tube (λ =2537 A°) for about 90 minutes before use to avoid contamination.

Anatomical scissors, scalpels, spatula, needles, and forceps used in aseptic operations were sterilized by flaming with absolute alcohol inside the cabinet.

(B) Surface sterilization of plant material

The flower buds of Gossypium hirsutum L. cv. Sankar-5 and Nicotiana tabacum L. var. Anand-2 were collected from the plants grown in the Botanical garden of the M. S. University of Baroda. The flower buds were thoroughly washed with tap water to remove adhered dust and stickiness. After rinsing with double glass distilled water, the buds were surface sterilized with 80% ethanol for 1 minute and then with 0.1% (w/v) mercuric chloride for 5 minutes. The flower buds were finally washed, copiously with sterile glass distilled water.

(C) Culture technique

i) Initiation of flower bud callus from tobacco

The flower buds sterilized as described above were cut

with a sharp scalpel lengthwise into two halves and planted on solid MS medium containing 2.0 mg/l each of IAA, NAA and KN. The cut surface of the floral bud was kept in contact with the medium.

ii) Initiation of anther callus from cotton

The surface sterilized flower buds of cotton were cut with the help of a scalpel. The anthers were then carefully excised and transferred onto MS medium containing 2% sucrose and supplemented with 2.0 mg/l each of IAA, NAA and KN.

(D) Incubation of the cultures

After inoculation of the explants onto the medium, the culture vessels were incubated in a constant temperature (26±2°) culture room. The cultures were exposed to continuous illumination by day light fluorescent tubes (Philips) at 3000 lux light intensity. Some of the experiments were carried out in total darkness as mentioned elsewhere in the text.

(E) Technique of sub-culture

Callus development occurred in 3-4 weeks and covered the explant completely.

The stock callus cultures were built up by transferring

callus masses regularly, once in every four weeks, to freshly made nutrient medium. These callus masses served as inocula for the experiments.

(6) Measurement of Growth

Growth measurements were made as a function of increase in fresh and dry weights of both tobacco and cotton callus tissues. Five replicate cultures were harvested at fixed intervals of time for growth measurements. Standard error was calculated to ascertain statistical significance of the growth data.

(A) Fresh weights

Five replicate cultures were harvested at fixed intervals of time for growth measurements. The callus mass was removed carefully from each flask and freed from specks of agar which adhered to the tissue. The tissue was sponged lightly with filter paper to remove water which might have accumulated as a consequence of condensation or otherwise. The callus tissue was then transferred onto pre-weighed aluminium foil and the weight determined on a single pan balance (E. Mettler, Zurich) or (K. Roy, Varanasi, India).

(B) Dry weights

Fresh callus masses which were weighed previously were oven-dried at 60° to a constant weight before recording

their dry weights.

(7) Biochemical parameters

During the course of growth and differentiation, the callus tissues of both cotton and tobacco were analysed for :

(A) Soluble total sugars,

(B) Reducing sugars,

(C) Extractable starch, and

(D) Soluble proteins.

The procedures adopted for the determination of each of the above are described :

(A) Extraction and estimation of soluble total sugars by Anthrone method

Anthrone (9-oxyanthracene) method (Yemm et al., 1954) was employed for the extraction and estimation of soluble total sugars. Anthrone has the capacity to react with all sugars giving a characteristic green colour. Soluble total sugars were extracted by grinding 50 mg dry callus tissue in a mortar with 2 ml of hot 80% (v/v) ethanol. The homogenate was centrifuged at 5000 rpm for 15 to 20 minutes. The supernatant was collected and the above process was repeated with the residue. Finally the residue was washed with 2 ml of ethanol and the supernatant was collected after centrifugation. The successive supernatants were combined and the final volume was made to 10 ml.

A suitable aliquot from the above ethanolic extract was diluted to 2.0 ml with glass distilled water. 4 ml of anthrone reagent (0.2 gms of anthrone dissolved in 100 ml of 95% sulphuric acid) was added and the tubes were kept in an ice bath. The contents were mixed thoroughly and placed in a boiling waterbath for 3 minutes and then cooled in ice. The colour intensity of the solution was measured at 620 nm. The extract was omitted during blank preparation. Standard curve was plotted using glucose and the concentrations of soluble total sugars estimated were expressed in terms of milligrams of this compound.

(B) Estimation of reducing sugars by Folin and Malmrose method

After following the extraction procedure as described above, reducing sugars were estimated by the method of Folin and Malmrose (1929). A suitable aliquot from the above ethanolic extract was diluted to 1 ml with distilled water, and then 2 ml of 0.4% potassium ferricyanide ($K_3Fe(CN)_6$) and 1 ml of carbonate cyanide reagent (8 gms of anhydrous Na_2CO_3 dissolved in 40-50 ml of water, and 15 ml of freshly prepared 1% sodium cyanide (NaCN) was added and diluted to 500 ml) were added. The samples were mixed and heated for 8 minutes in a boiling waterbath and then cooled for 2 minutes at room temperature. 5 ml of ferric iron (20 gm of gumghatti was suspended in water in a cheese cloth bag for 24 hours. To

this, a mixture of 5 gm of anhydrous $Fe_2(SO_4)_3$, 75 ml 85% (v/v) phosphoric acid (H_3PO_4) and 100 ml water were added. After mixing thoroughly 15 ml of 1% potassium permanganate was added slowly to destroy reducing materials present in the gumghatti and the solution was allowed to stand for a few days) was added to develop colour. The total volume was made upto 25 ml and the colour which developed was read at 520 nm. Standard curve was plotted using glucose and the concentrations of reducing sugars estimated were expressed in terms of milligrams of this compound.

(C) Extraction and estimation of starch

The starch was estimated by the method of Hassid and Newfeld (1964). The sugar free residue is heated 15 minutes with 1 ml of water in a boiling waterbath in order to gelatinize the starch. The suspension was cooled to room temperature and 1.3 ml of 52% perchloric acid was added while stirring. The tube was kept cool by immersion in a waterbath at about 25°. Stirring was continued for about 5 minutes and thereafter occasionally during 15 minutes. 1 ml of water was added and the mixture was centrifuged. The aqueous starch solution was decanted into a 10 ml volumetric flask. Extraction with perchloric acid was repeated without preliminary heating. The combined extracts and washes were diluted to the mark.

Suitable aliquot of this starch extract was taken in a test tube and the volume was made upto 2 ml with distilled water. 4 ml of anthrone reagent was added, heated for 3 minutes in a boiling waterbath and then cooled at room temperature. The colour of the solution was measured at an absorbance of 620 nm in a colorimeter. This reading was multiplied by 0.9 to determine the amount of starch present in it.

(D) Estimation of soluble proteins

Soluble proteins in the cell-free extracts were estimated by the procedure of Lowry et al. (1951) with bovine serum albumin as standard.

(8) Preparation of Cell-free extracts for the Enzyme Assays

The callus tissues from different flasks were pooled together, weighed and were homogenized in a chilled mortar, using neutral glass powder, 0.1% poly-vinyl polypyroledene (PVP) and 0.1 M phosphate buffer, pH 7.0. The extraction procedures were carried out at 0-4°. The tissue homogenates were then centrifuged at 10,000 rpm for 20 minutes in a refrigerated centrifuge (Model IB-20, International Equipment Co., U.S.A.). The supernatant was collected and volume made upto 10 ml with the same buffer. The supernatants were used for the assay of enzymes and proteins. pH optima,

temperature and substrate concentrations were standardized for each enzyme separately prior to the experimentation.

Enzyme Assays

(A) Amylase (E.C.3.2.1.1) :

$$\text{Starch} \xrightarrow{\text{Amylase}} \text{Maltose.}$$

The enzyme amylase was assayed with modification of the method described by Peter Bernfeld (1955). The assay system consisted of 50 μ moles of acetate buffer pH 5.6; and 4.0; 0.2 and 0.3 ml of 1.5% starch respectively for tobacco and cotton. The reaction was started by the addition of the suitable aliquot of enzyme extract. The blanks were terminated at zero time with 1 ml of 3-5,dinitrosalicylic acid. The final volume of the assay system was 2 ml. The reaction mixture was incubated for 30 minutes at 40°C. Immediately after the incubation of the reaction mixture the reaction was terminated by the addition of 1 ml of 3-5,dinitrosalicylic acid. The reaction mixture was heated in a boiling waterbath for 5 minutes and was cooled. It was then diluted with 10 ml of water. The colour was read in the colorimeter at 520 nm.

Standard curve was plotted using maltose and the concentrations of maltose were expressed in terms of

milligrams of this compound. Enzyme unit was described as the amount of enzyme required to produce 10 mg of maltose per 30 minutes under the conditions of assay. Specific activity was expressed as units per mg of protein.

(B) Invertase (β D Fructofuranoside fructohydrolase) (E. C. 3.2.1.26) :

The name "invertase" was given to the enzymes believed to hydrolyze sucrose to a mixture of glucose and fructose, thus producing an inversion of the sign of the optical rotation from positive to negative.

$$\text{Sucrose} + \text{water} \underset{\longleftarrow}{\overset{\text{Invertase}}{\longrightarrow}} \text{Glucose} + \text{Fructose}.$$

The activity of invertase was assayed according to the method of Bacon (1955). The reaction mixture contained 10 μ moles of phosphate buffer pH 7.0; 1.17 μ moles of the substrate, sucrose. The reaction was started by the addition of the suitable aliquot of enzyme extract. Blanks contained no substrate. The final volume of the assay system was 0.5 ml. The reaction mixture was incubated for one hour at 35°. The reaction was terminated by the addition of 1 ml of Nelson's copper reagent (50 gm of anhydrous sodium carbonate, 50 gm of Rochelle salt, 40 gm of sodium bicarbonate and 400 gm of anhydrous sodium sulphate were mixed in about 1600 ml of water and it was diluted to 2 liters. This solution was stored at

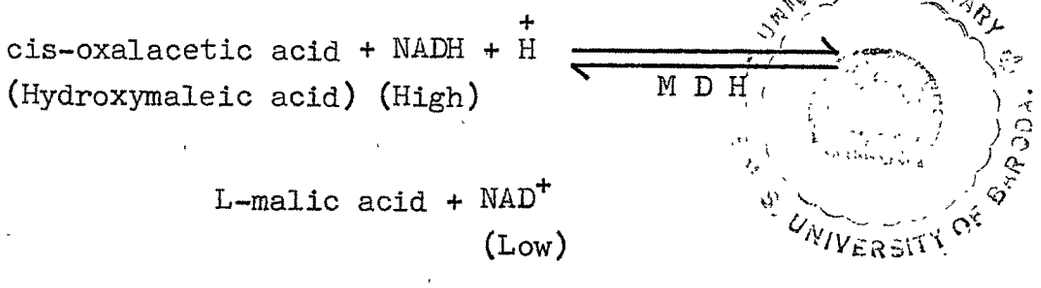
room temperature separately. 150 gm of $CuSO_4 5H_2O$ was dissolved in water and diluted to 1 liter. 0.5 ml of concentrated sulphuric acid was added to it and stored. 96 ml of above solution and 4 ml of $CuSO_4 5H_2O$ solution were mixed before use and 1 ml of this was added to 0.5 ml enzyme system).

The reaction mixture was kept in a boiling waterbath for 20 minutes. It was cooled and then 1 ml of Nelson's arsenomolybdate solution was added to it (Nelson, 1944; Somogyi, 1952). (25 gms of ammonium molybdate $(NH_4)_6 Mo_7O_{24} 4H_2O$ (analytical reagent) was dissolved in 450 ml of water and 21 ml of conc. H_2SO_4 was added and mixed. 3 gm of crystalline disodium arsenate ($Na_2H\ ASo_4 7H_2O$ AR) was dissolved in 2ml of water and added to it. After 48 hours at 37° the reagent was ready for use. The reaction mixture was diluted with 10 ml of distilled water. It was read in colorimeter at 520 nm. A standard curve was plotted using glucose and the concentrations of reducing sugars estimated were expressed in terms of milligrams (mg) of this compound. Enzyme unit was described as the amount of enzyme required to produce 1 mg of reducing sugars per hour (60 minutes) under the conditions of the assay. Specific activity was expressed as units per mg of protein.

(C) <u>NAD - Malate Dehydrogenase (MDH) (E.C. 1.1.37)</u> :

Malate dehydrogenase catalyzes the reduction of

oxalacetate to L-malate in the presence of NADH.



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The enzyme was assayed with modifications of the method described by Ochoa (1955). The assay system in a final volume of 2 ml contained 0.11 and 0.22 μ moles of nicotinamide adenine dinucleotide reduced (NADH), 0.6 and 0.4 μ moles of oxalacetic acid (neutralised), 150 μ moles of phosphate buffer pH 7.2 and 7.4 respectively for cotton and tobacco and suitable enzyme preparation. The mixture was inverted rapidly in a quartz cuvette and the decrease in O.D. as a result of oxidation of NADH was followed spectrophotometrically at 340 nm for 1 minute.

MDH activity was expressed in terms of units per culture. One unit of enzyme activity was defined as the enzyme required to cause a decrease in the optical density by a factor of 1 per minute under the assay conditions. Specific activity was expressed as units per mg of protein.

(D) Glucose-6-phosphate Dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase) (G-6-PDH) (E.C. 1.1.1.49) :

This enzyme catalyzes the reduction of $NADP^+$ by

glucose-6-phosphate according to -

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADPH} + H^{+}.$$

The enzyme was assayed with modifications of the method described by Filner and Klein (1968). The assay system in a final volume of 2 ml contained 0.496 μ moles of Nicotinamide adenine dinucleotide phosphate (monosodium salt) (NADP), 0.85 μ moles of Glucose-6-phosphate (Disodium salt, hydrate), 135 μ moles of tris HCl buffer pH 7.2, 1 μ mole of magnesium chloride, and enzyme preparation. After addition of the enzyme preparation, the mixture was inverted rapidly in a quartz cuvette, and the increase in O.D. as a result of reduction of NADP was followed spectrophotometrically at 340 nm for 1 minute.

G-6-PDH activity was expressed in terms of units per culture. One unit of enzyme activity was defined as the enzyme required to cause an increase in the optical density by a factor of 0.1 per minute under the conditions of assay. Specific activity was expressed as units per mg of protein.

(E) Fructose Diphosphate Aldolase (Fructose 1,6 diphosphate D-glyceraldehyde 3-phosphate-lyase) (FDPA) (E.C.4.1.2.13) :

$$\text{Fructose 1,6-diphosphate} \underset{\text{Hydrazine sulphate}}{\overset{\text{Aldolase}}{\rightleftharpoons}} \text{Dihydroxyacetone phosphate} + \text{Glyceraldehyde-3-phosphate}.$$

Fructose diphosphate aldolase catalyzes the reduction of fructose 1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The enzyme was assayed with modifications of the method described by Jagannathan et al. (1956). The assay system in a final volume of 2 ml contained 0.505 μ moles of fructose 1,6 diphosphate (trisodium salt), 1.5 μ moles of hydrazine sulphate, 160 μ moles of tris HCl buffer pH 7.4 and enzyme extraction. After addition of the enzyme preparation, the mixture was inverted rapidly in a quartz cuvette and the increase in O.D. was followed spectrophotometrically at 240 nm for 5 minutes.

FDPA activity was expressed in terms of units per culture. One unit of enzyme activity was defined as the enzyme required to cause an increase in the optical density by a factor of 0.1 per 5 minutes under the assay conditions. Specific activity was expressed as units per mg of protein.

(F) Glutamate-oxalacetate Transaminase (GOT) (E.C.2.6.1.1) :

Glutamate-oxalacetate transaminase catalyzes the transamination of L-aspartic acid and ∝-keto-glutaric acid to oxalacetic acid and L-glutamic acid, according to equation:

L-aspartic acid + ∝-ketoglutaric acid $\underset{\text{NADH}}{\overset{\text{GOT}}{\rightleftharpoons}}$

oxalacetic acid + L-glutamic acid + NAD^+.

The enzyme was assayed with modifications of the method

described by Williamson and Corkey (1969). The assay system in a final volume of 2 ml contained 0.15 μ moles of Nicotinamide adenine dinucleotide reduced (NADH), 2 μ moles of α-ketoglutaric acid, 140 μ moles of tris HCl buffer, 20 μ moles of L-aspartic acid, 1 μ mole of Ethylene diamine tetracetic acid (EDTA) and enzyme preparation. After adding enzyme preparation, the mixture was inverted rapidly in a quartz cuvette and the decrease in O.D. as a result of oxidation of NADH was followed spectrophotometrically at 340 nm for 1 minute.

GOT activity was expressed in terms of units per culture. One unit of enzyme activity was defined as the amount of enzyme required to cause a decrease in the optical density by a factor of 1 per minute under the assay conditions. Specific activity was expressed as units per mg of protein.

(G) NADP-malic enzyme (L-malate : NADP-oxidoreductase-oxalacetate decarboxylating) (E.C. 1.1.1.40) :

Malic enzyme catalyzes L-malic acid to pyruvate in the presence of NADP.

$$\text{L-Malic acid} + \text{NADP} \underset{\text{ME}}{\overset{Mn^{++}}{\rightleftharpoons}} \text{pyruvic acid} + CO_2 + \text{NADPH}$$

$$\text{oxalacetic acid} \xrightarrow{Mg^{++}} \text{pyruvic acid} + CO_2.$$

The enzyme was assayed with modifications of the method

described by Hsu and Lardy (1969). The assay system in a final volume of 2 ml contained 1.5 μ moles of L-malic acid, 165 μ moles of tris HCl buffer pH 7.2, 2 μ moles of manganese chloride, 0.131 μ moles of Nicotinamide adenine dinucleotide phosphate (NADP) and enzyme preparation. After addition of the enzyme preparation, the mixture was inverted rapidly in a quartz cuvette and the increase in O.D. was followed spectrophotometrically at 340 nm for 1 minute.

Activity of the enzyme was expressed in terms of units per culture. One unit of enzyme activity was defined as the enzyme required to cause an increase in the optical density by a factor of 0.1 per one minute under the assay conditions. Specific activity was expressed as units per mg of protein.

(H) Phosphoenolpyruvate carboxylase (orthophosphate : oxalacetate carboxylase (phosphorylating)) (PEPC) (E.C. 4.1.1.31) :

PEP carboxylase catalyzes phosphoenolpyruvate to oxalacetate in the presence of NADPH.

$$\text{Phosphoenolpyruvic acid} + \text{NADPH} \xrightleftharpoons[\text{PEPC}]{Mg^{++}} \text{oxalacetic acid} + \text{NADP}$$

The assay of PEP carboxylase was carried out with modifications of the method described by Taylor et al. (1974). The

assay system in a final volume of 2 ml contained 0.54 μ moles of EDTA, 1 μ mole of magnesium chloride, 0.85 μ moles of substrate phosphoenolpyruvate (trisodium salt, hydrate), 140 μ moles of tris HCl buffer pH 7.8, suitable amount of enzyme preparation and 0.12 μ moles of nicotinamide adenine dinucleotide phosphate reduced (NADPH). After the addition of enzyme preparation, the mixture was inverted rapidly in a quartz cuvette and the decrease in O.D. was followed spectrophotometrically at 340 nm for two minutes.

Activity of the enzyme was expressed in terms of units per culture. One unit of enzyme activity was defined as the enzyme required to cause a decrease in the optical density by a factor of 0.1 per two minutes under the conditions of assay. Specific activity was expressed as units per mg of protein.

(9) Photomicrography

Exakta and/or Contax camera/s were used to photograph the culture vessels showing growth, morphogenesis and differentiation. Orwo-Documentation Neg-Film was used for most of the work. Developer used was Kodak DA-163.

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