MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

1. Plant Material :-

The present investigations were carried out in vitro utilizing :

i) Callus tissues derived from the floral buds of Nicotiana tabacum L. var. Anand-2. The cultures were maintained on Murashige and Skoog's (1962) medium supplemented with 2.0 mg/l Indole-3-Acetic Acid (IAA), 2.0 mg/l 1-Naphthalene Acetic Acid (NAA) and 2.0 mg/l Kinetin (KN);

ii) anthers from young unopened flower buds of Nicotiana tabacum L. var. Anand-2. Haploid plantlets of pollen origin were initiated on Nitsch's (1969) medium supplemented with 15% (v/v) coconut milk (CCM) and 0.2 mg/l Indole-3-Acetic Acid (IAA);

iii) callus cultures initiated from the Shoot of haploid plantlets derived from the anthers of Nicotiana tabacum L. var. Anand-2. Cultures were maintained on Murashige and Skoog's (1962) medium supplemented with 2.0 mg/l Indole-3-Acetic Acid (IAA), 2.0 mg/l 1-Naphthalene Acetic Acid (NAA) and 2.0 mg/l Kinetin (KN);

iv) shoot, root and leaf explants of plantlets regenerated from the floral bud callus of Nicotiana tabacum L. var. Anand-2; and

v) shoot, root and leaf explants of haploid plantlets raised from the anthers of Nicotiana tabacum L. var. Anand-2, in culture.

Floral bud callus as well as the haploid callus initiated on Murashige and Skoog's (1962) medium supplemented with 2.0 mg/l each of IAA, NAA and KN were later on transferred and maintained on the same medium, subculturing after 30 day cultural cycles. For studies in morphogenesis and the biochemistry involved therein, auxins and kinetin were added separately into the medium.

2. Culture Medium :

(a) Chemicals

The chemicals used in experimentation were of the highest purity available and obtained from British Drug House (BDH, AnalR grade), E. Merck (Guaranteed reagents) and M/s. Sigma Chemical Co. (U.S.A.).

(b) Culture vessels

Depending upon the type of culture, tubes or Erlenmeyer flasks were used. Stock calli were maintained on agar medium

in 150 ml Erlenmeyer flasks.

All culture vessels and glassware used for the preparation of media or other biochemical assays were cleansed with chromic acid. The acid was washed off by thorough rinsing with running tap water. The glasswares were again washed with a detergent (Teepol) and running tap water. Finally, the glasswares were rinsed with double glass distilled water and oven dried at 60°.

(c) Preparation of Coconut Milk

Coconut milk was obtained from green coconuts purchased in the local market. The milk obtained was deproteinated by repeated boiling, cooling and filteration, till all precipitates were removed. Aliquotes of 100 ml were placed in boiling tubes and autoclaved, the tubes were later stored in a deep freeze.

(d) Preparation of Media

The basal medium was prepared from concentrated stock solutions prepared in double glass distilled water and stored at 5-10°.

In the present study, Murashige and Skoog's (MS, 1962) and Nitsch's (NM, 1969) media were used. Their composition is given in Tables I and 2 respectively. All supplements were added into the medium prior to the final adjustment of volume.

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	82.5	20	1650.00
B.	KNO_3	95.0	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.	$CaCl_2.2H_2O$	88.0	5	440.00
E.	$MgSO_4.7H_2O$	74.0		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	7.4	5	37.35
	$FeSO_4.7H_2O$	5.57		27.85
G.	Thiamine HCl	0.02		0.10
	Nicotinic Acid	0.1	5	0.50
	Pyridoxin-HCl	0.1		0.50
	Glycine	0.4		2.00

Addendum : Sucrose 20 gms/l, myo-inositol 100 mg/l
2.0 mg/l IAA; 2.0 mg/l NAA and 2.0 mg/l KN

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.

Table 2. J. P. NITSCH (1969) 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.	KNO_3	95.0	10	950.0
B.	NH_4NO_3	72.0	10	720.0
C.	$MgSO_4.7H_2O$	18.5	10	185.0
D.	$CaCl_2$	16.6	10	166.0
E.	KH_2PO_4	6.8	10	68.0
F.	Na_2EDTA	7.45	5	37.35
	$FeSO_4.7H_2O$	5.57		27.85
G.	$MnSO_4.4H_2O$	5.0		25.0
	H_3BO_3	2.0		10.0
	$ZnSO_4.7H_2O$	2.0	5	10.0
	$Na_2MoO_4.2H_2O$	0.05		0.25
	$CuSO_4.5H_2O$	0.005		0.025
H.	Myoinositol	20.0		100.0
	Nicotinic acid	1.0		5.0
	Glycine	0.4		2.0
	Pyridoxine HCl	0.1	5	0.5
	Thiamine HCl	0.1		0.5
	Folic acid	0.1		0.5
	Biotin	0.01		0.05

Addendum : Sucrose 20 gm/l

pH of the medium adjusted to 5.5.

The pH of the medium was measured and adjusted to 5.6 - 5.8 for MS medium and 5.5 for NM medium with 0.1 N NaOH or 0.1 N HCl as the case might be. The pH measurements were taken with Elicon pH meter. The medium used was solidified with 0.9% (w/v) Difco-Bacto, E. Merck or Centron agar.

(e) Sterilization of Media and Culture Vessels

After pH adjustments, fixed volumes of the media were transferred to oven dried culture vessels for autoclaving. The culture vessels were plugged with non-absorbant cotton wool bungs, wrapped by brown paper for protection from condensed water during autoclaving. The media and the culture vessels were sterilized by autoclaving at 15 psi for 20 minutes. The instruments were sterilized by imersing in absolute alcohol and subsequent flamming.

3. Aseptic Techniques :

All inoculations and manipulations involving sterile cultures or media were carried out in an inoculation chamber. The inoculation chamber was saturated with an aerosol of 2% thymol and 2% glycerine in 90% ethanol. The working table was cleaned with 4% formaldehyde. The interior of the transfer chamber was irradiated with ultraviolet light (=2537 A°) for 2 hours before use to avoid contamination.

(a) Surface Sterilization

The flower buds were collected from the Nicotiana tabacum L. var. Anand-2 plants grown in experimental plots in the Botanical garden. These flower buds were thoroughly washed with running tap water till they became free of adhered dust and stickiness. They were then rinsed with double glass distilled water, surface sterilized with 80% ethanol for 2 minutes and 0.1% (w/v) mercuric chloride for 4 minutes.

(b) Culture Technique

i) Initiation of Flower Bud Callus Cultures

The flower buds sterilized as described above were washed repeatedly with sterilized double glass distilled water, cut lengthwise into two identical halves and planted on solid MS (1962) medium supplemented with 2.0 mg/l each of IAA, NAA and KN. The cut surface of the flower bud was kept in contact with the medium. The culture vessels were then incubated in continuous light at 26±2°.

ii) Raising of Haploids by Anther Culture

Young, unopened flower buds, 6-8 mm long, were sterilized as above. The anthers were excised and inoculated on Nitsch's (1969) medium supplemented with

15% coconut milk and 0.2 mg/l IAA. Out of five anthers per flower bud, four were cultured and one was fixed in acetic alcohol (1 : 3) to determine the stage of pollen development. The cultures were incubated in continuous light at 26±2°.

iii) Initiation of Callus of Haploid Origin

Haploid plantlets (n=24) of anther origin were used as source for haploid callus tissues. Young shoots of the plantlets were excised under aseptic conditions and inoculated on MS (1962) agar medium supplemented with 2.0 mg/l IAA, 2.0 mg/l NAA and 2.0 mg/l KN to derive callus from them. The cultures were incubated in light at 26±2°.

iv) Culture of root, shoot and leaf explants of haploid and diploid plants

Root, shoot and leaf explants of haploid plants of anther origin and diploid plants differentiated from floral bud callus were excised under sterile conditions and cultured on MS (1962) agar medium supplemented with phytohormones and incubated in continuous light at 26±2°.

v) Stock Cultures

Callus cultures derived from different sources were

repeatedly transferred onto fresh medium to ensure dedifferentiation to the highest possible degree and from these callus masses, healthy, fast growing and green chlorophyllous tissues were used to build up stock cultures.

vi) Technique of Sub-Culture

The stock callus cultures built up by the above technique were transferred to fresh nutrient medium every 30 days. From these well-established, 'clonal' stocks, tissues were used as inocula for experimentations.

4. Measurements of Growth :

Growth measurements were made as a function of increase in fresh and dry weights of both diploid and haploid callus tissues. Of the total number of replica in culture, 5 were harvested at fixed intervals of time (every 3 days till the end of culture period) and growth measurements made. The growth curves were plotted after calculating and making amends for the standard error.

A. Fresh weight

The callus tissues grown on agar medium were carefully freed from specks of agar which adhered strongly at the points of contact and sponged lightly with filter paper to remove water which might have accumulated as a consequence of condensation or otherwise. The callus tissue was then removed onto pre-weighed aluminium foil and the weight determined on a single pan balance (Mettler, Zurich).

B. Dry weight

Pre-weight masses of fresh callus tissue was oven dried at 60° to a constant weight which was recorded as its dry weight.

5. Chemical Analysis

The chemicals used for the analytical work were obtained from British Drug House (India), E. Merck (India or Germany) and Sigma Chemical Co. (U.S.A.).

Callus tissues of haploid and diploid origin were assayed during growth and differentiation for :

A. Enzymes :

i) Peroxidase,

ii) Indole Acetic Acid Oxidase (IAA Oxidase),

iii) Phenylalanine Ammonia-Lyase (PAL) and

iv) Malate dehydrogenase (MDH).

B. Disc gel electrophoretic separation of Peroxidase Isoenzymes :

i) Anodic Peroxidase Isoenzymes, and

ii) Cathodic Peroxidase Isoenzymes.

C. Soluble Proteins and

D. Extractable Phenolic Compounds.

The methods used for their extraction and assay are as follows :-

Cell-free extracts for enzyme assays were prepared from callus tissues harvested and pooled after each treatment. The tissue (500 mg fresh weight) was homogenized in presence of 2% (w/w) Poly-Vinyl Pyroledene (PVP) and extracted with 0.1 M phosphate buffer, pH 7.0. The homogenate was centrifuged for 20 minutes at 18,000 x g. The supernatent was collected and volume made upto 10 ml with the same buffer. All operations were carried out at 0-4°. The resultant cell free extract was used for the assay of Peroxidase, IAA oxidase, PAL, MDH, proteins and electrophoric separation of peroxidase isoenzymes.

A. Enzyme Assays :

i) Peroxidase :

The enzyme peroxidase was assayed with modification of

the method discribed by Machly and Chance (1954). Oxidation of guaiacol was followed colorimetrically at 470 nm. The enzyme system in a colorimeter tube consisted of 20 mM guaiacol, 0.1 M phosphate buffer (pH 6.5) and an aliquote of cell-free extract in a total volume of 10 ml. At zero time, 0.1 ml of 10 mM H_2O_2 solution was added and the tube invested rapidly. The increase in colour intensity was followed at an interval of 15 seconds, with readings taken at 15, 30, 45, 60 and 75 seconds. The cell-free extract aliquot in the system was varied from time to time to ensure linear development of colour over this span of time. Peroxidase activity is expressed as units per culture, and one unit of enzyme activity is defined as the enzyme required to cause an increase in optical density by a factor of 10 per minute under the assay conditions. Specific activity is expressed as units per mg of protein.

ii) IAA Oxidase

The assay system in a final volume of 3 ml contained 200 ug IAA, 100 uM 2,4-dichlorophenol, 66 μM $MnCl_2$, and 0.1 M citrate phosphate buffer (pH 4.0), besides an aliquot of cell-free extract. The assay mixture was incubated at 37° in a water bath for 25 minutes. The residual IAA was estimated with modified Salkowski

reagent (Gordon and Webber, 1951). The modified Salkowski reagent contained 100 ml of 35% perchloric acid and 2 ml of 0.5 M $FeCl_3$. 6 ml of Salkowski reagent was added to the enzyme assay system and incubated further for 20 minutes at 37° before reading the colour developed colorimetrically at 530 nm. A calibration curve for the determination of the residual IAA was linear between 10-200 μ gms IAA. The enzyme activity is expressed as units per culture and one unit is defined as the amount of enzyme required to destroy 1 mg of IAA in 25 minutes under the experimental conditions. Specific activity is expressed as units per mg of protein.

iii) Phenylalanine ammonia-lyase (PAL)

The reaction mixture containing 50 μm L-phenylalanine, 25 mM tris-HCl buffer (pH 8.8) and enzyme extract in a total system of 2 ml was incubated in a water bath at 37° for 60 minutes. The reaction was terminated by the addition of 1 ml 0.5 M HCl. The reaction product, cinnamate, was measured at 278 nm in a spectrophotometer (C.Z. Instrumentation, Germany model VSUZ-P). A calibrated curve for the determination of cinnamate was constructed over a range of 2-100 μg of trans-cinnamic acid. Enzyme activity is expressed as

units per culture.One unit of enzyme activity is defined as the amount of enzyme required for the formation of 10 μM of cinnamate at 37° in 60 minutes. Specific activity is expressed as units per mg of protein.

iv) Malate Dehydrogenase (MDH)

The enzyme was assayed with modifications of the method described by Ochoa (1955). The assay system in a final volume of 2 ml contained 20 μM Nicotinamide adenine dinucleotide reduced (NADH), 40 μM oxalacetic acid (neutralized) and 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.

B. Disc Gel Electrophoretic Separation of Peroxidase Isoenzymes

i) Anodic Peroxidase Isoenzymes

For the electrophoretic separation of anodic

Table 3. Constituents for basic Polyacrylamide Gel (pH 8.9) preparation* (Davis, B.J., 1964)

STOCK A :	(i)	1N HCl	48 ml
	(ii)	TRIS	36.6 g
	(iii)	TEMED	0.23 g
		Water added to make 100 ml.	
		pH 8.9	
STOCK B :	(i)	Acrylamide	28.0 g
	(ii)	Bis	0.735 g
		Water added to make 100 ml.	
STOCK C :		Ammonium persulfate	0.14 g
		Water added to make 100 ml.	
STOCK D :	Buffer for electrophoresis reserviors :		
	(a)	TRIS	60.0 g
	(b)	Glycine	28.8 g
		Water added to make 1 litre.	
		pH 8.3	

Separation gel was prepared by mixing :

1 part	STOCK A.
2 parts	STOCK B.
4 parts	STOCK C.
0.45 parts	Water

* The above composition is with modification of the original. Gel formed is of 7.5% acrylamide.

peroxidase isoenzymes, the method used was essentially that of Davis (1964). The electrophoresis tubes were filled with narrow pore separation gel and layered with a drop of water. The separation gel contained 7.5% (w/v) acrylamide and its pH was 8.9. The gels were allowed to polymerize in light. On the completion of polymerization the enzyme extract containing approximately 100 µg protein was mixed with an equal amount of 60% (w/v) sucrose and layered on top of the gel. The electrophoresis was performed in Tris-glycine buffer, pH 8.3, by applying a current of 2 mA per gel.

Bromophenol blue served as the marker dye. The completion of electrophoresis could be observed when the bromophenol blue layer had migrated to the bottom of the gel tube. The gels were removed carefully from the tubes by a water jet and stained for peroxidase isoenzymes with 1.5% Benzidine hydrochloride in 25% acetic acid and 5% hydrogen peroxide (100 volumes). The peroxidase isoenzymes appear as blue bands with Benzinidine hydrochloride H_2O_2 staining. within a minute or so of incubation of gels in the stain. All runs were carried toward anode.

ii) Cathodic Peroxidase Isoenzymes

The method followed for the electrophoretic separation

of cathodic isoperoxidases was that used by Reisfeld, et al. (1962) for the sèparation of basic proteins and peptides. The electrophoresis tubes were filled with small pore separation gel and layered with a drop of water. The separation gel, pH 4.3, contained 7.5% (w/v) acrylamide. The gels were polymerized in light, and enzyme extract - carrying ca. 100 µg protein - was mixed with equal volume of 60% sucrose and layered on top of the gel after removal of the water layer at the gel top. Electrophoresis was carried out in β-Alanine-acetic acid buffer, pH 4.5, for 90 minutes, by applying a current of 2 mA per gel. The gels were removed from the electrophoresis tubes and stained immediately. 1.5% Benzidine hydrochloride in 25% acetic acid and 5% H_2O_2 was used to stain the gels. Blue bands develop in Benzidine hydrochloride - H_2O_2 stain within a minute of incubation of the gels in the stains. All runs were carried towards the cathode.

Constituents for the preparation of gels for the separation of anodic and cathodic isoperoxidases are given in Tables 3 and 4 respectively.

Results of these exercises were presented as a series of detailed diagrams of stained isoperoxidase bands drawn to actual scale.

Table 4. Constituents for acidic Polyacrylamide gel (pH 4.3) preparation* (Reisfeld et al., 1962).

STOCK A :	(i)	1N KOH	48 ml
	(ii)	Acetic acid (glacial)	17.2 ml
	(iii)	TEMED	4.0 ml
		Water added to make 100 ml	
		pH 4.3	
STOCK B :	(i)	Acrylamide	28.0 g
	(ii)	Bis	0.735 g
		Water added to make 100 ml	
STOCK C :	Ammonium persulfate		0.14 g
		Water added to make 100 ml	
STOCK D :	Buffer for electrophoresis reserviors :		
	(a)	β - Alanine	31.2 g
	(b)	Acetic acid (glacial)	8.0 ml
		Water added to make 1 litre.	
		pH 4.5	

Separation gel was prepared by mixing :

1 part	STOCK A.
2 parts	STOCK B.
4 parts	STOCK C.
0.45 parts	water

* The above composition is with modification of the original. Gel formed is of 7.5% acrylamide.

Full chemical name of reagents used for Polyacrylamide gel preparations :

Bis : N,N' - Methylenebisacrylamide

TRIS : 2-Amino-2-(hydroxymethyl)-1,3-propanediol
also known as
tris (hydroxymethyl) aminomethane (TRIZMA BASE)

TEMED : N,N,N',N' - Tetramethylethylene diamine

C. 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.

D. Extractable Phenolic Compounds

Phenolic compounds were extracted by grinding 50 mg (dry wt) samples of tissue using a chilled pestle and mortor with an aliquot of ice-cold 80% (v/v) ethanol. The homogenate was centrifuged at 13,000 x g for 15 minutes. The supernatent was collected and the residual pallet was extracted twice more as above. The supernatent of all three extractions was pooled and volume made upto 25 ml with 80% ethanol.

The ethanol extractable phenolic compounds were estimated by the Folin method of Swain and Hillis (1959). A suitable aliquot of the ethanolic extract was diluted with distilled water to 8.5 ml, 0.5 ml of Folin-phenol reagent was added and the contents mixed well. 3 minutes later 1 ml of saturated Sodium carbonate (1 gm/3 ml H_2O) was added and the mixture shaken thoroughly. Colour was allowed to develop over 60 minutes and read at 725 nm. Standard curve was prepared using chlorogenic acid and all the concentrations were expressed in terms of mgs of this compound.

6. Cytological Methods :-

To determine the stages of pollen embryogenesis, cultured anthers of Nicotiana tabacum var. Anand-2 were harvested at weekly intervals. The anthers were cut open at the two ends and incubated for 15 minutes in 0.1 N HCl at 60° to carry out hydrolysis. The hydrolysed anthers were then stained with 2% aceto-carmine. The pollen grains were teased out of the anthers in a drop of 1% aceto-carmine placed on a glass slide. A cover slip was dropped into place and sealed with paraffin wax.

To determine the chromosome number of anther derived plantlets, root tips were collected from them in early morning hours and pretreated with 0.1% cholchicine for 2 hours. Root tips were later thoroughly rinsed with distilled water. Hydrolysis of root tips was carried out by incubating them in 0.1 N HCl at 60° for 10 minutes. These hydrolysed root tips were stained with 2% aceto-carmine and the squashes prepared in 1% aceto-carmine.

7. Photomicrography :-

Exakta and/or Contax camera 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. Steindroff microscope fitted with X10 or X15 projection eyepiece and 10/0.3, 45/0.65 or 100/(oil emersion) objectives were used.