CHAPTER II

MATERIALS AND METHODS

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1. Plant Material

The experiments described in the present thesis were conducted with the callus tissues derived from:

(i) the excised anthers of *Datura metel* L., maintained on *Datura metel* L. medium (Rao and Mehta, 1968) supplemented with 10% coconut milk and 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D);

(ii) the anther callus tissues of *Cassia fistula* L. and

(iii) the anther callus of cotton, *Gossypium hirsutum* Linn. Variety 'Sankar 4'.

Cassia (ii) and cotton (iii) tissues were initiated and maintained on Murashige and Skoog's complex medium (Bagde and Mehta unpublished) supplemented with 10% coconut milk, 2.0 mg/l 2,4-D, 2.0 mg/l Indoleacetic acid (IAA) and 2.0 mg/l kinetin.

2. Culture Media

The different culture media on which tissues were initiated and maintained are shown in Tables 1 and 2. The completely defined medium evolved in the present investigation and which supported the rapid and continuous growth of all the 3 tissues is given in Table 3.

Table 1 : *Datura metel* L. Medium (Rao & Mehta, 1968)

Constituents		Concentration in 1 litre of complete medium expressed as mg of hydrated salt
I. Inorganic Salts (Hellers, 1953)		
Calcium chloride	$CaCl_2.6H_2O$	112.00
Magnesium sulphate	$MgSO_4.7H_2O$	250.00
Potassium chloride	KCl	750.00
Sodium nitrate	$NaNO_3$	600.00
Sodium dihydrogen orthophosphate	$NaH_2PO_4.2H_2O$	125.00
II. Microelements and Vitamins (White, 1954)		
*Ferric citrate	$FeC_6H_5O_7.5H_2O$	2.00
Boric acid	H_3BO_3	1.50
Manganese sulphate	$MnSO_4.4H_2O$	6.65
Potassium iodide	KI	0.75
Zinc sulphate	$ZnSO_4.7H_2O$	2.68
Glycine		3.00
Nicotinic acid		0.50
Pyridoxine hydrochloride		0.10
Thiamine hydrochloride		0.11
III. Supplements		
Sucrose		20.00 g
Coconut milk		100.00 ml
2,4-dichlorophenoxyacetic acid (2,4-D)		2.00 mg
Casein hydrolysate		300.00 mg
Water (double glass distilled) to make up		1 litre

*Ferric citrate was used instead of Ferric chloride.

Table 2 : Murashige and Skoog's Complex Medium

Stock soln.	Constituents	Conc. in stock soln. gm/l	Volume 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	1900.0
C	H_3BO_3	1.24	5	6.2
	KH_2PO_4	34.00		170.0
	KI	0.166		0.83
	$Na_2MoO_4.2H_2O$	0.005		0.025
	$CoCl_2.2H_2O$	0.005		0.025
D	$CaCl_2.2H_2O$	88.0	5	440.0
E	$MgSO_4.7H_2O$	74.0	5	370.0
	$MnSO_4.H_2O$	3.37		16.9
	$ZnSO_4.7H_2O$	1.72		8.6
	$CuSO_4.5H_2O$	0.005		0.025
F*	$Na_2.EDTA$	7.45	5	37.25
	$FeSO_4.7H_2O$	5.57		27.85
G	Thiamine-HCl	0.02	5	0.1
	Nicotinic acid	0.1		0.5
	Pyridoxine-HCl	0.1		0.5
	Glycine	0.4		2.0

Addendum : Sucrose 20 gm/l,
Indole-3-acetic acid 2 mg/l, kinetin 2 mg/l, 2,4-D 2mg/l, coconut milk 100 ml/l.

The stock solutions A-G were prepared and stored in a refrigerator (never more than 4-6 weeks) and mixed just before preparing the final medium.

*The $FeSO_4.7H_2O$ is dissolved in Ca 200 ml double distilled water. The $Na_2.EDTA$ is dissolved in Ca 200 ml double distilled water heated and mixed (under continuous stirring) with the $FeSO_4.7H_2O$ solution. After cooling, the volume is adjusted to 1000 ml. Heating and stirring result in a more stable Fe EDTA complex.

Table 3 : Modified Murashige and Skoog's (1962) Medium

Constituents		Concentration in 1 litre of complete medium expressed as mg of hydrated salt
I. Inorganic salts		
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2.2H_2O$	440.00
Magnesium sulphate	$MgSO_4.7H_2O$	370.00
Potassium dihydrogen phosphate	KH_2PO_4	170.00
II. Microelements and Vitamins		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Sodium Molybdate	$Na_2MoO_4.2H_2O$	0.025
Cobalt chloride	$CoCl_2.2H_2O$	0.025
Manganese sulphate	$MnSO_4.4H_2O$	22.3
Zink sulphate	$ZnSO_4.7H_2O$	8.6
Copper sulphate	$CuSO_4.5H_2O$	0.025
*Ferric sulphate	$FeSO_4.7H_2O$	27.85
**Na_2EDTA		37.25
Thiamine-hydrochloride		1.0
Nicotinic acid		1.0
Pyridoxine-hydrochloride		1.0
Glycine		4.0
III. Supplements		
Sucrose		20.0 gms
myo-Inositol		100.0 mg
Kinetin		0.4 mg
2,4-D		2.0 mg

*The $FeSO_4.7H_2O$ was dissolved in approximately 200 ml of double glass distilled water.

**The Na_2EDTA was dissolved in 200 ml of double glass distilled water, heated and mixed (under continuous stirring) with the $FeSO_4.7H_2O$ solution. After cooling the volume was adjusted to 100 ml. Heating and stirring resulted in a more stable FeEDTA complex.

A. Preparation of the Media

The chemicals used in the preparation of the media were of research grade purity and were obtained from British Drug Houses (Analar grade) or E. Merck (Guaranteed reagent).

The basic media were prepared from concentrated stock solutions which had been stored at 2-4°C. Double glass distilled water was used for preparing the medium as well as the stock solutions. Wherever hydrates of any salts were used appropriate corrections were made in weights. The constituents of the three media were added in the order shown in Tables 1,2 and 3. The supplements to be incorporated into basic media were added before the final adjustment of the volume. The pH of the medium was measured and adjusted to 5.5 with a Beckman pH meter with the help of 0.1 N HCl or 0.1 N NaOH as found necessary. The medium was solidified with Difco-Bacto agar.

B. Culture Vessels

Erlenmeyer flasks (100 ml and 150 ml) made of Corning glass were used as culture vessels. The flasks containing sterile cultures were covered with two layers of aluminium foil which had been sterilized in a flame.

All culture vessels and glassware used in the preparation of the media and for other purposes were cleaned in chromic acid (Potassium dichromate in sulphuric acid). The acid was removed by prolonged rinsing with tap water. Next the glassware was washed with detergent Teepol (B.D.H.) which was then removed by thorough washing with tap water. The glassware was finally rinsed with double glass distilled water and dried in an oven.

C. Sterilization of Media and Culture Vessels

After adjustment of pH, known volumes of media were transferred to culture vessels for autoclaving. The mouth of the culture vessels was closed with non-absorbent cotton-wool plugs covered by gauze. Brown papers were wrapped to protect them from condenced water during autoclaving. The media and glassware were sterilized by autoclaving at a pressure of 15 lb/in^2 for 20 minutes. The instruments used were sterilized by flaming with absolute alcohol.

3. Aseptic Techniques

All inoculations and manipulations involving sterile cultures or media were carried out in a transfer cobinet. The working surface was cleaned with 4%

formaldehyde. The interior of the cabinet was sprayed with absolute alcohol and irradiated with ultra-violet (λ = 2537 A°) light for 60 minutes before use to reduce contamination.

4. Culture Techniques

A. Stock Cultures

Tissue fragments from the same callus mass were, by repeated transfers to fresh media, used to build up into stocks of 'clonal' material. Every 4 weeks the healthy looking and fast growing callus pieces from the 'clonal' stocks were regularly subcultured to freshly made nutritive media. These well established tissues were then used as inocula for the experiments.

B. Initiation of Suspension Cultures

Cell suspensions were obtained by transferring pieces of callus tissues from 'clonal' stocks maintained on agar media to the liquid media contained in Erlenmeyer flasks. The latter were continuously agitated on a horizontal rotary shaker in an illuminated constant temperature (26$\pm$2°C) culture room.

Routinely, the suspension cultures were subcultured every 4 weeks. 4 ml aliquots of cell suspension were

pipetted out into 50 ml of freshly made liquid medium of the same composition. Cell suspensions so obtained were used as inocula for experiments.

An environmental growth chamber (Instrumentation Specialities Company, U.S.A.), where temperature, light and humidity could be programmed was used for the incubation of cultures in some experiments.

5. Measurements of Growth

Growth was measured by determining increase in fresh and dry weights. Of the total number of replicates inoculated, 5 to 6 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

The cells grown in liquid media were collected by filtration through previously weighed sinter glass Gooch crucibles and their fresh weight was measured on a Mettler balance (Zurich). In the case of callus cultures grown on solid media, the tissue was carefully removed on to a previously weighed aluminium foil and the weight was determined on a Mettler balance.

B. Dry Weights

Dry weights of the cells were determined by drying a weighed mass of tissue to a constant weight at 60°C in an oven.

6. Chemical Analysis

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

A. Extraction and Estimation of Phenolic Compounds

For the estimation of total polyphenols, 1 g (F.Wt.) samples of tissue were stirred with 8 ml aliquots of 80% (v/v) ethanol over a period of 35 min using Thomas tissue homogenizer equipped with Teflon plunger. The successive extracts from each sample were combined and made up to 25 ml.

Total polyphenols were estimated by the Folin method of Swain and Hillis (1959). A suitable aliquot of the ethanolic extract containing 0.5 to 1.0 ml was diluted with water to about 7 ml in a 10 ml graduated test tube. The contents were well mixed, 0.5 ml of Folin-Denis reagent was added and the tubes were thoroughly shaken again.

Exactly 3 minutes later, 1 ml of saturated sodium carbonate solution was added and the final volume was made upto 10 ml with good mixing. The colour developed was measured at 725 mμ after standing for 60 minutes. The standard curve was prepared using chlorogenic acid as the standard and all the concentrations were expressed in terms of μgrams of this compound.

7. Enzyme Assays

In the present study, the progressive changes in the pattern of peroxidase, indoleacetic acid oxidase (IAA oxidase) and phenylalanine ammonia-lyase (PAL) enzymes were examined.

A. Peroxidase

Cell-free extracts were prepared from tissues collected and pooled after each treatment. All preparations were carried out at 0 to 4°C. The tissue was homogenised and extracted with 0.1M phosphate buffer (pH 7.0). The supernatant was collected after centrifugation at 15000 x g. The resultant cell-free extract was assayed for total peroxidase activity.

The peroxidase activity was measured by the modification of the technique described by Maehly and Chance (1954).

Oxidation of guaiacol was followed colorimetrically at a wavelength of 470 mμ. An aliquot of the cell-free extract was placed in a colorimeter tube; 20 mM of guaiacol and sufficient buffer were added to bring the volume to 10 ml; at time zero 0.1 ml of hydrogen peroxide (10 mM) was added and the tube rapidly inverted. Following the addition of hydrogen peroxide, the increase in colour intensity was followed in the colorimeter with readings at 20, 40 and 60 seconds, since the rate of colour formation over one minute was linear within this time.

Peroxidase activity was expressed as the rate of increase in optical density per minute at 470 mμ.

B. Indoleacetic acid Oxidase (IAA Oxidase)

For the preparation of cell-free extracts of IAA oxidase, the tissues were homogenised in chilled mortar and pestle with ice cold 0.1M phosphate buffer (pH 6.1). The homogenate was centrifuged in cold at 20,000 x g and the clear supernatant was dialyzed for 24 hours against 4 litres of deionized water. All operations were done at 2 to 4°C. The dialyzed extract was centrifuged and the clear supernatant was used as the enzyme source.

IAA oxidase determinations were made as described by Gortner and Kent (1953).

Assay for IAA oxidase was carried out in 50 ml Erlenmeyer flasks in a water bath at 37°C. An aliquot (1 ml approx.) of the dialysed cell-free extract and 1 ml of substrate containing 200 µg of indoleacetic acid, 0.1 mM 2,4-dichlorophenol, 0.1 mM $MnCl_2$ were placed in a 50 ml flask and enough buffer was added to bring the final volume to 5 ml. Duplicate number of flasks were incubated in the water bath at 37°C. After ~~20~~ 25 minutes, the reaction was stopped by adding 3 ml of 1:4 perchloric acid.

The residual IAA was determined colorimetrically on a 1 ml aliquot by the modification of the method of Gordon and Weber (1951). The modified Salkowski reagent contained 50 ml of 35 per cent perchloric acid and 1 ml of 0.5 M ferric chloride. To the 1 ml aliquot were added 2 ml of Salkowski reagent and the mixture was incubated for 20 minutes at 37°C. 7 ml of 1:4 perchloric acid were then added and the solution was read against blank in a colorimeter at a wavelength of 530 mµ. A calibration curve for the determination of the residual IAA was constructed over a range of 10 to 100 µg of IAA.

The proteins in the original extract were precipitated with 10% (w/v) trichloroacetic acid (TCA) and estimated by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard.

Enzyme activity was expressed as µg of IAA destroyed/mg dry weight of the tissue in ~~20~~ 25 minutes.

Specific activity: µg of IAA destroyed/µg protein.

C. Phenylalanine ammonia-lyase (PAL)

Phenylalanine ammonia-lyase (PAL) assay was carried out using acetone powders.

The collected tissues whose fresh weight was 1-2 grams were frozen at -20°C and ground with 25 ml of acetone previously chilled to -20°C. The homogenate was filtered on a Buchner funnel and the residue was washed twice with chilled acetone and spread to dry at room temperature, yielding about 100 to 120 mg of powder. The powders were transferred to vials and stored at -25°C. The powders were stable for several months.

The PAL activity was determined according to the method of Zucker (1965).

The acetone powder (at 80 mg/25 ml) was suspended in cold 0.1M borate buffer at pH 8.8, and the mixture was

stirred for 15 minutes in an ice bath. The extract was then centrifuged in cold at 10,000 x g for 20 minutes. The supernatant was used as the crude enzyme extract. The crude extract was not stable and must be assayed within short period.

The reaction mixture consisted of 200 µmoles of borate buffer, pH 8.8; 60 µmoles of L-phenylalanine; 1 ml of enzyme extract (containing 200-300 µg of protein) and enough water to bring the total volume to 4 ml. The reaction was carried out at 40°C using water bath. The rate of reaction was calculated from measurements of the absorption at 290 mµ taken at 20 minutes intervals for at least one hour, after the addition of phenylalanine. An extinction coefficient of 16,000 was determined for cinnamic acid at 290 mµ in pH 8.8 borate buffer.

Enzyme activity was expressed as the micromoles of cinnamic acid produced at 40°C/hr/100 mg of acetone powder.

8. Photomicrography

EXakta and/or Contax camera were used to photograph the culture vessels showing growth and morphogenetic responses of the callus. Orwo-Documentation Neg-film was used for most of the work. Developer used was Kodak DA-163.