CHAPTER II

MATERIALS AND METHODS

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1. Plant Material

The callus tissue was initiated from the anthers of *Datura*. The anthers of *Datura metel*, Linn. were collected from the plants growing in the Botanical garden of the Department. The callus tissues propagated on Heller's (1953) macroelement salts, White's (1954) microelements and vitamins, and supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) and coconut milk (Table 4) were used as experimental material for the investigations presented in this thesis.

2. Culture Media

The different media tested for induction and growth of callus are shown in Tables 1, 2 and 3. The medium which supported the rapid and continuous growth of *Datura* anther callus is shown in Table 4. It consisted of :

A. Heller's (1953) macroelement salts (as given in Table 2),
B. White's (1954) microelements and vitamins (as given in Table 1),
C. 10% (v/v) Coconut milk,
D. 2.0 mg/l 2,4-D, and
E. 300 mg/l Casein hydrolysate.

Table 1 : White's (1954) Medium

I. Inorganic Salts		Concentration in 1 litre of complete medium expressed as mg of hydrated salt
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	288.00
Magnesium sulphate	$MgSO_4.7H_2O$	738.00
Potassium chloride	KCL	65.00
Potassium nitrate	KNO_3	80.00
Sodium sulphate	$Na_2SO_4.10H_2O$	454.00
Sodium dihydrogen orthophosphate	$NaH_2PO_4.2H_2O$	24.20
II. Microelements and Vitamins		
*Ferric citrate		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 ml
2,4-dichlorophenoxy acetic acid(2,4-D)		2.00 mg
Water (double glass distilled) to make up		1 litre

* Iron was supplied as ferric citrate instead of ferric chloride.

Table 2 : Heller's (1953) Medium

I. Inorganic Salts		Concentrations in 1 litre of complete medium expressed as mg of hydrated salt
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

The microelements, vitamins and growth factors supplied were the same as in White's medium (Table 1).

Table 3 : Murashige and Skoog (1962) Medium

I. Inorganic Salts		Concentrations in 1 litre of complete medium expressed as mg of hydrated salt
Ammonium nitrate	NH_4NO_3	1440.00
Calcium chloride	$CaCl_2.6H_2O$	655.00
Magnesium sulphate	$MgSO_4.7H_2O$	370.00
Potassium nitrate	KNO_3	1990.00
Potassium dihydrogen phosphate	KH_2PO_4	134.00

The microelements, vitamins and growth factors supplied were the same as in White's medium (Table 1).

Table 4 : *Datura metel* Linn. Medium

I. Inorganic Salts (Heller, 1953)		Concentration in 1 litre of complete medium expressed as mg of hydrated salt
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		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 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.

A. Preparation of the medium

The basic media were prepared from stock solutions which had been stored at 2°C. When the constituents were added in the order shown in the Tables, precipitation of the iron by the concentrated inorganic salts was prevented. All the constituents of the standard media were obtained from either British Drug House (B.D.H. Analar grade) or E. Merck (Guaranteed reagents).

The green coconuts were obtained from a local dealer. The milk released from these coconuts was boiled and after cooling it was filtered through Whatman No.1 filter paper in order to remove precipitated protein. This process was repeated until no more protein precipitated. 100 ml aliquots of the filtrate were placed in 150 ml capacity Pyrex tubes. The mouth of the tubes were closed by using non-absorbent cotton. They were autoclaved at a pressure of 15 lbs/in^2 for 15 minutes. The tubes were stored in a deep freeze.

Media were solidified by using Difco Bacto Agar (8.0 g/l). After the final adjustment of the volume, the pH of the media was measured with a Beckman pH meter and adjusted to 5.5, using 0.1 N HCl or 0.1 N NaOH as found necessary.

B. Culture vessels

Callus cultures were maintained in 100 ml or 250 ml Erlenmeyer flasks made of either Corning or Pyrex glass. The mouth of these 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 medium and 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 thoroughly washing the glassware with tap water. The glassware was finally rinsed with double glass distilled water.

C. Sterilization of the medium

After adjustment of the pH, known volumes of media were distributed in culture vessels for autoclaving. The mouths of the culture vessels were closed with non-absorbent cotton-wool plugs covered by guaze. Brown paper coverings were made use of to protect them from condensed water during autoclaving. The media and glassware were sterilized by autoclaving at a pressure of 15 lbs/in^2 for 20 minutes. Instruments were sterilized by flaming in absolute alcohol.

3. Aseptic Techniques

A. Transfer cabinet

All inoculations and manipulations involving sterile cultures or media were carried out in a transfer cabinet. The working surface was cleaned with 4% formaldehyde. The interior of the cabinet was sprayed with absolute alcohol and irradiated with ultra violet light (λ =2537 A°) for one hour before use to reduce contamination.

B. Surface Sterilization of Anthers

Excised anthers of Datura were surface sterilized by shaking with 0.1% (w/v) mercuric chloride solution for three minutes and repeatedly washed in sterile distilled water.

C. Initiation of Callus cultures from Anthers

These sterile anthers were cut into two to three mm long bits with a sterile scalpel and transferred to the medium which was, by preliminary experiments, found to be suitable for the initiation of callus growth. The culture vessels were incubated at 26±2°C in light. Anthers over grown with callus tissues were cut into two to three equal pieces with a sterile scalpel and transferred to flasks containing freshly prepared media.

4. Culture Techniques

Routinely the callus tissues were subcultured every four weeks. For subculturing each callus mass was cut by a sterile scalpel into two to four equal parts and the later transferred aseptically to other flasks containing equal volumes of freshly made media. Clones of callus cultures were established by selecting a healthy looking and fast growing pieces of callus and regularly subculturing it until a large amount of callus tissue was obtained. For inoculation of an experiment weighed amounts of callus pieces were used from such stock of clonal material.

5. Measurements of Growth

A. Fresh weight

In case of tissues used for inoculating the experiment, fresh weights were determined by weighing aseptically on the Torsion balance (Varmax, Polland). Final fresh weights were determined on a Mettler balance (Zurich) after gently pressing the callus tissue between the folds of blotting paper to remove the excess of surface water.

B. Dry weight

Dry weights of callus tissues were determined by drying a weighed mass of callus to a constant weight at 60°C.

C. Total cell Number

Growth measurements by weights were supplemented by determination of total cell number of the callus culture. Out of total cell mass from replicate culture flasks harvested at different intervals of time, fixed weight of tissue was used for the determination of cell number. 100 mg of fresh weight was suspended in 10 ml of 5 per cent (w/v) chromium trioxide solution. The suspension was then put in an oven at 60°C for an hour and then shaken overnight on a rotary shaker. This method effectively separated cells of aggregates but did not bring about cell disintegration. After removing the chromic acid, the contents were diluted to a fixed volume with water, the number of cells in each ml of suspension was determined. 0.01 ml aliquots were next pipetted onto a clean slide and the slide scanned under a microscope, counting all the cells. From the mean of 10 such counts, the total number of cells in 1 ml was calculated.

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. Sugar estimation

Known amounts of dry tissues were used in estimating total and free sugars. Aqueous extract was prepared for free sugars and kept in boiling water bath for one hour. For total sugar the extract was prepared in 6 N hydrochloric acid and kept in boiling water bath for half an hour. The extracts were then filtered through Whatman No.1 filter paper and the sugars were estimated from the filtrate by Nelson's (1955) method. The difference between total and free sugars was taken as bound sugars.

B. Nitrogen estimation

A method previously described by Givan and Collin (1967) for the assay of total, soluble and insoluble nitrogen has been used with some modifications.

I. About 0.5 g of tissue was homogenised with 5 ml of ice-cold 10 per cent (w/v) trichloroacetic acid (TCA), and the homogenate transferred to ice-cold test tubes. After stirring the contents for five minutes with the tubes immersed in ice, the TCA extract was separated from the cell debris by filtering with Whatman No.1 filter paper. The filtrate was collected in a filter tube. Extraction was completed by using 3 ml of 10 per cent

TCA to wash out the test tube and cell debris on the filter.

II. The contents of filter tube were transferred in micro-kjeldahl flask. The blank was prepared from 8 ml of 10 per cent TCA and evaporated. For both total and TCA soluble nitrogen determinations, the dried contents of the Kjeldahl flasks were digested for 5-6 hours with 3 ml of concentrated H_2SO_4 and 0.5 g of catalyst mixture (K_2SO_4 and $CuSO_4.5H_2O$ in 1 : 1 ratio). When the hot digest became green and turned colourless on cooling, it was transferred quantitatively and diluted to 25 ml in volumetric flask.

III. 5 ml aliquots from this flask were distilled with 10 ml of 40 per cent NaOH in a Markham still and the released ammonia collected in 10 ml of 3 per cent boric acid (H_3BO_4) containing a mixed indicator (3 parts 0.1 per cent bromocresol green and 2 parts 0.1 per cent methyl red in ethanol).

IV. The boric acid was titrated against 0.02N sulphuric acid to pink colour. Insoluble nitrogen was taken as the difference between total nitrogen and TCA soluble nitrogen. Protein values were obtained by

multiplying with 6.25 values obtained for total nitrogen.

7. Preparation of Cell free extracts

Cell free extracts were prepared from tissues collected and pooled after each harvest. All preparations were carried out at 0 to 4°C. The tissue was ground in chilled mortar and pestel with half of its weight of acid-washed glass powder and 5 volumes of 0.1 M phosphate buffer pH 7.5. The homogenised material was then centrifuged at 3000 x g for 15 minutes and the supernatent was used for enzyme assay.

For the preparation of cell free extracts of nitrate reductase 0.1 M Tris HCl buffer pH 7.4 with 0.001 M cysteine was used in place of phosphate buffer. For the preparation of cell free extracts of acid phosphatase 0.95% potassium chloride solution was used in place of phosphate buffer.

Protein in the cell free extracts was estimated by the method of Lowry et al. (1951).

8. Enzyme assays

A. Acid Phosphatase

Acid phosphatase activity was determined by the

estimation of the liberated inorganic phosphorous (Shinowara et al., 1942).

The assay system consisted of 240 µ moles of sodium glycerophosphate 150 µ moles of sodium acetate buffer (pH 5.5), 10 µ micromoles of magnesium acetate and 0.5 ml of enzyme extract in total volume of 5 ml. The blanks were run along with the test system. The system was incubated at 37°C for 90 minutes. The reaction was stopped by addition of 1.0 ml of 30 per cent cold trichloroacetic acid and centrifuged. The supernatents were analysed for inorganic phosphorus by the method of Fiske and Subbarow (1925).

A unit of enzyme is that amount which liberates 1 µ g of inorganic phosphorous in 90 minutes at 37°C. Specific activity is defined as units per mg protein.

B. Invertase

Invertase activity was determined by measuring quantitatively the hexose formed by the method of Nelson (1955).

The reaction mixture contained 150 µ moles of sodium acetate buffer (pH 5.0), 29.2 µ moles of sucrose and 0.5 ml of 20% enzyme extract in a total volume of

4.0 ml. Blank tubes contained boiled enzyme. Incubation was carried out at 37°C for 60 minutes and the reaction was stopped by adding 3.0 ml 0.5 M dibasic sodium phosphate and heating in a boiling water bath for 2 minutes. Precipitated protein was filtered and aliquotes of the filtrates were analysed for the reducing sugars.

A unit of invertase activity is that amount which liberates 1 µ g of reducing sugar in 60 minutes at 37°C.

C. Glutamic Oxaloacetic Transaminase (GOT or Asparto Amino Transferase)

GOT activity was determined by estimating the glutamic acid formed by transamination (Karmon et al., 1955). The assay system used in this method consisted of 100 µ moles of phosphate buffer pH 8.3, 12 µ moles of aspartate, 50 µ moles of 2-oxo-glutarate, 10 µ g of pyridoxal phosphate and 0.5 ml of enzyme extract in a total volume of 2.0 ml. The system was incubated at 37°C for 1 hour. The reaction was stopped by the addition of 0.2 ml of 100% TCA. The precipitate formed was centrifuged off and a sample of the supernatant was spotted on Whatman No.1 sheet and developed butanol-acetic acid water (40 : 5 : 7) for 16 hours in the horizontal phase Glutamic acid formed was visualized by ninhydrin

and estimated by the method of Giri et al. (1953).

A unit of enzyme is that amount of the enzyme which produces 1 μg of glutamate in 1 hour at 37°C.

D. Nitrate Reductase

The method used was basically that described by Nason and Evans (1953 and 1955) and Cove (1966).

The activity of a sample to produce nitrite from nitrate was tested and nitrite formed determined colorimetrically. The assay system contains 22.5 mg KNO_3, 0.2 mg NADPH, 3.5 μg FAD, 0.05 M potassium phosphate buffer (pH 7.5) and 0.5 ml of the enzyme extract in a final volume of 3.0 ml. After 15 minutes incubation at 30°C the reaction was stopped by the addition of sulphanilamide, followed by 0.5 ml of the naphthylenediamine reagent to develop the colour. After 10 minutes the density of the colour was read on a colorimeter with 540 mμ filter. Control tubes lacking NADPH were used to correct for nitrite present in the extract, and any turbidity caused by the sample.

A unit of enzyme is defined as the amount of enzyme which can reduce to 1 mμ mole of substrate in 1 hour.

9. Histological Procedure

To determine the origin of the callus in the anther segments and to examine the structural organization within the callus histological preparations were made. Tissues were fixed in Formalin Acetic acid Alcohol (FAA) made up according to Johansen (1940). After 24 hours in fixative, they were washed several times in 70 per cent alcohol. After dehydrating the tissues by passing through alcohol Xylene series, they were embedded in paraffin wax. Serial sections were cut at 10 µ. Sections were stained in safranin fast-green. After usual dehydration through ethyle alcohol and xylene, the sections were mounted in Canada Balsam.

10. Photomicrographs

The phtomicrographs of the histological preparations were taken on Leitz photomicrography equipment of the reflex type. Exakta and/or Contax camera was used for graph copying. Orwo-Documentation Neg.-film used for most of the work. Developer used was Kodak DA-163. The Steindor microscope fitted with x10 or x5 projection eyepiece and 10/0.30 objective was used.