CHAPTER II

MATERIALS AND METHODS

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1. Plant Material

The experiments described in the present thesis were conducted with the seedlings of *Crotalaria juncea* L. (sun hemp, family Papilionaceae) and the callus tissues derived from the seedling.

Seeds of Crotalaria were collected from the local field and surface sterilized in 0.1% (w/v) mercuric chloride solution for five minutes. After thorough washing with distilled water, they were transferred to moistened filter papers in petri dishes. The seeds were allowed to germinate in light at 26± 2°C. *In vivo* experiments were conducted during germination of these seeds for initial five days.

In vitro experiments were conducted with the callus tissues derived aseptically from the above seedling. Callus was initiated on Murashige and Skoog (MS) medium (1962) supplemented with 2.0 mg/l 2, 4-D, 2.0 mg/l indoleacetic acid (IAA) and 2.0 mg/l kinetin (Shah and Mehta, 1975). After initial subcultures the

callus was transferred and maintained on MS medium supplemented with 2.0 mg/l 2,4-D alone.

2. Culture Media

The completely defined medium evolved in the present investigation and which supported rapid and continuous growth of the tissue is given in Table 1. The other medium tested is given in Table 2.

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). Fine chemicals were purchased from Sigma Chemical Co., U.S.A. The basic media were prepared from concentrated stock solutions which had been stored at 2-4^{o}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 medium were added in the order shown in Table 1. The supplements to be incorporated into

Table 1 : Modified Murashige and Skoog's (1962) 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	1650.0
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
	$Na_2MoO_4.2H_2O$	0.05		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	5	370.0
	$MnSO_4.4H_2O$	4.46		22.3
	$ZnSO_4.7H_2O$	1.72		8.6
	$CuSO_4.5H_2O$	0.005		0.025
* F	Na_2.EDTA	7.4	5	37.35
	$FeSO_4.7H_2O$	5.57		27.85
G	Thiamine HCl	0.2	5	1.0
	Nicotinic acid	0.2		1.0
	Pyridoxin HCl	0.2		1.0
	Glycine	0.8		4.0

Addenum : Sucrose 20 g/l (20 g/l in original), Myoinositol 100 mg/l, 2,4-Dichlorophenoxy-acetic acid 2 mg/l and Agar 8 g/l.

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

* The $FeSO_4.7H_2O$ is dissolved in approx. 200 ml distilled water. The Na_2EDTA is dissolved in approx. 200 ml distilled water separately and heated and mixed (under continuous stirring) with $FeSO_4.7H_2O$ soln. After cooling the volume is adjusted to 1000 ml. Heating and stirring result in a more stable Fe EDTA complex.

Table 2 : White's (1954) Medium

Constituents		Concentration in 1 litre of complete medium expressed as mg of hydrated salts
I. Inorganic salts		
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	$FeC_6H_5O_7.5H_2O$	2.00
Boric acid	H_3BO_3	1.50
Mangenese sulphate	$MnSO_4.4H_2O$	6.65
Potassium iodide	KI	0.75
Zinc sulphate	$SnSO_4.7H_2O$	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-Dichlophenoxyacetic 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.

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 Elico pH meter with the help of 0.1 N HCl or 0.1 N NaOH as found necessary. The medium was solidified with E. Merk (India) 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 either two layers of aluminium foil which had been sterilized in a flame or non-absorbent cotton-wool plugs covered with gauge.

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 the pH, known volumes of media were distributed in culture vessels for autoclaving. The mouth of the culture vessels was closed with non-adsorbent cotton-wool plugs covered by gauge. Brown paper coverings 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 cabinet. The working surface was cleaned with 4.0% formaldehyde. The interior of the cabinet was sprayed with absolute alcohol and irradiated with ultra violet (λ =2537 A^o) light for 60 minutes before use to avoid contamination.

4. Culture Techniques

A. Initiation of Seedling cultures :

The seeds of Crotalaria juncea L. were soaked overnight in distilled water. They were surface sterilized in 0.1 % (w/v) mercuric chloride solution for five minutes and after thorough washing with sterile distilled water, they were transferred aseptically to moistened sterile filter papers in sterile petri dishes. The seeds were allowed to germinate in dark at 26± 2°C for 3-4 days. On the 4th day of germination, seedlings were cut into pieces of 3-5 mm length and transferred aseptically on the surface of solid media in Erlenmeyer flasks. The flasks were incubated in continuous light at 26± 2°C. The best initiated callus was used for further experiments.

B. Stock Cultures :

Tissue fragments from the 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.

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 (speed 120 rpm, model of Emenvce, Poona) in an illuminated constant temperature ($26 \pm 2^{o}C$) culture room. The suspension cultures were maintained by subculturing every 4 weeks. 5 ml aliquots of cell suspension were pipetted out into 40 ml of freshly made liquid medium of the same composition. Cell suspensions so obtained were used as inocula for 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 tissue grown on agar media was carefully

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

B. Dry weights :

Dry weights of the callus 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 (India and Germany) or Sigma Chemical Co. (U.S.A.).

A. Extraction and Estimation of Phenolic Compounds :

Total polyphenols were extracted by grinding 25 mg (dry wt.) samples of tissue using mortor and pestle with 8 ml aliquot of ice-cold 80 % (v/v) ethanol. The homogenate was centrifuged at 5000 X g for 10 minutes.

The extract was collected and process was repeated with the residue. Finally, the residue was washed with 8 ml aliquot of ice-cold 80 % (v/v) ethanol. The successive extracts 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 was diluted with distilled water to 8.5 ml. 0.5 ml of Folin-phenol reagent was added and the contents were well mixed. 3 minutes later, 1 ml saturated sodium bicarbonate solution was added and the tubes were thoroughly shaken. The colour development was measured at 725 nm after standing for 60 minutes. The standard curve was prepared using chlorogenic acid and all the concentrations were expressed in terms of µgrams of this compound.

7. Enzyme Assays

The seedlings as well as the cultured tissues were assayed for the following enzymes :

A. Peroxidase,

B. IAA oxidase,

C. Ammonia-lyases : Phenylalanine ammonia-lyase and Tyrosine ammonia-lyase,

D. p-Coumarate : CoA ligase (Cinnamyl CoA synthetase), and

E. Transaminases : Phenylalanine transaminase and Tyrosine transaminase.

The methods adopted for their extraction and estimation are as follows :-

A. Peroxidase :

Cell-free extracts were prepared from tissues collected and pooled after each treatment. The tissue (1 g F. Wt.) was homogenized and extracted with 0.1 M phosphate buffer, pH 6.2. The supernatant was collected and made to 25 ml with the same buffer after centrifugation at 15,000 X g. The resultant cell-free extract was used for the assay of total peroxidase activity. All the operations were carried out at 0 to 4°C.

The enzyme peroxidase was assayed by the modification of the technique described by Maehly and Chance (1954). Oxidation of guaiacol was followed colorimetrically at

470 nm. The enzyme system, in a colorimeter tube, consisted of 30 mM guaiacol, 0.1 M phosphat buffer, pH 6.2 and an aliquot of cell-free extract in a total volume of 10 ml. At zero time, 0.1 ml of 10 mM hydrogen peroxide solution was added and the tube was rapidly inverted. The increase in colour intensity was followed at 470 nm in the colorimeter (Lichtelcktrisches Kolorimeter, Modell J. nach Dr. B. Lange, Germany) with readings at 15, 30, 45, 60 and 75 seconds, since the rate of colour development over this time is linear. Peroxidase activity is expressed as units per culture. One unit of the enzyme activity is defined as the amount of enzyme required to cause an increase in optical density of 10 per minute under the conditions of assay. Specific activity is expressed as units per mg of protein.

B. Indoleacetic acid oxidase (IAA oxidase) :

1 g of fresh tissue was extracted in a mortor and pestle with 100 mg neutral glass powder and 0.1 M phosphate buffer pH 6.2. The slurry was centrifuged at 15,000 X g for 20 minutes. The supernatant was collected

and made to 25 ml. All operations were done at 0 to 40° C. The clear supernatant was used as the enzyme source.

The assay system in a total volume of 3 ml consisted of 200 µgrams of IAA, 0.1 mM 2,4-dichlorophenol, 0.1 mM $MnCl_2$ and 0.1 M citrate-phosphate buffer pH 4. This was incubated in a water bath at 37° C for 25 minutes. The residual IAA was estimated with the modified Salkowski reagent (Gordon and Webber, 1951). The modified Salkowski reagent contained 50 ml of 35% perchloric acid and 1 ml of 0.5 M ferric chloride. To the system was added 6 ml of Salkowski reagent and the mixture was incubated for 20 minutes at 37°C in a water bath. The colour of the solution was read against blank in a colorimeter (Dr. B. Lange's) at 530 nm. A calibration curve for the determination of the residual IAA was constructed over a range of 10 to 100 µgrams of IAA. The enzyme activity is expressed as units per culture. One unit is defined as the amount of enzyme required to destroy one mgram of IAA under the conditions of assay. Specific activity is expressed as units per mg of protein.

C. Ammonia-lyases : Phenylalanine ammonia-lyase and Tyrosine ammonia-lyase :

Phenylalanine ammonia-lyase (PAL) and Tyrosine ammonia-lyase (TAL) assays were carried out using acetone powders.

Acetone Powder Preparation

The collected samples (2-3 g by F. Wt.) were frozen at -20°C and ground with 25 ml of acetone previously chilled to -20°C. The slurry was filtered on a sinter glass Gooch crucible under vacuum and the residue was washed twice with chilled acetone and spread to dry at room temperature. The dried powders were transferred to vials and stored at -20°C. The powders were stable for several months.

Enzyme Extraction

The enzyme was extracted by grinding acetone powder (25 mg/10 ml) in a precooled mortor along with glass powder and 25 mM tris-HCl buffer, pH 8.8. The extract was centrifuged in cold at 10,000 X g for 20 minutes. The supernatant was used as the crude enzyme source.

Assay of PAL and TAL

The reaction mixture containing 2.5 mM L-Phenylalanine or L-tyrosine, 25 mM tris-HCl buffer, pH 8.8 and enzyme preparation in a total volume of 2 ml was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 1.0 ml of 0.5 M HCl. The product of the reaction, cinnamate or p-coumarate, respectively, was measured at 278 or 308 nm in a spectrophotometer (C.Z. Instrumentation, Germany, Model VSU2-P). The molar extinction coefficients for connamic acid and p-coumaric acid were 20,100 and 15,000 litre mol^{-1} cm^{-1} respectively. Enzyme activity is expressed as units per culture. One unit of the enzyme is defined as the amount of enzyme required for the formation of ten μmole of cinnamate or coumarate at 37°/hr. Specific activity is expressed as units per mg of protein.

D. p-Coumarate : CoA ligase (Cinnamyl CoA synthetase) :

The samples were macerated at 0°C in a precooled mortar along with neutral glass powder, phosphate-KOH

buffer (0.1 M, pH 8.0) which also contained 0.25 M sucrose, 1 mM EDTA and 0.02 M ascorbate. The macerate was filtered through cheese cloth. After the pH had been adjusted to 8.0, the filtrate was centrifuged at 15,000 X g for 20 minutes at $0^{o}C$. The clear supernatant was used as crude enzyme source.

p-Coumarate : Coa ligase was assayed according to the procedure of Walton and Butt (1971) as described for unsubstituted cinnamic acid. Two cuvettes, each containing 130 μmoles of tris-HCl buffer (0.1 M, pH 8.5), 20 μmoles of ascorbate, 5 μmoles of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 5 μmoles of ATP, 0.3 μmoles of CoA and the enzyme preparation in a total volume of 2.0 ml, were flushed with N_2 and stoppered. The reaction was followed after addition of 5 μmoles of cinnamic acid (dissolved in 1% $KHCO_3$) to one cuvette, by the increase in absorbancy at 311 nm. The amount of cinnamyl CoA formed were calculated from the extinction coefficients given by Gross and Zenk (1966). The enzyme activity is expressed as the units per culture per hour. One unit is defined as the amount of enzyme required for the formation one μmole of cinnamyl CoA under the

conditions of assay. Specific activity is expressed as units/hr/mg of protein.

E. Transaminases : Phenylalanine transaminase and Tyrosine transaminase :

Tissue samples (1 g F. Wt.) were ground with glass powder and 0.1 M borate buffer, pH 8.0. The extract was centrifuged at 15,000 x g for 15 minutes and the supernatant was collected and made to 10 ml. This clear supernatant was used directly as enzyme source.

In each experiment 50 μmoles of keto acid (α-keto-glutaric acid), 50 μmoles of amino acid (L-phenyl-alanine or L-tyrosine), 50 μg of pyridoxal phosphate, 0.1 M borate buffer, pH 8.0 and an enzyme extract in a total volume of 3 ml was incubated for 60 minutes at 37^{o}C in glass tubes closed by cotton. The amino acid and keto acid solutions were adjusted to pH 7.5 to 8.0 before use. After incubation the mixture was treated with 0.1 ml of 100% (w/v) TCA. The precipitated proteins were removed by centrifugation. The optical density values of clear incubation mixtures were read in a spectrophotometer (VSU2-P model) at 300 nm for phenyl-

pyruvic acid and 310 nm for p-hydroxyphenylpyruvic acid. The molar extinction coefficients of 6650 for phenylpyruvic acid and 5350 for p-hydroxyphenylpyruvic acid were determined under these conditions and the amount of phenylpyruvic acid and p-hydroxyphenylpyruvic acid were calculated from these extinction coefficients. The enzyme activity is expressed as units per culture at 37°C per hour. One unit is defined as the amount of enzyme required for the formation of 100 µmoles of phenylpyruvic acid or hydroxyphenylpyruvic acid under the conditions of assay. Specific activity is expressed as units/hr/mg of protein at 37°C.

8. Polyacrylamide Disc Gel Electrophoresis

To determine the purity of the purified PAL, disc gel electrophoresis was performed by the method of Davis (1964). Samples (50-100 g of protein) were applied to the stacking gel in 60% sucrose. Gels (7.5% crosslinked polyacrylamide) were subjected to 4 ma/tube at 4°C. Bromophenol was the marker dye, and after removal, the protein in the gels was stained with Coomassie Brilliant Blue R. The gels were freed of

unbound dye with 7.5% acetic acid.

9. Molecular weight Estimation by Gel Filtration

The molecular weight of the enzyme (PAL) was determined by gel filtration using Sephadex G-200 (40-200 μ) from which the fine particles in suspension have been decanted. The Sephadex G-200 was equilibrated in 25 mM tris-HCl (pH 8.8) and 10 mM 2-mercaptoethanol and packed to give a column dimension of 3 X 100 cm. The column was calibrated with the following standards : Blue dextran (Sigma : Mol. Wt. 2,000,000), urease (Sigma : Mol. Wt. 483,000, type IV), catalase (Sigma : Mol. Wt. 244,000), bovine serum albumin, monomer (Sigma : Mol. Wt. 68,000) and ovalbumin (Sigma : Mol. Wt. 43,000). Fractions (1.0 ml) were collected and assayed for enzyme activities. Urease was assayed by nesslerization. Standard procedures were used to determine the activities of catalase (Beers and Sizer, 1952), bovine serum albumin (procedure of Reinhold et al., 1950 as described by Oser, 1965) and ovalbumin (spectrophotometric estimation). All the standards listed above plus phenyl-

alanine ammonia-lyase, in a total volume of 1.0 ml, were layered on the gel column and was eluted with tris-HCl (pH 8.8) buffer.

10. PAL Subunits

The method of Weber and Osborn (1969) with 7.5% (w/v) gels was followed for polyacrylamide gel electrophoresis of proteins in sodium dodecyl sulphate (SDS). The standard marker polypeptides for this purpose were y-globulin (unreduced), Mol. Wt. 150,000; bovine serum albumin, monomer Mol. Wt. 68,000; catalase, monomer Mol. Wt. 58,000 and ovalbumin, Mol. Wt. 43,000. To determine the subunit Mol. Wt. of PAL, these standards and the experimental molecules were treated as described below : The subunit Mol. Wts. of the standards were plotted logarithmically versus their mobility towards the anode after disc gel electrophoresis. The resultant graph described a linear relationship which was used to interpret the mobility of PAL subunits in terms of their molecular weights.

Each protein was dissociated into subunits by treatment with sodium dodecyl sulphate (1%, w/v)

containing 2-mercaptoethanol (1%, v/v) at 100°C for 2 minutes. Electrophoresis was performed at a constant current of 4 ma/gel with the positive electrode in the lower chamber. Bromophenol Blue was the marker dye and the gels were stained with Coomassie Brilliant Blue R.

11. Determination of Protein

In all the procedures described above, protein was determined by the method of Lowry, et al. (1951) with bovine serum albumin as standard.

12. Photomicrography

Exakta and/or Contax cameras were used to photograph the culture vessels showing growth. Orwo-documentation Neg-film was used for most of the work. Developer used was Kodak DA-163. Microfilm-xerography technique was used for line drawings.