CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

(1) Plant Material

Evolvulus alsinoides L., a member of the family Convolvulaceae, is a common weed growing all over India, all the year round. It is a profusely branched herbaceous plant spreading over the ground with blue flowers. Plant material for "In vivo" and "In vitro" studies were collected from the University Campus. Callus was initiated from the young leaves in Murashige and Skoog's medium supplemented with 2.0 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.4 mg/l kinetin (Table I).

(2) Culture Medium

The medium used for the initiation and growth of tissues comprised of the combinations of macroelements, microelements and vitamins as formulated by Murashige and Skoog (1962) and depicted in Table I .

(A) Preparation of the media

The basic medium was prepared from concentrated stock solutions stored in Refrigerator at 5-10°C. The medium as well as stock solutions were prepared in double glass distilled water. The supplements to be incorporated in the media were added before the adjustment of the final volume. The pH of the medium was adjusted to 5.8 with an Elicon pH

Table - 2. Murashige and Skoog's (1962) Modified 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	1900.00
C	H_3BO_3	1.24		6.2
	KH_2PO_4	34.00		170.0
	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	440.0
	$MgSO_4.4H_2O$	74.0		370.0
E	$MnSO_4.4H_2O$	4.46	5	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	0.02		0.1
	Nicotinic acid	0.1	5	0.5
	Pyridoxine-HCl	0.1		0.5
	Glycine	0.4		2.0

Addendum : Sucrose 20 gm/l, Inositol 100 mg/l.

The stock solutions A - G were prepared and stored in a refrigerator (not more than 4 weeks) and mixed while preparing the final medium.

* The $FeSO_4.7H_2O$ is dissolved in approximately 20 ml distilled water. The Na_2EDTA is dissolved in approximately 20 ml distilled water, separately and heated and mixed (under continuous stirring) with $FeSO_4.7H_2O$ soln. After cooling the volume is adjusted to 100 ml. Heating and stirring result in a more stable Fe EDTA complex.

meter. HCl (0.1 N) or NaOH (0.1 N) were used to adjust the pH. The medium was solidified using 0.8% of Centron agar.

The chemicals used for preparing the media were of research grade and were obtained from S. M. (Guaranteed reagent), British Drug House (Analar grade) or E. Merck (guaranteed reagent). Auxins, Vitamins, cytokinins and amino-acids were purchased from the Sigma Chemical Co., U.S.A. Mesoinositol and a few aminoacids were also obtained from Loba Chemicals, Austria.

(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 aluminium foils sterilized in a flame.

The glasswares 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 continuous rinsing with tap water. Further the glassware was washed with detergent Teepol (B.D.H.) and then thoroughly washed with tap water to remove the teepol completely. Finally the glassware was sterilized by autoclaving at a pressure of 15 lbs/in^2 for 15 minutes and then rinsed with double glass distilled water and dried in an oven.

(C) Sterilization of Media and Culture Vessels

Known volumes of the medium (pH adjusted to 5.8),

semisolid or liquid, were distributed in culture vessels. The mouth of the culture vessels was closed with non-absorbent cotton plugs specially prepared with bandage cloth. These vessels were covered by brown paper so as 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 15 minutes. The instruments used for inoculation were sterilized by flaming them with absolute alcohol.

3. Aseptic Techniques

(A) Inoculation Chamber

All inoculations involving sterile cultures or media or seed germination were carried out in an inoculation chamber. The working space in the inoculation chamber was cleaned with 4% formaldehyde. The interior of the cabinet was saturated with an acrosol of 2% thymol and 2% glycerine in 90% ethanol.

The Instruments used in aseptic operations were sterilized by flaming with absolute alcohol inside the cabinet. Just before the use, the cabinet was exposed to ultra violet light (λ = 2537 A°) for about 2 hours before inoculation to reduce contamination.

(B) Surface Sterilization

Evolvulus seeds for germination experiments were collected from the plants growing in the University Campus. The seeds were kept in water for about 2 hours so that the

seeds imbibed water and then they were washed thoroughly with glass distilled water. These seeds were surface sterilized by putting them in 0.1% (w/v) mercuric chloride solution for about 3 minutes, shaking vigorously. These seeds were repeatedly washed for 2-3 times with sterile distilled water to remove the mercuric chloride. The seeds were then transferred into the sterilized petri dishes containing wetted filter papers for germination studies. The petri dishes were incubated in a culture room at 25±2°C in light.

In the case of leaves, they were thoroughly washed with running tap water to remove soil and other dust particles. They were then rinsed with glass distilled water. The leaves were first sterilized with 70% ethanol for 1-3 minutes and then with mercuric chloride (0.1%) for 5 minutes. After repeated washing for 2-3 times with sterile distilled water to remove mercuric chloride, the leaf explants were ready for culture.

4. Culture Techniques

(A) Initiation of Leaf Cultures

Fresh young leaves surface sterilized and then repeatedly washed as stated above were cut into discs and asceptically transferred into 150 ml Erlenmeyer flasks containing 40 ml agar medium for the initiation of callus cultures. The culture vessels were incubated at 25±2°C. Leaves developed callus within a week's period.

(B) Stock cultures

Stock cultures were maintained by repeatedly sub-culturing the tissue in fresh media after every 30 days. These well-developed callus tissues were used as inocula for the experiments.

(C) Suspension cultures

Cell suspensions were obtained by transferring pieces of callus tissues from agar media to the liquid media (of the same composition) kept in 150 ml Erlenmeyer flasks. These flasks were continuously agitated on a horizontal rotary shaker (speed 120 rpm) at 25±2°C in a constantly illuminated culture room. 3000 Lux light intensity was provided to the cultures. The suspension cultures were sub-cultured every 30 days. Measured aliquots of cell suspension (5 ml) were transferred with the help of a sterilized test tube into 40 ml of freshly prepared liquid medium of the same constitution. Cell suspensions so obtained were used as inocula for experiments.

5. Measurement of Growth

Growth was measured by determining increase in fresh and dry weights of the tissue. For growth measurements five replicate cultures were harvested at fixed intervals of time. Standard error was calculated to ascertain statistical significance of growth data.

(A) Fresh Weight

In the case of callus cultures grown on solid medium, the tissue was removed onto a previously weighed aluminium foil and the weight was determined on a semimicro single pan balance (K. Roy, K-15). In case of suspension cultures, the cells/tissue was collected by filtration through a Sinter glass Gooch crucible and the fresh weight was measured as in the case of static cultures.

(B) Dry Weight

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

6. Chemical Analysis

(A) Extraction and Estimation of Total Alkaloid

200 mg of dry tissue from static or suspension cultures was powdered and macerated with 0.4 ml of ammonium hydroxide, 0.5 ml of ethanol and 0.5 ml of diethyl ether for overnight. This was mixed in chloroform (25 ml) and boiled for about 20 minutes and then extracted through a specially prepared glass column (Fig. 5). The extraction was repeated for 2-3 times. Soxhlet extraction was also tried to compare the results. Extraction through a glass column gave similar results with that obtained in soxhlet extraction, and hence

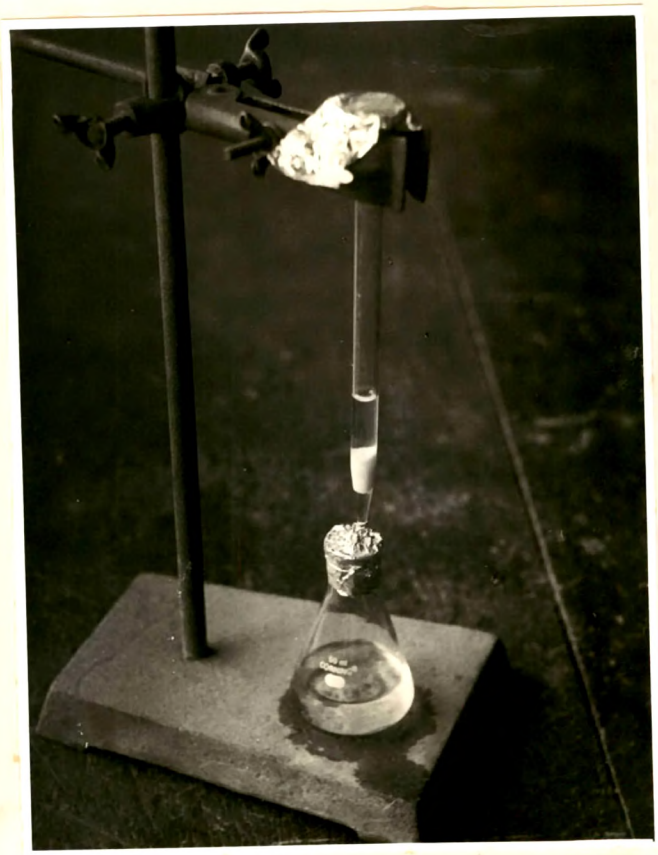

FIG. 5. Glass column for extraction of Alkaloids.

the former method was used for further alkaloid estimation. Besides, this method had an advantage over soxhlet extraction as it took less time and so more samples could be examined in a short period. Soxhlet extraction was, however, carried out for "in vivo" studies as large quantities of powder could not be extracted through the glass column. In this case 5 gms of different plant parts were dried separately and the dried powder was evaporated for overnight in ammonium hydroxide (10 ml), Ethanol (12.5 ml) and diethyl ether (12.5 ml) before soxhlet extraction. The chloroform extract was dried in vacuo and the residue was dissolved in ethanol and tested for ergot alkaloids with Van urk's reagent. In case of quantitative studies preparative Thin layer chromatography was used.

(B) Chromatographic procedures

(i) Thin-Layer-Chromatography (TLC) for Alkaloid

The alkaloids were estimated quantitatively by Thin-Layer-Chromatography. Glass plates (20 X 20 cm) were used for TLC. Slurry of Kieselgel. G (Typ 60) (15 gms in 30 ml distilled water) was prepared and spread on the glass plates using an applicator. The thickness was maintained at 250 μ. These plates were dried in the air and then activated at 60°C for 30 minutes. The chloroform extract of tissue was evaporated to dryness and redissolved in ethanol. This was

applied on a spot, 1 cm from the lower edge of the plate, and run for 15 cm using the solvent system : Chloroform : Ethanol (9 : 1). The plates were air dried and sprayed with modified Van urk's reagent. Two spots with characteristic blue colour of ergot alkaloids were observed. These spots were scraped out and dissolved in ethanol and the absorbancy was read in a Lichtelektrisches Kolorimeter Modell. J at 540 nm. Total alkaloids were estimated against Ergocristine as a standard.

(ii) Paper Chromatography for Amino acids

Whatman No. 1 paper (20 cm X 20 cm) was used for analysis of amino acids qualitatively and quantitatively. The tissue was analysed, qualitatively and quantitatively for their amino acid contents. Extracts were prepared in 80% ethanol. 50 mg of dry tissue was homogenized in equeous alcohol and incubated for 24 hours. The supernatant of this was loaded on the corner of the chromatographic paper, 1 cm from the lower side of the paper for two dimensional chromatography. The solvent systems used were as described by Brenner et al. (1964) :-

Solvent 1. Butanol : Glacial Acetic acid : Water, (12 : 3 : 5)

Solvent 2. Phenol : Water (3 : 1, by weight).

125 ml of water was added to a 500 gm bottle of phenol,

replaced the stopper and it was allowed to stand overnight. Before use, a few drops of ammonia was added and mixed well.

The air dried papers were sprayed with Ninhydrin reagent (Tribetohydrindene hydrate) and developed at 60°C for 10 minutes. The spots of amino acids where quantitative study was to be done, were then eluted separately and dissolved in 80% ethanol. The colour intensity was measured at 540 m μ on a colorimeter. Similarly, the standard amino acids were also run, eluted out and assayed coloriemetrically.

Enzyme Assays

In the present investigation, the progressive changes in the pattern of tryptophan synthetase was examined.

(A) Tryptophan synthetase (EC. 4.4.1.20)

The enzyme was assayed by modification of the method described by Charles Yano Fsby (1955). This is based on the caloriemetric determination of residual Indole. Dilute enzyme extracts were prepared with 0.5 M, phosphate buffer (pH 7.8) from 500 mg of fresh tissue. The enzyme system consisted of 0.08 ml of 0.005 M Indole soln., 0.4 ml of 0.2 M DL-serine, 0.1 ml pyridoxal phosphate soln. (200 ϒ ammonium pyridoxal phosphate per millilitre), 0.02 ml of 0.05 M glutathione (oxidised) soln., and 0.12 ml of 0.5 M phosphate buffer 9(pH 7.8) in a test tube. 0.2 ml of enzyme extract was added and 0.08 ml of distilled water was also

added to bring the final volume in the tube to 1 ml. The assay mixture was incubated at 37°C in a waterbath for 60 minutes. Reaction was terminated by addition of 0.2 ml of 5% NaOH after 60 minutes incubation at 37°C. Indole was extracted with the help of 4 ml toluene and estimated by addition of 4 ml of ethanol and 2 ml of colour reagent (PDAB). Colour reagent was prepared by dissolving 36 gms of p-dimethylaminobenzaldehyde in 500 ml of ethanol. 180 ml of con. HCl was added and after cooling the volume was brought to 1 litre with ethanol. The colour was allowed to develop for 60 minutes before reading the colour developed colorie-metrically at 540 nm. The enzyme activity is expressed as units per culture and one unit is defined as the amount of enzyme required to convert 1 micromole of Indole to 1 μ mole of tryptophan under the experimental conditions. Specific activity is expressed as units per mg of protein.

(B) Proteins

Proteins were estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Photography

Exakta and/or Contax cameras were used to photograph the culture vessels showing response under various treatments. 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.

*****