MATERIALS & METHODS

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1. PLANT MATERIAL

The work presented in the present thesis was carried out on two angiosperms. They are :

i) ***Boerhaavia diffusa L.***

Family : Nyctaginaceae, and

ii) ***Achyranthes aspera L.***

Family : Amaranthaceae

Plants growing in the M.S.University Campus and other localities were collected, when they were in their fertile stage of development. Herbarium sheet of each specimen viz., *B. diffusa L.* and *A. aspera L.* were prepared and maintained in the Herbarium of Botany Department.

2. CHEMICALS

The chemicals used in this study were of high purity (A.R.grade) obtained from Sisco Research Laboratories, S.D.Fine Chemicals, India and British Drug House, England. Fine chemicals were obtained from Sigma Chemicals, U.S.A. Authentic samples of punarnavine and achyranthine were obtained from Banaras Hindu University, Varanasi and Punjab University, Chandigarh respectively.

3. CULTURE VESSELS

Culture flasks (100/150/250/500 ml), culture tubes (25 x 150 mm) and all other glassware used were of Borosil brand. They were washed with chromic acid (40%) and thoroughly cleaned under running tap water until the acid was removed. They

were washed with teepol and finally rinsed with distilled water. Glasswares were dried in oven at 60°C for 24 h.

4. CHEMICAL ANALYSIS

Healthy mature plants of *B. diffusa* L. and *A. aspera* L. which were collected from various localities were washed under tap water, rinsed in distilled water and dried in oven at 40°C. These plants were separated in their individual organs *viz.*, root, stem, leaves, flowers, fruits and seeds. Using Sumeet grinder coarse powder of each sample was made and stored in glass bottles.

(i) Extraction of Alkaloid/s

Powder (10 g) of each sample of *B. diffusa* plants was extracted in soxhlet with ethanol (90%), following the procedures described by Singh and Udupa (1972). The extract was dried by evaporation and it was redissolved in water. Using separating funnel, the aqueous extract was separated into the water soluble and insoluble fractions. The presence of alkaloid/s in aqueous fraction was detected with Dragendorff's reagent. Rest of the extract was used for isolation and identification of the alkaloids.

A.aspera, each of the powder samples (10 g) was soxhlet extracted with ethanol (90%) separately, following the procedures described by Kapoor and Singh (1967). The extract was concentrated (¼ volume), acidified with sulphuric acid and mixed with chloroform in a separating funnel. Chloroform layer was discarded which contained the non-alkaloidal matter. The acidic layer was treated with alkaline sodium hydroxide and once again treated with chloroform. A portion of chloroform

layer when tested with Dragendorff's reagent gave positive test for the presence of alkaloid. The remaining portion of the extract was used for isolation and identification of the alkaloid/s.

(ii) Identification of Alkaloid/s

A thin layer chromatography (TLC) procedure was used to identify alkaloid/s. An authentic sample of punarnavine served as the control. Chromatographic plates (20 x 10 and 15 x 15 cm) were prepared with silica gel G (with binder). The plates were activated at 100°C for 40 min before use. The extract of each sample was spotted on the TLC plates along with the authentic sample of punarnavine. The TLC plates were run in chloroform : ethanol (2:1) solvent system. When Dragendorff's reagent was sprayed on these plates orange coloured spots of the isolated alkaloid fraction were developed. The Rf values of the samples along with that of the authentic punarnavine were calculated. Each spot of the alkaloid was eluted and kept in a vial for further purification.

For *A. aspera*, extract was spotted on TLC plates along with the authentic sample of achyranthine. Plates were run in n-butanol : acetic acid : water (4:1:5) solvent system. Plates were further sprayed with Dragendorff's reagent and Rf values of the alkaloid and of the authentic sample were calculated. The coloured spots were eluted with chloroform and kept in a vial.

(iii) Quantitative Estimation of Alkaloid/s

The quantitative estimation of alkaloid/s *viz.*, punarnavine and achyranthine, was conducted following the method described by Huber (1967). Powdered sample of

each plant material (0.2 g) was extracted with glacial acetic acid mixed with acetic anhydride (40 : 10 ml v/v) to which 0.5 g of mercuric acetate was added. This solution was titrated with 0.1 N acetus perchloric acid using crystal violet as an indicator [Acetus perchloric acid was prepared by mixing 8.5 ml of perchloric acid (70%) with 500 ml of glacial acetic acid and 21 ml of acetic anhydride, the volume was made to 1000 ml with glacial acetic acid]. Readings were taken when the colour of the solution changed to emerald green. Alkaloid content were expressed on percentage dry weight basis using the formulae :

(1) $N_1V_1 = N_2V_2$

Where N_1 = Normality of acetus perchloric acid

N_2 = Normality of plant extract

V_1 = Volume of acetus perchloric acid used

V_2 = Volume of plant extract

(2) Punarnavine/Achyranthine g/litre = N_2 X equivalent weight of Punarnavine/Achyranthine

5. COMPOSITION AND PREPARATION OF MEDIA

The basal media used were of White's (1954) and Murashige and Skoog's (1962) whose compositions have been given in Table I and II. Each medium was prepared from concentrated stock solutions which were prepared in glass double-distilled water and stored in glass bottles at 2-4°C. The supplements *viz.*, sucrose and phytohormones (Kn, BAP, $AdSO_4$, IAA, NAA, IBA, 2,4-D) were added to the basal medium before the final adjustment of the volume. The pH of the medium was adjusted to 5.8 using either 0.1 N HCl or 0.1 N NaOH.

TABLE I : White's (1954) Medium

I. Macroelements

		Concentration of salts in mg./litre of medium
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	288.00
Magneium 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 dihyrogen-orthophosphate	$NaH_2PO_4.2H_2O$	24.00

II. Microelements

Ferrous sulphate	$FeSO_4.7H_2O$	2.50
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

III. Vitamins

Glycine	3.00
Nicotinic acid	0.50
Pyridoxine hydrochloride	0.10
Thiamin hydrochloride	0.11

IV. Supplements Sucrose,Phytohormones

Table II : Murashige and Skoog's (1962) medium

I. Macroelements

		Concentration of salts in mg./litre of medium
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 orthophosphate	KH_2PO_4	170.00

II. Microelements

Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Manganese sulphate	$MnSO_4.H_2O$	22.3
Zinc sulphate	$ZnSO_4.7H_2O$	8.6
Copper sulphate	$CuSO_4.5H_2O$	0.025
*Ferrous sulphate	$FeSO_4.7H_2O$	27.85
**Disodium ethylene diamine tetracetic acid	Na_2EDTA	37.35

III. Vitamins

Thiamine hydrochloride	0.1
Nicotinic acid	0.5
Pyridoxine hydrochloride	0.5
Glycine	2.0
Myoinositol	100.0
IV. Supplements	Sucrose, Phytohormones

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

** The Na_2EDTA was dissolved in 200 ml of glass double distilled water, heated and mixed (under continuous stirring) with the $FeSO_4.7H_2O$ solution. After cooling the volume was adjusted to 1000 ml. Heating and more stirring resulted in a more stable FeEDTA complex.

6. STERILIZATION OF MEDIA AND CULTURE VESSELS

The medium was distributed (15/30 ml) in test tubes/Erlenmeyer flasks (150/250 ml) and the mouths of these culture vessels were plugged with non-absorbent cotton covered with gauze. They were wrapped with brown paper to prevent absorption of moisture during sterilization. A few empty flasks, petri-dishes, pipettes were wrapped in brown paper. These were sterilized by autoclaving at 15 lb/sq.in. pressure (120°C) for 20 min.

Amino acids used were sterilized with millipore membrane filter (0.22 μ) under aseptic conditions and added to the sterilized medium.

7. ASEPTIC TECHNIQUES

All aseptic manipulations were carried out in Laminar Flow Cabinet (Klenzaids, India). The cabinet was sprayed with aerosol and the working table was wiped with cotton swab soaked in Dettol. The culture flasks and other required material were transferred to the table. Stainless steel instruments *viz.*, scalpel, spatula, forceps, needles were dipped in alcohol and were flammed before use. The cabinet was sterilized by switching on UV light (λ- 2357 A°) for 30 min before use.

(i) Surface Sterilization of Explants

Seeds of `elite' plants were collected, surface sterilized with $HgCl_2$ (0.1%) and after washing with sterile distilled water they were germinated in sterile petri-dishes containing filter-papers. Root with tips (1 to 2 cm) of germinated seeds were excised and inoculated in liquid media for establishment of excised root-culture.

`Elite' plants of *B. diffusa* L. and *A. aspera* L. were collected in flasks containing distilled water. They were separated into individual organs (roots, stem, leaves) and were washed under tap water rinsed in distilled water and surface sterlized as mentioned above. Further, they were taken in petri-dishes and giving proper cuts, each explant *viz.*, root with tip (1-2 cm), stem (1-2 cm) and leaves (3-4 pieces) was inoculated on culture media.

(ii) Incubation

Culture flasks/tubes inoculated with explants were maintained in culture room at 25 ± 2°C under fluorescence light 16 h photoperiod. Root cultures were maintained on gyratory shaker (120 rpm) in culture room.

8. ESTABLISHMENT AND MAINTENANCE OF CULTURES

(i) Root Culture

Root cultures were initiated from roots with the tips intact 1-2 cm, from aseptically germinated seeds of *B. diffusa* L. The medium employed was (40 ml) of White's/MS liquid medium with sucrose and requisite dose of phytohormones (0.1 to 8 μM/1). Root clones established were regularly sub-cultured in fresh medium of the same composition at four weeks interval, which were maintained on gyratory shaker (120 rpm) in culture room 25 ± 2°C.

(ii) Callus cultures

B. diffusa L. and *A. aspera* L. `elite' plants were used for the induction of callus tissues from their stem/leaf/floral buds. Healthy fast growing callus masses (300 ± 20 mg) were subcultured regularly at four weeks interval to fresh medium

(30 ml) so that a ready callus stock was available for the experiments.

9. MEASUREMENT OF GROWTH

i) **Length** : Cultured roots were taken out from the flask, the length of the main axis of the root was measured with a thread which was determined with the help of scale.

ii)**Lateral Roots** : The number of lateral roots visible (0.5 cm in length) were counted from each sample of cultured roots and recorded .

iii)**Fresh Weight** : Fresh weight of roots/callus were determined by weighing them on preweighed aluminum foil taking care no liquid medium/agar remained adhered to root/callus.

(iv) **Dry Weight** : Dry weights were determined after drying the weighed amounts of harvested roots/callus on aluminum foil in an oven (60°C) until constant weights were recorded.

10. CHEMICAL ANALYSIS OF ROOTS/CALLUS IN CULTURE

Alkaloid fraction/s from dried samples of roots/callus of each treatment was determined according to the procedures standardized and employed for plant material. Quantity of alkaloid was expressed on percentage dry weight basis.

11. HISTOLOGICAL STUDIES

For histological studies, procedures described by Jensen (1962) were followed. Callus with morphogenesis induced were fixed in FAA (40% formalin : glacial acetic acid : 50% ethyl alcohol ; 5 : 5 : 90 ml) for 24 h and dehydrated in tertiary butyl alcohol series. Blocks were prepared in paraffin (M.P. 58°C). Ribbons were

cut at 10 μ on rotary microtome and mounted on slide using egg albumin. Sections were stained with toluidine blue and mounted in DPX (dextrene plasticizer of xylene).

12. PHOTOGRAPHY

Photomicrographs were taken on Carl Zeiss microscope with automatic photographic equipment using ORWO black and white or Konica coloured negative films.