MATERIALS AND METHODS

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1. PLANT MATERIAL

The work presented in this thesis has been carried out on *Withania somnifera* (L.) Dunal, (Family : Solanaceae) a perennial shrub.

Plants growing in the M S University Campus and various localities of Baroda *viz.*, Ellora Park, Karelibag, Chhani and Harni were collected. A Herbarium specimen was prepared and maintained in the Departmental Herbarium.

2. CHEMICALS

The chemicals used in this study were of high purity (A.R. grade) obtained from Sisco Research Laboratories, India and British Drug House (BDH) England. Fine chemicals including authentic sample of tropine were obtained from Sigma Chemicals, U.S.A.

3. CULTURE VESSELS

Culture flasks (100/150/250/500 ml), 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 then washed with teepol, rinsed with distilled water and dried in oven (60°C) for 24 h.

4. CHEMICAL ANALYSIS

Healthy, fertile plants of *W.somnifera* (L.) Dunal were collected from various localities, washed under running tap water and dried at room temperature. The individual organs of each plant were separated into *viz.*, roots, stem, leaves, fruits and were powdered using Sumeet grinder. Powder of each sample was stored in glass bottles.

(i) Extraction of alkaloid/s

One g of each sample of *W.somnifera* plants was extracted following the procedure described by Roberts and James (1947). The sample was treated with a few drops of H_2SO_4 (0.05N), and 15 ml of ether was added to it. The ether layer was slowly decanted and again washed with ether (15 ml). The extract was dried by keeping it in water bath. This powder was mixed with a few drops of NH_4OH (10%), treated with benzene (50 ml), and further maintained on the water bath for few minutes. The mixture was firmly packed in a column and benzene was percolated through it. The filtrate was passed through alumina column (2-4 cm) and silica gel column (10-12 cm) in sequence, and further it was treated with NH_4OH (20%) mixed with chloroform. The chloroform soluble fraction was used for isolation and identification of the alkaloid/s.

(ii) Isolation and identification of alkaloid/s

Chromatographic plates (20 x 10 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

authentic sample of Tropine. The TLC plates were run in chloroform : ethanol (9:1) solvent system. As soon as Dragendorff's reagent was sprayed, orange coloured spots developed of the alkaloid and isolated sample. The R_f values of the isolated alkaloid/s and of the authentic tropine were calculated.

(iii) Quantitative estimation of alkaloid/s

The quantitative estimation of the tropine alkaloid was done according to the method described by Roberts and James (1947).

The chloroform soluble fraction was evaporated to dryness and the powder was acidified with 1 ml of H_2SO_4 (0.02 N) and double-distilled water (14 ml). This mixture was titrated with NaOH (0.01 N) using methyl red as an indicator. End point was recorded when the colour of the solution changed to yellow. Alkaloid content was expressed on percentage dry weight basis using the following formula:-

(1) $N_1V_1 = N_2V_2$

Where,

N_1 = Normality of NaOH

N_2 = Normality of plant extract

V_1 = Volume of 0.01 N NaOH

V_2 = Volume of plant extract

(2) Tropine g/litre = N_2 x equivalent weight of Tropine

5. COMPOSITION AND PREPARATION OF MEDIA

The basal media used were White (1954) and Murashige and Skoog (1962) whose compositions have been given in Table I and II. Each medium

Table I : White's (1954) Medium

			Concentration of salts in mg/litre of medium
I.	**Macroelements**		
	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	$Na_2SO_4 \cdot 10H_2O$	454.00
	Sodium dihydrogen-orthophosphate	$NaH_2PO_4 \cdot 2H_2O$	24.00
II.	**Microelements**		
	Ferrous sulphate	$FeSO_4 \cdot 7H_2O$	2.50
	Boric acid	H_3BO_3	1.50
	Manganese sulphate	$MnSO_4 \cdot 4H_2O$	6.65
	Potassium iodide	KI	0.75
	Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	2.68
III.	**Vitamins**		
	Glycine		3.00
	Nicotinic acid		0.50
	Pyridoxine hydrochloride		0.10
	Thiamin hydrochloride		0.11
IV.	**Supplements**		
	Sucrose		20000 ?

Table 1 : Murashige and Skoog's (1962) Medium

			Concentration of salts in mg/litre of medium
I.	**Macroelements**		
	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	$Na_2MoO_4.2H_4O$	0.25
	Cobalt chloride	$CoCl_26H_2O$	0.025
	Manganese sulphate	$MnSO_4.H_2O$	22.3
	Zinc sulphate	$ZnSO_47H_2O$	8.6
	Copper sulphate	$CuSO_4.5H_2O$	0.025
*	Ferrous sulphate	$FeSO_4.7H_2O$	27.85
**	Disodium ethylene diamine tetracetic acid	$Na_2EDTA.2H_2O$	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 30000 mg

Cytokinins Kn/BAP

Auxins IAA/IBA/NAA/2,4-D

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* $FeSO_4$. $7H_2O$ 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.

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, phytohormones (Kn, BAP, IAA, NAA, IBA, 2, 4-D) and coconut water milk 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.

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 guaze. They were wrapped with paper to prevent absorption of moisture during sterilization. A few empty flasks, petridishes, pipettes wrapped in brown paper and tied with thread were sterilized by autoclaving at 15 lb/in^2 pressure (120°C) for 20 min.

Amino acid used was sterilized with millipore membrane filter (0.22 µm) under aseptic conditions and added to the sterilized medium.

7. ASEPTIC TECHNIQUE

All aseptic manipulations were carried out in Laminar Air Flow Cabinet (Klenzaids, India). The cabinet was sprayed with aerosol and the working table was wiped with cotton swab soaked in Dettol. The stainless steel instruments, viz., scalpel, spatula, forceps, and needles were dipped in alcohol and flamed before use. The cabinet was sterilized by UV light (λ2537Å) for 30 min before use.

(i) Surface sterilization of explants

Seeds from 'elite' plants were collected, surface sterilized with $HgCl_2$ (0.1%), and after washing with sterile distilled water, they were germinated in sterile petridishes containing wet filter papers. Roots (1-2 cm long) with their tips intact were excised and inoculated in the various media (40 ml) for establishment of exised root-culture. Also hypocotyls / stem / leaves were excised and inoculated on solidified MS medium (20/30 ml) for establishment of callus-cultures.

Young floral buds of the first flowering of garden-grown *W.somnifera* plants were collected and surface sterilized as mentioned above. The anthers were excised from the floral buds and inoculated on solidified medium (20/30 ml) for establishment of haploid culture, after checking them for uninucleate microspore stage following meiosis.

(ii) Incubation

Culture flasks/tubes with explants were maintained in culture room (25 ± 2°C) under fluorescent light in 16 h photoperiod (1000 lux). Root cultures were maintained on gyratory shaker (120 rpm) in culture room.

8. ESTABLISHMENT AND MAINTENANCE OF CULTURES

(i) Root cultures

Root cultures established in culture media, White's/MS (40 ml), from excised roots were maintained on gyratory shaker. The root clones established were regularly sub-cultured in fresh medium of the same composition at four weeks interval.

(ii) Callus cultures

Callus tissues initiated from the hypocotyl/ /leaf explants (300 ± 20 mg) were subcultured regularly at four weeks interval to fresh medium (30 ml) to keep a ready callus stock for the experimental work.

(iii) Suspension cultures

Suspension cultures of leaf callus were initiated by transferring the healthy callus pieces to MS liquid medium (30 ml) of the same composition and the culture flasks were maintained on gyratory shaker (120 rpm) in culture room for 16 h photoperiod. Within a period of two to four weeks callus separated out into single cells or group of cells which were filtered with nylon mesh (45 μ pore) aseptically and 5 ml of the filtrate containing mostly single cells were transferred to MS liquid medium (25 ml) of the same composition. This resulted in the establishment of uniform homogenous cell suspension cultures. These cultures were maintained by regular subculturing to fresh medium at an interval of three weeks.

9. CYTOLOGICAL METHODS

(i) Stage of pollen grains

The stages of pollen grains collected from anthers of young unopened floral buds were determined by hydrolysing them for 15 minutes in HCl (0.1N) at 60°C. They were stained with aceto-carmine (2%) by teasing the anthers in a drop of it. Mostly anthers containing pollens at uninucleate stage of development were inoculated for the establishment of haploids.

(ii) Chromosome determination

Excised root tips were pretreated in colchicine (0.2%) for 2-3 hours at 4-5°C, washed in distilled water and fixed in glacial acetic acid : ethyl alcohol (1:3) mixture for 24 hours. Root tips were hydrolysed with HCl (0.1 N) at 60°C and staining was done with acetocarmine (1%).

10. DIPLODISATION

Colchicine treatment (0.5%) for a certain period was given to axillary buds of haploids developed *in vitro*.

11. PREPARATION OF COCONUT MILK

Coconut milk collected from green coconuts was filtered through cotton wool, then boiled for the precipitation of proteins. It was cooled and filtered through filter paper and added to the medium as per requirement.

12. MEASUREMENT OF GROWTH

(i) Root length

The length of main axis of the root was measured with a thread after taking it out from the culture medium.

(ii) Lateral roots

The number of visible lateral roots were counted and recorded.

(iii) Fresh weight

Fresh weight of root/callus were determined by weighing them on preweighed aluminium foil taking care so that no liquid medium/agar

remained adhered to root/callus. In suspension cultures, the cells were harvested by suction filtration in a previously weighed whatman filter paper No.1 and the weights were recorded.

(iv) Dry weight

These were determined after drying the weighed amounts of harvested roots/callus on aluminium foil and cells from suspension on whatman filter paper No.1, in an oven (60°C), until constant weights were recorded.

(v) Cell viability

Viability of cells from suspension cultures was determined by staining them with Evan's blue (0.025%) (Gaff and Ogola, 1971).

(vi) Cell number

Cells in suspension cultures were counted using haemocytometer and recorded.

Total cell number = average number of cells in unit volume of haemocytometer x total volume of the sample.

(vii) Packed cell volume (PCV)

A known volume of homogeneous cell suspension was transferred to graduated centrifuge tube (15 ml) and spun for 5 min. PCV was calculated according to the formula described by Dixon (1985).

$$PCV = \frac{\text{Volume of pellet}}{\text{Volume of culture}} \times 100$$

(viii) Cell plating

Uniform homogeneous cell suspension containing single cells or cell units (4-6 cells) was used for cell plating following the technique described by Bergmann (1977).

13. CHEMICAL ANALYSIS OF CULTURED ROOTS/CALLUS

Alkaloid fraction/s after drying each sample of roots/callus from various treatments was determined following the procedures standardised for plant material. Quantity of alkaloid was expressed on percentage dry weight basis.

14. HISTOLOGICAL STUDIES

Histological studies for initiation of root primordium and callus exhibiting morphogenesis were done according to the procedures described by Jensen (1962). The tissues were fixed in FAA (40% formaldehyde 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 (MP 58°C). Ribbons were cut at 10 µ on rotary microtome and mounted on slide using egg albumen. Sections were stained with toluidine blue and mounted in DPX (dextrene plasticizer of xylene).

15. PHOTOGRAPHY

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