Chapter II

ROUTINE PROCEDURE FOR TISSUE CULTURE TECHNIQUES

ROUTINE PROCEDURE FOR PLANT TISSUE CULTURE TECHNIQUES

Material and methods:

The general requirements, procedure for initiation and maintenance of cultures for the micropropagation of selected plants were as:

Chemicals: All the chemicals used were of high purity grade (AR grade), obtained from SISCO Research Laboratories (SRL), Rankem (Ranbaxy), LOBA, Glaxo (Qualigen), S.D. Fine Chemicals, India, E. Merch. All the plant growth regulators and vitamins were obtained from Sigma Chemical Ltd (USA) through the agencies in India.

Culture vessel: All the glassware including culture vessels viz culture flasks, test tubes, petri dishes, measuring cylinders used were either of borosil or corning make. Erlenmeyer flasks (150 ml) test tubes (50 ml) and jam bottles (200 ml) were used as culture vessels. The culture vessels were plugged with cotton-plugs made of non-absorbent cotton wrapped in 2 layers of gauze cloth, where as bottles were capped with the autoclavable plastic lids. The advantages of using these jam bottles are:

(a) more inoculum per bottle
(b) cost effective
(c) easily available.

All the new glassware used for media preparation, culture initiation and maintenance were first dipped (30 min) in chromic acid-sulphuric acid mixture (100g potassium dichromate in 500 ml concentrated sulphuric acid and diluted to 2:1 with distilled water) and washed with a mild detergent (Teepol). The glassware were thoroughly washed first with tap-water and then followed by a rinse in distilled water. Lastly, all the glassware were dried (overnight) in the oven (50 – 60° C).

Composition and preparation of media: The Murashige and Skoog MS medium (1962) with various combinations of plant growth regulators were used for

micropropagation studies of all the plants presented in the thesis. The chemical composition of the MS medium and corresponding stock solutions are given in the Table-2.1.

Table-2.1: Chemical composition and stocks of Murashige and Skoog's (1962) medium

CHEMICALS	CONCENTRATION (mg /l)	STOCK (mg/l)	VOLUME OF STOCK (ml/l)
(A) MACRONUTRIENTS			
NH_4NO_3	1650	33,000	50
KNO_3	1900	38,000	
$CaCl_2 2H_2O$	440	8,800	
KH_2PO_4	170	3,400	
$MgSO_4$	370	74,00	
(B) MICRONUTRIENTS			
$MnSO_4 4H_2O$	22.3	4460	5
$ZnSO_4 7H_2O$	8.6	1720	
H_3BO_3	6.2	1240	
KI	0.25	166	
$Na_2MoO_4 2H_2O$	0.83	50	
$CuSO_4 7H_2O$	0.025	5	
$CoCl_2 6H_2O$	0.025	5	
(C) IRON SOURCE*			
$FeSO_4 7H_2O$	27.8	5560	5
Na_2 EDTA $2H_2O$	37.3	7460	
(D) ORGANIC CONSTITUENTS			
Myo-inositol	100	20,000	5
Nicotinic acid	0.5	100	
Pyridoxine-HCl	0.5	100	
Thiamine- HCl	0.1	10	
Glycine	2	400	
pH			5.8
Sucrose			30 g/ l

*The $FeSO_4 7H_2O$ and Na_2 EDTA$2H_2O$ were dissolved separately in approximately 200 ml of warm distilled water. The two solutions were mixed with continuous stirring and volume was then adjusted (1000 ml) with distilled water. The Fe-EDTA solution prepared by this method could be stored upto two months without precipitation, in amber coloured bottle, under refrigeration.

The medium was prepared from the concentrated stock solutions of micro-elements, macro-elements, vitamins and iron stocks that were made in the double glassed distilled water and stored in amber coloured glass bottles (2-4°C). The growth regulators and supplements were added to the basal medium before the final pH (5.7±0.1) was adjusted with either HCl (0.1 N) or KOH (0.1 N) solutions.

For semi-solid media, 0.8 % (w/v) agar-agar (Glaxo, bacteriolgical grade) was added to the mixture and dissolved by gentle heating with constant stirring. On complete dissolution of agar, the medium was dispensed into culture vessels Erlenmeyer flasks (30 ml each), test-tubes (15 ml each) and jam glass bottles (50 ml each).

Sterilization: All the instruments, culture media including explants were sterilized before inoculation of explants on MS medium.

1) Culture medium and glass ware: All the glassware i.e.

(a) Culture tubes, Erlenmeyer flasks with dispensed medium were tightly closed with cotton-plugs (non-absorbent cotton wrapped in gauze cloth) or jam bottles with plastic caps.

(b) Pipettes, petri-plates, dissection instruments such as pair of forceps, scalpel-blade handle, all wrapped in paper.

(c) Plastic-pots needed for acclimatization of plants stacked over one another in a beaker and wrapped with paper were autoclaved at 121° C (15-20 min).

After autoclaving, empty vessels and dissection instruments were kept in an oven (60° C) till they were taken to the Laminar-Flow-Hood (LFH) for inoculation procedure. The vessels containing medium were kept in a culture-room before being inoculated.

The sterilized culture vessels were used for inoculation only after 24 hrs of incubation to confirm that there is no contamination.

2) Plant material: For establishing contamination free cultures, the explants were subjected to surface disinfection much before the inoculation, by the following method –

The twigs were excised and immediately their cut ends were dipped in distilled water. In the laboratory they were washed under running tap-water to remove the dust particles. The material was then cut into explants, rinsed with mild detergent (Teepol; 0.5 %) and washed thoroughly with tap-water. The explants were then pre-treated with 70 % (v/v) ethanol (60-90 sec) and 1% chlorabendazim and chloramphenicol (0.05%) on shaker (1-15 hr). Before their inoculation, such treated explants were surface sterilized with 0.05-0.1 % (w/v) $HgCl_2$ (2-6 min) or 0.1-3% (v/v) NaOCl (2-5 min).

Aseptic conditions: All the inoculations and manipulations for establishment of fresh cultures as well for sub-culture or transfer of culture were carried out in a LFH cabinet (Klenzaids, Mumbai / ADC, Baroda). Prior to the use, the working area in LFH was first wiped with dettol and then by 90 % (v/v) ethanol. All the culture vessels were also wiped with cotton swab dipped in 90% (v/v) ethanol. Later on, the culture vessels were placed on the LFH bench. All the instruments required for inoculation were also kept with their working-end immersed in 70 % (v/v) ethanol in a wide mouth vessel-coupling jar. Before inoculation, the LFH was left on (15 min) with UV light. During the inoculations all the operations were performed near the source of air-flow and gas-flame. The instruments were flame-sterilized intermittently during manipulations.

Culture conditions: All the cultures were maintained in a culture-room with $25\pm1^{\circ}C$ temperature and 16 hrs photoperiod provided by cool white fluorescent tubes of 50-60 $\mu Em^{-2}\ s^{-1}$ photo flux (Philips, India). The liquid cultures in flasks were maintained on a gyratory shaker (100 rpm). In the absence of greenhouse, the *in vitro*

raised plantlets were acclimatized in the same culture conditions before their transfer to the field.

Subcultures: Multiple shoots generated from explants of *Bougainvillea / Mussaenda / Schefflera / Syngonium / Aglaonema* were harvested and dissected into *in-vitro* nodal explants, which were transferred to fresh media for further multiplication. In case of *Syngonium / Aglaonema* the bunch of 2-3 multiples were isolated and subcultured for further multiplication.

Rooting: Various methods were used for the induction of roots on well grown *in vitro* shoots viz pulse treatment with auxins (IBA), continuous presence of auxin in MS basal medium, *ex-vitro* initiation of roots. Details of rooting method and procedure followed are described in respective chapters.

Acclimatization: Before their transfer to the field, the *in-vitro* raised plantlets were hardened. The rooted shoots were transferred under sterile conditions, to sterile net pots (2 cm diameter) containing vermiculite and perlite mixture and kept covered with polythene bag for about 15-20 days in the culture room. During this period the high humidity was maintained by periodically spraying sterile distilled water. For further hardening, the plantlets were either kept in a tray (20 pot tray) or in an indigenously made chamber from acrylic (11.5 x 12.5 cm; 12 pots capacity) and fully covered with the polythene sheet. The base of the chamber or tray was lined with three layers of filter paper soaked with sterile distilled water. Initially, each plant in the pot was irrigated with 3 ml of 1/4 strength MS basal medium without sucrose. The plants were gradually exposed by making small holes (4 mm diameter) after 5-7 days, which were subsequently increased in number. After 15-20 days, the well-developed plantlets were transferred to the earthen pots (8 cm diameter) containing a mixture of sterile garden soil, sand and compost and kept in the culture room for about 10-15 days before their transfer to the field for further growth.

Field evaluation:

In the field, initially the potted plants were kept under the shade of trees for about 7 to 10 days. The plants when became sturdy enough, were transferred to earthen pots (8cm diameter) exposed gradually to sunlight. After transfer of the regenerated plants to the field, the growth and the morphology of the *in vitro* regenerated plants were compared with the parent plant, for the study of their true-to-type to their parent stock.

Design of Experiments:

Optimization of media composition for optimum response was done by designing the experiments in light of other reports and literature. Each combination in an experiment was replicated by a minimum of seven replicates and each experiment was repeated for a minimum of three times.

Statistical analysis:

The collected data was computed and presented as mean + standard error(S.E.) of the replicates. The data was also subjected to one way and two way ANOVA analysis (Fischer Behranes test). The significant variations among the means were analyzed by Duncan's Multiple Range test (Duncan, 1952). All the computations and analyses were carried out on a computer using statistical package-SPSS (Windows 6.0 program).