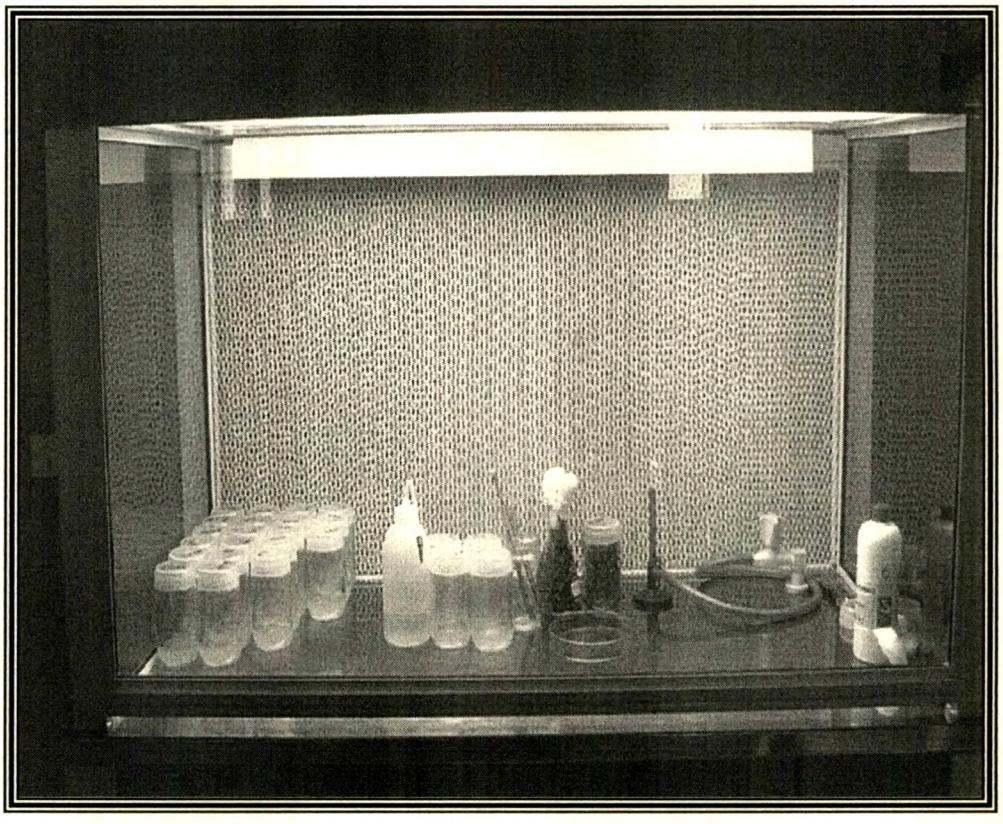
MATERIALS AND METHODS



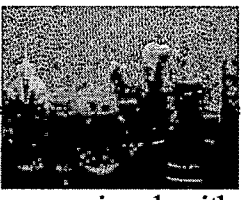
The general procedure for the initiation and maintenance of cultures and associated methods employed for the present investigations are explained in this chapter. The description of materials namely plant material, chemicals and glasswares are also discussed.

Explant

The plants growing in the Botanical Garden of the Maharaja Sayajirao University of Baroda served as the source for initial explants. It is always advisable to select a young actively growing plant for isolating explants. Younger the developmental stage, greater is the shoot regenerative capability. Also, it is not possible to obtain more than one or few terminal buds from a particular plant at a given period of time, therefore axillary buds were used as explants. Various other explants (apical tip, leaf, rhizome, internode) were also explored for the present study for multiplication of the selected plants (*Aloe vera, Bacopa monnieri, Centella asiatica, Plumbago zeylanica* and *Withania somnifera*).

Culture vessels

Erlenmeyer flasks (100 and 150 ml capacity), long-neck Jam bottles (250 ml) and culture tubes (150 mm X 26 mm) were used as culture vessels. The culture vessels (culture bottles, culture flasks, culture tubes, petridishes, measuring cylinders etc.) used were of standard make (Borosil, India). Long-neck bottles used were of local supplier. The culture vessels like Erlenmeyer flask and test tubes were plugged with cotton plugs, which were made of non-absorbent cotton wrapped with 2 layers of gauze cloth. All the glassware, before use, were cleaned with liquid detergent (teepol)



and thorough washing with tap water. The cleaned glassware were rinsed with distilled water and air-dried in the oven (60°C).

Chemicals

All chemicals used in the preparation of culture media and for various analyses were of analytical grade either from E. Merck (India), Glaxo (Qualigens), SRL, Loba, or Sigma (St. louis, USA). Plant growth regulators and vitamins were obtained from Sigma or Glaxo (Qualigens) through local suppliers.

For phytochemical analysis, petroleum ether, methanol, ammonia, ethyl acetate, chloroform, formic acid, toluene, sulphuric acid etc used were of Glaxo make. TLC Silica gel (GF 254) precoated (0.2mm thickness) plates for TLC was supplied by E. Merck (India).

Culture media preparation

Basal medium (Table-1), formulated by Murashige and Skoog (1962) with various combinations of plant growth regulators, was used for all experiments.

Concentrated stock solutions of media components were prepared and stored at 5-10°C in the refrigerator. The MS basal medium was prepared by mixing the stock solutions in a precise manner and the supplements were added prior to the adjustment of the final volume. The pH of the media was adjusted to 5.8 with KOH (0.1N) or HCl (0.1N). For semi-solid media 0.8% (w/v) agar-agar (Qualigen-bacteriological grade) was dissolved by gentle heating with constant stirring. The medium was dispensed in pre-determined aliquots to 150 ml erlenmeyer flasks (30 ml), 250 ml culture bottle (30 ml) and 50 ml culture tubes (15 ml).

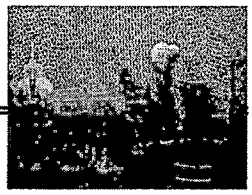
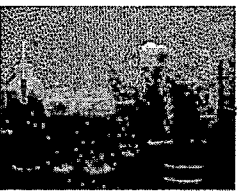


Table-1 Chemical composition of Murashige and Skoog (MS) medium (1962) used for *in vitro* multiplication of selected plants

	Constituents	Concentration (mg l^{-1})
(a)	**Macronutrients**	
	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.H_2O$	440
	$MgSO_4,7H_2O$	370
	KH_2PO_4	170
(b)	**Micronutrients**	
	$MnSO_4,4H_20$	22.3
	$ZnSO_4,7H_2O$	8.6
	H_3BO_3	6.2
	KI	0.83
	$Na_2MO_4,2H_2O$	0.25
	$CuSO_4,7H_2O$	0.025
	$CoCl_2,6H_2O$	0.025
(c)	**Organic supplements**	
	Myo-inositol	100
	Nicotinic acid	0.5
	Pyridoxine HCl	0.5
	Thiamine HCl	0.1
	Glycine	2.00
(d)	**Iron source**	
	$FeSO_4,7H_2O$	27.8
	Na_2EDTA	37.3
(e)	**Carbon source**	
	Sucrose	30000



Inorganic nutrients

A melange of inorganic elements are required for the over all growth and life cycle of a plant beside carbon, hydrogen and oxygen. Essential elements required in more concentration than 0.5 mmol l^{-1} are referred as macronutrients and those less than 0.5 mmol l^{-1} as micronutrients (Razdan, 1993).

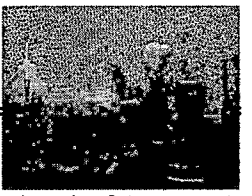
Macronutrients:

It includes six major elements in the form of salts of Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (M) and sulphur (S).

Micronutrients:

Inorganic elements included in the tissue culture media are iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) cobalt (Co), iodine (I) and molybdenum (Mo).

The elements (nitrogen, phosphorus and sulphur) are covalently bonded to carbon compounds. They are vital constituents of the macromolecules, DNA, RNA and proteins. All other elements (potassium, magnesium, calcium, sodium, manganese, iron, copper, zinc, molybdenum, boron, chlorine) participate in a variety of often overlapping functions. This includes regulation of osmotic and electric gradients, protein conformation and oxidation–reduction reaction of metalloproteins (Clarkson and Hanson, 1980). Nitrogen added in the highest quantity among all elements is supplied in the form of ammonium or nitrate ion or a combination of these ions. Ammonium is responsible for the drop in pH, which may restrict the availability of nitrogen. The inclusion of 20-40 mM potassium nitrate can prevent the extreme fluctuations of pH and is essential for activation of many enzymes. Phosphorus and Sulphur are usually supplied as phosphate and sulphate. Phosphate is the primary



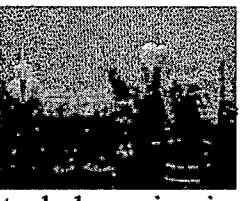
buffering constituent in the tissue culture medium. Phosphorus at a level of 1.25 mM serves as the near optimal level (Murashige and Skoog, 1962). Calcium and magnesium are antagonistic to each other. It has been noted that an increase in the concentration of one element increased the requirement for the other (Heller, 1965; Ohira et al, 1973). Molybdenum is a component of nitrate reductase and is very essential for plant nutrition. Iron is not easily available at high pH due to precipitation, therefore iron is supplied as an EDTA complex. Iodine is found to be beneficial for the growth. Cobalt has been demonstrated for its effects on plant metabolism. Sodium and chlorine are included in the media in the form of salts of other elements.

Organic nutrients

Vitamins:

Vitamins have catalytic functions in enzyme systems and are required only in trace amounts. Commonly used vitamins include thiamine, nicotinic acid and pyridoxine HCl. Myo-inositol is an important carbohydrate more because of its ability to serve as a carbon source. Though myo-inositol is not considered to be essential, its absence was found to bring about 50% decline in the growth rate of tobacco tissue (Linsmaier and Skoog, 1965). Similarly Paul's scarlet rose showed 90% decline in its normal growth rate (Nesius et al, 1972).

The most important and widely used vitamin is Thiamine, which is generally added in the range of 0.1-0.4 mg/l. Pyridoxine and nicotinic acid are essential in smaller quantities. Biotin, folic acid, pantothenic acid, ascorbic acid, choline chloride and riboflavin are other vitamins chiefly used for specific cultures (Gamborg and



Shyluk, 1981). Ascorbic acid in combination with citric acid retards browning in freshly excised tissue (Dodds and Roberts, 1985).

Plant growth regulators

The most critical organic component of any plant propagation medium is auxin and cytokinin alone or in combinations. The growth, differentiation and organogenesis of tissues become feasible on the addition of plant growth regulators. Broad classess of growth regulators used in cultures are namely auxins, cytokinins, gibberellins, abscissic acid and others.

1. **Auxins:**

The auxins are the class of compounds that stimulate shoot cell elongation, apical dominance, abscission and rooting. They differ in stability, effectiveness and their influence on organogenesis. Indole-3-acetic acid (IAA) is a natural auxin. IAA is the weakest among the auxins and is inactivated readily in some tissue cultures but is preferred over other auxins as it has minimum adverse effects on organ formation. Napthalene acetic acid (NAA) and 2,4-Dichlorophenoxy acetic acid (2,4-D) are synthetic auxins. It may be necessary during the establishment of the aseptic cultures but should be avoided in the protocol where the propagules are put for multiplication. The other synthetic auxins frequently employed in tissue culture are 2,4,5 trichlorophenoxy acetic acid (2,4,5-T), 2 methyl,4-chlorophenoxy acetic acid (MCPA), 4-amino-3,5,6-trichloropicolinic acid (Picloram) and 3,6-dichloro-2-methoxybenzoic acid (Dicamba).

2. **Cytokinins:**

They are six substituted purine compounds. They are known for stimulating cell division, modification of apical dominance and shoot differentiation in tissue cultures. The natural cytokinins are zeatin and N^6–isopentyladenine (2iP) while the synthetic ones are 6-furfuryl amino purine (Kinetin-KN) and N^6-benzyl adenine (BA).

The initiation of roots and shoots in cultures depend upon the interaction between auxin and cytokinin. The pattern of organogenesis depends largely on their relative concentrations. Auxins when added in relatively high concentrations induce root formation and suppress shoot formation. Similarly when cytokinin is added in relatively high concentrations, it favours shoot initiation and suppress rooting. Hence, in order to achieve the desired results, the auxins and cytokinin balance need be properly manipulated.

3. **Gibberellins and other classes of growth regulators:**

Gibberellins are known to promote growth of cells in culture at low density and enhance callus growth. It inhibits root formation in the presence of light. However, it stimulates root formation in darkness. While other classes of growth regulators are abscisic acid (ABA) known to stimulate or inhibit the callus growth. Etheylene (ETH) is known to influence the growth of plant tissues *in vitro.*

Carbon and Energy Source

Culture media require the source of a carbon and energy for better growth and response. The carbohydrate concentration not only has a pronounced effect on growth and morphogenesis (Lu et al, 1981) but also play an important role in the regulation of the external osmotic potential (Brown and Thorpe, 1980). The carbohydrate

requirement is satisfied by the inclusion of most preferred - sucrose in the nutrient media in the range of 2-3%.

Solidifying Agents

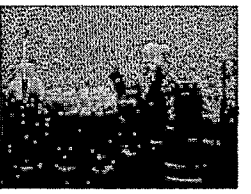
They are used for preparing semi-solid tissue culture media. Agar-Agar, a polysaccharide obtained from seaweeds, is routinely used in all tissue culture experiments undertaken for the study.

The Hydrogen ion concentration of the medium

The hydrogen ion concentration of the nutrient medium is also a critical factor. It determines many important aspects of the structure and activity of biological macromolecules. The hydrogen ion concentration (pH) is adjusted to 5.6-5.8 normally. The pH adjustment is accomplished prior to autoclaving. However, there is slight drop in pH after autoclaving. A wide variation in the pH may affect the stability of IAA and may cause precipitation of phosphates and heavy metal salts. It also influences the gelatilization of agar-agar and the destruction of thiamine, pantothenic acid, cysteine, tryptophan and gibberellic acid during sterilization by autoclaving. Intracellular pH depends upon the pH of the medium. At low and high pH values, the growth of plant tissues get inhibited. At lower pHs more fluid agar is attained whereas at higher pHs comparatively harder gels are formed.

Sterilization of the culture vessels and medium

All the culture vessels with dispensed medium were closed with cotton plugs. Other glassware such as petridishes, conical flasks and dissection instruments like pair



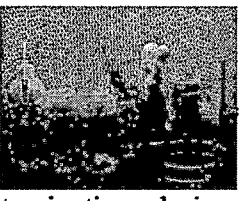
of forceps, scalpel- blade holder, needles, spatula were wrapped in paper and autoclaved at 121°C (20-45 min). After the sterilization process, the glassware without medium and the dissection instruments were stored in the oven (60°C). At the same time culture vessels containing medium were kept in the culture room. The culture vessels with media were used for inoculation only after 24 hrs to confirm that there is no contamination. The dissection instruments such as pair of forceps and scalpel handle with blade were kept immersed in 70% ethanol and flame sterilized frequently during the inoculation manipulations

Sterilization of plant material

In order to avoid bacterial and fungal growth, explants were subjected to surface sterilization. The explants were collected carefully, washed thoroughly under running tap water and then treated with mild detergent (Teepol). Pretreatment was also given to the explants before their surface sterilization. (Details for the individual plants are explained in the respective chapters). Before inoculation the explants were surface sterilized with 0.05 – 0.2.5 % (w/v) $HgCl_2$ (2-6 min). The explants were thoroughly rinsed with sterilized distilled water. If required antibiotic treatment was followed. The explants were trimmed according to the size required (1cm) before their inoculation into culture vessels containing medium.

Antibiotics

To prevent the growth of systemic infection of microorganisms it is essential to treat the explants in antibiotic solution before inoculation. The explants were treated in the 100-mg/l mixture of antibiotics containing streptomycin, rifampillicin



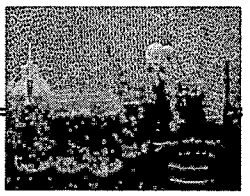
and ampillicin each (10 min) to avoid heavy bacterial contamination during establishment of sterile cultures.

Sterile conditions

All steps leading to the establishment of cultures namely surface sterilization, inoculation and sub-cultures were carried out in a Laminar Airflow hood (LAB India/ADC, Vadodara). Initially the chamber was cleaned with labolene Liquid (Glaxo) and the culture vessels were wiped with 70% (v/v) ethanol. The culture vessels and the dissection instruments were arranged neatly on the flow hood bench. Laminar airflow hood was left on with UV lamp (20 min) in the chamber to disinfect the surface of the vessels, instruments and even the bench. The inoculation operations were conducted well within the laminar airflow near the gas flame. The dissection instruments used for inoculation and subculture were flamed-sterilized intermittently during manipulations. Before carrying out any exercise on laminar airflow hood, hands were thoroughly washed with soap and then rinsed with hand disinfectant (Glaxo, India). During manipulations in the Laminar Airflow Hood, face mask and head-cap were used for extra care.

Culture maintenance

The major factors influencing the tissue culture environment are light, temperature, humidity and gaseous environment. All cultures were maintained in a culture room with 25 ± 2°C temperature, 14-hour photoperiod using cool white fluorescent tubes (Phillips, India) at a light intensity of 50 $\mu Em^{-2}s^{-1}$ routinely.



Subcultures

Single node segments were dissected out from the isolated shoots, defoliated and implanted vertically on MS medium. Multiple shoots generated from such nodal explants were harvested at each passage and *in vitro* nodal explants were subcultured at every 4-week interval for further multiplication.

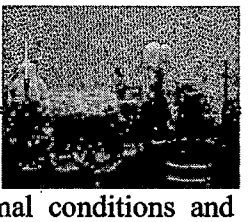
Rooting

Well-grown *in vitro* shoots (3-8 cm length) having 4-5 nodes were separated and transferred to the root induction medium. Details of the rooting procedure are mentioned in the respective chapters.

Acclimatization

The ultimate success of *in vitro* propagation lies in successful establishment of plants in the soil. *In vitro* generated plantlets were removed from the culture vessels and washed gently with water to remove agar. The regenerated plantlets (4-6 cm) were transferred to small sized plastic pots/ bags/ portray containing various potting mixtures in different ratios.

Plantlets of *Centella asiatica* and *Aloe vera* were transferred to portrays or plastic bags containing sterile potting mixture depending on the size of the plantlets and kept in greenhouse for 2-3 weeks. While, the plantlets of *Withania somnifera, Bacopa monnieri* and *Plumbago zeylanica* were kept directly in the Agri net house for acclimatization (2-3 weeks) before their subsequent transfer to the field. Thus, economizing the cost and time of greenhouse hardening. Humidity was maintained by regular sprinkling of water and irrigating the regenerated plantlets during daytime



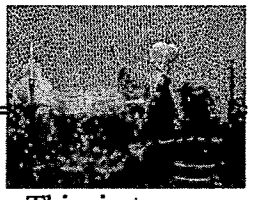
(Jasrai et al, 1999). Plants were gradually exposed to the normal conditions and transferred to the Medicinal Garden of GSFC Science Foundation.

Statistical analysis

All experiments for the selected five plants (*Aloe vera, Bacopa monnieri, Centella asiatica, Plumbago zeylanica* and *Withania somnifera*) were performed in replicates of ten for each type of explants and whole experiment was repeated three times. Experiments were set up in a Randomized block design (RBD) for *Withania* plants. The growth responses of the explants were examined periodically for visual observations of morphological changes. The data pertaining to the mean percentage of cultures responding to bud formation, mean number of shoot/culture, mean percentage of root induction, mean number of roots/shoot, percentage survival of shoots in greenhouse and subsequent on the field were statistically calculated.

Phytochemical analysis

For medicinal species, it is highly essential to retain the quality and quantity of desired secondary metabolites in the progeny. The therapeutic activity of the medicinal plant lies in the constituent organic molecules possessing pharmacological properties. With the advent of new analytical tools and sophisticated instrumental technology, it has become possible to carry out practicable quality assurance profiles for a crude drug, its bioactive constituents and the formulations themselves. Some modern techniques such as UV-spectroscopy, IR-Spectroscopy, Atomic absorption Spectroscopy (AAS), thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC)



can be very well utilized for the development of chemical markers. This, in turn, can be used for identification and quality assurance (Joliffe, 1977).

The phytochemical studies were conducted to compare the tissue culture raised plant, mother plant and market sample for their active principle contents in the selected plants through various qualitative (TLC) and quantitative methods (HPLC). Further details on phytochemical analysis for respective plants have been included in the respective chapters.