CHAPTER 2:
MATERIALS AND METHODS

This chapter deals with the general laboratory techniques routinely followed in various plant tissue culture aspects of the present study. Other important specific methodologies followed will be discussed separately in the respective chapters.

2.1. Glassware

Glassware used in all the experiments was procured from "Borosil", India. Test tubes (25 mm x 150 mm), glass bottles (70 mm x 125 mm), petridishes (85 mm x 15 mm; 55 mm x 15 mm), conical flasks (100, 250 & 500 ml; 1, 2 & 5 L capacity) and pipettes (1, 2, 5, 10 and 25 ml capacity) were used during the course of study.

2.2. Preparation of Glassware

Glassware used for all the experiments was cleaned by boiling in a saturated solution of Sodium bicarbonate for 1h followed by repeated washing in tap water. Thereafter, it was immersed in 30% HNO_3 solution for 30 min followed by repeated washing in tap water. Washed glassware was thereafter dried at room temperature. Test tubes and flasks were plugged with absorbent cotton (Mamta Surgical Cotton Industries Ltd., Rajastan, India). Autoclaving of the glassware and above items was done at 121°C, 15 psi for 1 h.

2.3. Plasticware

Sterile disposable filter sterilization units (0.22 micron) and petridishes (55 mm and 85 mm diameter) were procured from "Laxbro", India. Eppendorf tubes (1.5 ml and 2 ml capacity), microtips (10, 200 and 1000 µl capacity) and PCR tubes (0.2 ml and 0.5 ml capacity) were obtained from "Tarsons" and "Axygen", India.

2.4. Chemicals

All chemicals used in the tissue culture study were of analytical grade (AR) and were obtained from "Qualigens", "S.D. Fine Chemicals" or "HiMedia", India. The chemicals used in molecular biology study were obtained from "Sigma Chemical Co.," USA. Growth regulators, vitamins, antibiotics and phytagel were also obtained from "Sigma Chemical Co.", USA and HiMedia, India. Cefotaxime was procured from Alkem Laboratories, Mumbai, India. Sucrose, glucose and agar-agar were obtained from "Hi-Media". Bacto-Agar for microbial work was obtained from "DIFCO" laboratories, USA.

2.5. Preparation of culture media

Sterile single distilled water was used for preparation of all media used in the present study. After addition of all macro and micro-nutrients, vitamins, growth regulators and necessary carbohydrate sources like sucrose, the pH of the media was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl. Volume was made with sterile distilled water and gelling agent was added as per requirement. The medium was steamed to melt the gelling agent. Melted medium was then dispensed into test tubes, flasks and thereafter sterilized by autoclaving at 121°C at 15 psi for 20 min. Thermolabile growth regulators and antibiotics were filter sterilized through a sterile disposable filters of 0.22 µm pore size (Laxbro India, Mumbai). The filter sterilized growth compounds were added to autoclaved medium before dispensing. Compositions of different basal media used in the present study are given in Table 2.1.

2.6. Preparation of growth regulators and antibiotics used in the study

All the thermo-stable growth regulators namely N^6-benzyladenine (BA), 6-(gamma, gamma-Dimethylallylamino) purine (2ip), Kinetin (KIN), 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), alpha-Naphthaleneacetic acid (NAA), Adenine Sulphate (AdS), (±)-cis, trans-Abscisic acid (ABA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), beta-Naphthoxyacetic acid Free acid (NOA), Picloram and Casein Acid Hydrolysate were added to the media before autoclaving and thermo labile compounds like 3,6-Dichloro-o-anisic acid (Dicamba), Gibberellic acid (GA_3) etc. were filter sterilized and added to the media after autoclaving. The details of the solubility and usage of different growth regulators was given in Table 2.2.

After addition of all the components, media were autoclaved at 121°C temperature and 15 psi pressure for 20 min.

Table 2.1: Composition of macro and micro nutrients and vitamins of various basal media used in the study

Component	CP	Eriksson	B5	WPM	MS	NN	ER
$CoCl_2.6H_2O$	0.025	0.0025	0.025	0.00	0.025	0.00	0.025
$CuSO_4.5H_2O$	0.025	0.0025	0.025	0.25	0.025	0.025	0.025
FeNaEDTA	36.70	36.70	36.70	36.70	36.70	36.70	0.00
H_3BO_3	6.20	0.63	3.00	6.20	6.20	10.00	0.50
$MnSO_4.H_2O$	0.85	1.69	10.00	22.30	16.90	18.94	3.00
$Na_2MoO_4.2H_2O$	0.25	0.025	0.25	0.25	0.25	0.25	0.025
$ZnSO_4.7H_2O$	8.60	0.00	2.00	8.60	8.60	10.00	0.50
$ZnNa_2EDTA$	0.00	15.00	0.00	0.00	0.00	0.00	0.00
$Ca(NO_3)_2.2H_2O$	492.30	0.00	0.00	471.26	0.00	0.00	600.00
KH_2PO_4	170.00	340.00	0.00	170.00	170.00	68.00	0.00
KNO_3	1900.00	1900.00	2500.00	0.00	1900.00	950.00	160.00
$MgSO_4.7H_2O$	180.54	180.54	121.56	180.54	180.54	90.27	750.00
NH_4NO_3	1650.00	4006.61	0.00	400.00	1650.00	720.00	360.00
$NaH_2PO_4.H_2O$	0.00	0.00	130.44	0.00	0.00	0.00	19.00
KI	0.00	0.00	0.75	0.00	0.83	0.00	0.00
$(NH4)_2SO_4$	0.00	0.00	134.0	0.00	0.00	0.00	0.00
K_2SO_4	0.00	0.00	0.00	990.00	0.00	0.00	0.00
Na_2SO_4	0.00	0.00	0.00	0.00	0.00	0.00	200.00
$CaCl_2$	0.00	332.02	113.23	72.50	332.02	166.00	0.00
KCl	0.00	0.00	0.00	0.00	0.00	0.00	65.00
Iron citrate	0.00	0.00	0.00	0.00	0.00	0.00	10.00
Myo-inositol	10.00	0.00	100.00	100.00	100.00	100.00	50.00
Glycine	0.00	2.00	0.00	2.00	2.00	2.00	3.00
Biotin	0.00	0.00	0.00	0.00	0.00	0.05	0.00
Niacin	1.00	0.50	1.00	0.50	0.50	5.00	0.00
Pyridoxin	1.00	0.50	1.00	0.50	0.50	0.50	0.25
Thiamin HCl	1.00	0.50	10.00	1.00	0.10	0.50	0.25
Folic acid	0.00	0.00	0.00	0.00	0.00	0.50	0.00
Ca-pantothenat	0.00	0.00	0.00	0.00	0.00	0.00	0.25
Casein Acid Hydrolysate	0.00	0.00	0.00	0.00	0.00	0.00	50.00
L-Cystein	0.00	0.00	0.00	0.00	0.00	0.00	211.60

Table 2.2: List of growth regulators and their solubility

Growth regulator	Solvent	Diluent
2,4-Dichlorophenoxyacetic acid Sodium salt	Water	—
Indole-3-acetic acid Free acid (IAA)	EtOH/1N NaOH	Water
Indole-3-butyric acid (IBA)	EtOH/1N NaOH	Water
alpha-Naphthaleneacetic acid Free acid (NAA)	1N NaOH	Water
beta-Naphthoxyacetic acid Free acid (NOA)	1N NaOH	Water
Picloram	DMSO	—
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	EtOH	—
Adenine Free base	1.0 HCl	Water
N^6-Benzyladenine (BA)	1N NaOH	Water
6-(gamma, gamma-Dimethylallylamino) purine (2iP)	1N NaOH	Water
Kinetin	1N NaOH	Water
1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea	DMSO	—
Zeatin	1N NaOH	Water
trans-Zeatin riboside	1N NaOH	Water
(±)-cis,trans-Abscisic acid (ABA)	1N NaOH	Water
3,6-Dichloro-o-anisic acid (Dicamba)	EtOH/Water	—
Gibberellic acid (GA_3)	EtOH	—

2.7. Collection of plant material

Plant materials of the grapevine cultivar Crimson Seedless used in the present study were collected from the vineyard of National Research Centre for Grapes, Pune. Twigs of the cultivar for preparation of single node segments were collected during all the seasons of the year but quality of the material was better during new flush appearance immediately after pruning i.e., during May and November. Young tender light green tendrils were collected during flowering stage of the vines. Young inflorescences were collected for immature anthers at 10 d prior to anthesis where as selfed immature berries were collected from the field at 35, 45 and 55 d post anthesis during fruiting. Plant materials collected in the polyethylene bags were transferred immediately to a cold room maintained at 9^oC until use.

2.8. Preparation of plant materials

For *in vitro* propagation, twigs were defoliated and made into single node segments (1.5-2 cm). In case of tendrils, inflorescence and immature berries, whole material was used for surface sterilization.

2.9. Surface sterilization of *ex vitro* material

Single node segments and other plant materials were surface sterilized by soaking them in liquid soap solution for 10 min followed by thorough rinses with running tap water. The explants were then submerged in 0.1% Carbendazim fungicide solution (Bavistin™, BASF, India) for one hour followed by 2-3 washes with sterile distilled water. Then the explants were treated with 0.1% (w/v) Mercuric chloride for 10 min followed by several rinses with sterile distilled water in a laminar air-flow hood (Microfilt, India). Excess water was removed by blotting dry the explants on a sterile filter paper.

2.10. Inoculations

Disinfected explants were inoculated on media in a Laminar air-flow hood. Sub cultures were also carried out on sterile filter papers with the help of sterile scalpels and forceps. Scalpels and forceps were autoclaved and heated on flame prior to inoculation and also in between by dipping in rectified spirit. Surgical blades (No. 10 and No. 11) were used for all the inoculations and sub cultures. Sterile filter paper bridges (Whatman No.1) were used as supports for explants cultured in liquid media under static conditions.

Further information about the inoculation and culture of explants has been described in the respective chapters.

2.11. Statistical analysis

Standard deviations for the data were calculated and were analyzed statistically using one-way or/ two-way or/ three-way analysis of variance (Snedecor *et al*., 1989).

2.12. Culture conditions

The cultures were incubated in culture room at $25^{o}\pm2^{o}$C in dark or light. The details of the incubation conditions have been mentioned in each section separately.

2.13. Histology

Histological study was carried out by fixing the plant specimens like multiple shoot clumps, somatic embryos etc. in 5-10 ml of FAA (Formalin : acetic acid : 70% ethanol by volume) (5:5:90) in 15 ml capacity screw capped vials (Borosil®, India) for 48 h at room temperature.

2.13.1. Fixing

Tissues were fixed in FAA for 48 h

FAA= Formaldehyde: Glacial Acetic acid: 70% Alcohol (2.5:2.5:95)

	Water	**Alcohol**	**TBA**
Day1	50 parts	40 parts	10 parts
Day2	30	50	20
Day3	15	50	35
Day4	0	45	55
Day5	0	25	75
Day6	0	0	100
Day7	0	0	100
Day8	0	0	100

On 8th day, 2 ml TBA + wax (full) was added and melted at 60°C.

Day 9, wax was added and the tissues were incubated at 60°C

Day 10, fresh wax was added

Day 11, wax was poured into block maker and tissues were placed in block maker as required and marked their position. Block after solidification was ready for sectioning in a Microtome.

***TBA** – **T**ertiary **B**utyl **A**lcohol

2.13.2. Staining procedure

1. Xylene (60 ml) - 2 min
2. Xylene (60 ml) - 1 min
3. xylene:Alcohol 1:1, (70 ml) - 1 min
4. Abs. Alcohol (70 ml) – 1min
5. 70% alcohol (70 ml) - 1 min
6. 40% alcohol (70 ml) - 1 min
7. 20% alcohol - 1 min
8. dd water dip
9. 4% mordant (Ammonium Ferric sulphate) - 2-5 min
10. dd water dip
11. Haematoxylin - 6-8 min
12. dd water dip
13. 2% mordant - (less time)
14. dd water dip
15. 20% alcohol - 1 min
16. 40% alcohol - 1 min
17. 70% alcohol - 1 min

18. Abs. Alcohol - 1 min
19. 10% Eosin in alcohol - 6-8 min
20. Abs. alcohol - 1 min
21. Abs. Alcohol - 1 min
22. Xylene : alcohol (1:1) - 1 min
23. xylene - 1 min
24. xylene - 1 min

Mounting: The sections were covered with cover slips and mounted using DPX Mountant (Qualigens Fine Chemicals, Mumbai, India.)

2.14. Hardening of the plantlets

In vitro rooted shoots, plantlets from germinated somatic embryos and directly rooted nodal segments were carefully taken out of the test tubes and gently washed under tap water so as to remove the medium sticking to them. The shoots were dipped in 0.1 % aqueous solution of Bavistin for 10-15 min and then washed with tap water. These *in vitro* produced plantlets and plantlets originated from somatic embryos were transferred to plastic cups containing a mixture of soil and sand (1:1). In case of *ex vitro* rooting, shoots (>5 cm) were transferred to soil : sand : peat (1:1:1) mixture. For hardening of *in vitro* and *ex vitro* rooted shoots and nodal segments with direct rooting, sachet technique described by Ravindra and Thomas (1995) and Bharathy *et al*. (2003) was followed. After transfer to plastic cups, plantlets were covered with thin, transparent polythene sachets and kept in growth room having 24 h light with intensity of 24.4 μ mol m^{-2} s^{-1} at $25^o \pm 2^oC$ for 2 weeks. After 2 weeks, plantlets were shifted to another room having ambient temperature conditions. Here, sachets were cut at top corners and were removed completely after another 2 weeks. After that plants could be transferred to a polyhouse for acclimatization.