Chapter-2

MATERIALS & METHODS-GENERAL

General Glassware:

All glassware used for culture media preparation and bio-chemical assays etc were of Borosil make. For initiation, establishment and maintenance of cultures, Erlenmeyer flasks (100 & 150 ml) or culture tubes (25 X 150 ml) were used. Before their use all glassware were dipped in dilute chromic acid (12 hrs) and then thoroughly washed with non-ionic detergent (Teepol, National Organic Chemical Industries Ltd., Mumbai) followed by thorough wash with tap water. Finally, they were rinsed with double distilled water and dried in hot air oven (60° C).

Chemicals:

All chemicals used were of high purity (AR grade) except Sucrose (Pure Tissue Culture grade). They were obtained from SISCO Research Laboratories (SRL), E-Merk, Qualigens India and Loba-Chemie. All plant growth regulators used were procured from Sigma Chemical Company, St. Louis (USA).

Water:

The water employed in all tissue culture media, bio-chemical assays including water used during the culture procedure (washing and sterilization of explants etc) was double glass-distilled water.

Medium matrix:

The culture medium was prepared as either liquid or semi-solid matrix. For stationary cultures, agar-agar (Qualigens, India) at 0.8 % (w/v) was used as gelling agent, particularly for the initiation of cultures from explants and subculturing of callus. For liquid cultures, medium (without agar-agar) in 100 or 150 ml Erhlenmeyer flasks were used on a gyrotatory shaker (100 rpm).

Carbon source:

In all experiments, media were supplemented with sucrose as a carbon and energy source. Sucrose (Pure, Tissue Culture grade, SRL India) was added in the concentration range of 2-3 % (w/v).

Osmotica:

Sucrose at high concentration upto 5 % was used as osmotica in liquid media for maturation of the somatic embryos.

Medium composition and preparation:

The medium employed through out the present study was MS (Murashige and Skoog 1962; Table-2) with slight modifications whenever required like reducing the strength or concentration of salts to half or one-fourth of its original level. The stocks were prepared out of AR grade reagents in fresh double glass distilled water and stored in plastic (opaque) or amber coloured bottles under refrigeration (4° C). Four different stocks were prepared for MS medium as Macronutrients (MS-A:20X), Micronutrients (MS-B:100X), Vitamins (MS-G:100X), and iron stock (MS-F:200X) and were added in required proportions for making the media. Potassium dihydrogen phosphate (KH_2PO_4), myoinositol and sucrose were added directly after mixing the stocks. Stock solutions of growth regulators were prepared as 100 uM stocks. The required amount of growth regulators was weighed out carefully using electronic balance (Sartorius, Germany) and dissolved in a little amount of ethanol (95 %) or KOH solution (0.1N). The final volume was made with distilled water and stored in amber coloured bottles under refrigeration (4° C).

Normally, growth regulators were added prior to sterilization by autoclave but certain heat-labile growth regulators like ABA were added to the sterilized culture medium after filter sterilization using a stainless steel filter sterilization unit having a cellulose nitrate filter of pore size 0.22 (Sartorius, Germany). In liquid media the filter sterilized plant growth regulators were added just before the inoculation.

The pH of all media was adjusted to 5.8 $\pm$ 0.1 with 1 N HCl or 1 N KOH (freshly prepared) using a digital pH meter (Digitronics India). Then agar-agar was added if the medium was semisolid and brought it to boil with constant stirring. The medium was then dispensed in oven dried glassware and the mouth of the vessels was plugged with non-absorbent cotton plugs wrapped with gauze cloth. The media and other things required for aseptic operations (petridishes, forceps, spatula, scalpel etc were wrapped in newspaper) and were autoclaved at 121^0 C (1.5 Kg / cm^2 pressure) for 15 minutes. The autoclaved media were then kept in a dust free room before inoculations.

Aseptic operations were carried out in a laminar airflow hood (Klenzaids, India; ADC, Baroda). The working bench was wiped with 70 % alcohol or dilute dettol. After arranging all the culture vessels (media) and other instruments on the bench, it was exposed to UV irradiation for 30 minutes. The instruments were frequently flame sterilized with alcohol during manipulations.

Culture Conditions:

The cultures were maintained in a culture room with controlled conditions of temperature (25 $\pm$ 1^0 C), light (50-60 uEm^{-2} S^{-1}) and photo period (10 /14 hr day / night). The liquid cultures in Erlenmeyer flasks (25, 50, 100, 150 ml) were kept on a gyrotatory shaker (90-100 rpm). Subculturing of the cultures was done every 3 weeks.

Storage:

The artificial seeds were stored in an airtight desiccator in small petridishes (3 cm dia) sealed with parafilm at low temperature (5^0 C).

Germination:

The artificial seeds were scored for germination in semi-solid media, in culture vessels containing mixture of vermiculite/ perlite and in soil. Except in media the artificial seeds were irrigated with basal liquid MS medium (half-strength).

Statistical analysis:

Averages were represented as mean $\pm$ standard error of the replicates. The data were analyzed by one way and two way ANOVA and 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 **SPSS for Windows 6.0** statistical package.

Table-2: Chemical composition of Murashige & Skoog medium (1962)

Constituents	Amount (mg/lt)	Stock Volume/lt (ml/lt)
Macro Nutrients (MS-A)		50 ml /lt (Stock)
1. NH_4NO_3	1650	
2. KNO_3	1900	
3. $CaCl_2$	440	
4. $MgSO_4$	370	
Micro Nutrients (MS-B)		10 ml /lt (Stock)
1. H_3BO_3	6.2	
2. $MnSO_4.H_2O$	16.9	
3. $ZnSO_4.7H_2O$	8.6	
4. KI	0.83	
5. $Na_2MoO_4.2H_2O$	0.25	
6. $CuSo_4.5H_2O$	0.025	
7. $CoCl_2.6H_2O$	0.025	
Organic constituents (MS-G)		10ml/lt (Stock)
1. Nicotinic acid	0.5	
2. Pyridoxine-HCl	0.5	
3. Thiamine-HCl	0.1	
4. Glycine	2	
Iron Source		5ml/lt (Stock)
1. $FeSO_4.7H_2O$	27.8	
2. $Na_2EDTA.2H_2O$	37.3	
KH_2PO_4	170	(added directly)
Myoinositol	100	
Sucrose	3×10^4	
pH	5.8	

*The $FeSO_4.7H_2O$ and Na_2 EDTA.$2H_2O$ were dissolved separately in approximately 200 ml of double distilled water. Both the solutions were heated until dissolved completely. The FeSO4 was added to the warm EDTA solution with continuos stirring. The volume was then adjusted to 1000 ml with double distilled water. The Fe-EDTA solution prepared by this method could be stored in amber coloured bottle under refrigeration upto 2 months without precipitation.