

Chapter: 2

2.0: Materials and Methods

2.1A: Glassware

All the glassware used were of borosil. Bottles of (300,500ml) and flasks of (1000,2000 ml) were used according to the requirement for respective experiments. Culture tubes (25x150mm), beakers, micropipettes of different precision measurements (1000-5000 μ l) and Petridishes(100mm) were used.

All the glasswares were dipped in chromic solution(Potassium dichromate+Sulphuric acid) and next day washed with liquid soap followed by washing with tap water. Finally rinsed with distilled water and kept for drying in hot air oven at 60°C.

2.1B: Chemicals

All the chemicals used were of high purity (AR grade). These were procured from Merck India and Loba chemie. All PGRs were obtained from Himedia.

- Inorganic salts and vitamins
- BAP(6-Benzyl adenine) extrapure AR
- Kinetin (6-furfurylaminopurine) extrapure AR
- TDZ(Thiadiazuron)
- NAA
- IBA
- IAA
- GA₃
- Streptomycin sulphate
- HgCl₂
- Ascorbic acid
- Citric acid
- Labolene

2.1C: Water

Water used for preparation of media was distilled water.

2.1D: Carbon source

Sucrose pure

2.1E: Gelling agent

Agar agar

2.1F: Equipments used

- pH meter
- Electronic balance
- Autoclave
- Laminar air flow
- Glass Bead steriliser

2.1G: Plant tissue culture media

The medium used in plant tissue culture may be classified into two broad categories- chemically defined media and complex media.

Chemically defined media: A medium whose exact chemical composition is known. It contains one or more synthetic purified compounds.

Complex medium: The exact chemical composition of the constituents of this type of medium is not known. It contains natural compounds and their extracts viz. coconut milk, wheat germ extract, casein hydrolysate, yeast extract etc.

The *in vitro* growth of the plant depends on the media selected. The media should contain a combination of suitable minerals, a carbon source, vitamins and growth regulators. There are different types of plants, their requirements are also different and therefore, different types of media are used. No single media can be designed so as to meet the requirement of all types of plants. Even different parts of the same plant may have different requirements. Some media are

high salt while others are either low salt or medium salt. the most commonly used basal media are Heller's medium (1953), Murashige and Skoog's medium (1962), White's medium (1963), Eriksson's medium (1965), Gamborg B5 medium (1968), Nitsch's medium (1969), Nagata and Takebe medium (1971) etc.

Depending on the requirements of the plant under study, a suitable basal media is selected. This media was further modified according to need of the plant selected for its culture.

2.1H: Plant Growth Regulators

The important growth regulators in plant tissue culture are; the auxins, cytokinins, gibberellins. Skoog & Miller were the first to report that the concentration of auxin to cytokinin determined the type and extent of organogenesis in plant cell cultures. Both an auxin and cytokinin are usually added to culture media in order to obtain morphogenesis, although the ratio of hormones required for root and shoot induction is not universally the same. Considerable variability exists among genera, species, and even cultivars in the type and amount of auxin and cytokinin required for induction of morphogenesis. Cytokinins plant hormones promoting cell division and differentiation (Mok and Mok, 2001). The cytokinins include natural and synthetic compounds, adenine derivatives and phenyl urea derivatives. Adenine derivatives are represented by Kinetin, Zeatin and 6-Benzylaminopurine. Phenylurea derivatives include Diphenyl urea or Thidiazuron. Purkayastha *et al.*, (2008) tested various cytokinins (BAP, Kinetin, Thidiazuron and 2-Isopentyl adenine) in MS medium for *in vitro* multiplication and plant regeneration from nodal explants of *Andrographis paniculata*.

Table2.1: Some commonly used plant hormones and their molecular weights.

Hormone	Molecular Weight
Auxins	
Indole-3- Acetic Acid (IAA)	175.18
3- Indole Butyric Acid (IBA)	203.3
∞ - Napthalene Acetic Acid (NAA)	186.20
2,4 – Dichlorophenoxy Acetic Acid (2,4-D)	221.04
2,4,5 – Trichlorophenoxyacetic Acid (2,4,5-T)	255.49
4- Amino – 3,5,6 – Trichloropicolinic Acid (Picloram)	241.46
Cytokinins	
Adenine (Ad)	189.13
Adenine Sulphate (AdSO ₄)	404.37
6 – Benzyl Adenine or 6 – Benzyl amino purine (BAP)	225.20
6 - γ, γ - Dimethylallyl amino purine or N – isopentenyl amino purine (2 – ip)	203.3
6 – Furfuryl amino purine (kinetin)	215.21
n- phenyl – N- 1,2,3—thiadiazol – 5 urea (thiadiazuron)	220.20
6 – (4-Hydroxy-3 methyl but-2-enylamino)purine (zeatin)	219.20
Gibberellins	
Gibberellic Acid (GA ₃)	346.37

2.1I: Medium composition and preparation

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium. Several media formulations are commonly used for the majority of all cell and tissue culture work. These media formulations include those described by the scientist White, Murashige and Skoog, Gamborg *et al.* (1968), Schenk and Hilderbrandt, Nitsch and Nitsch and Lloyd and McCown. Murashige & Skoog's (MS medium) (1962) Schenk and Hildebrand's (SH medium) (1972), Lloyd and McCown's Woody plant Medium (WPM) (1980) and Gamborg's (B-5) (1968) medium are all high in macronutrients. Gantait *et al.* (2011) accelerated *in vitro* propagation of medicinal plant, *Aloe vera* L.

In the present study three media were used, their composition given as follows:

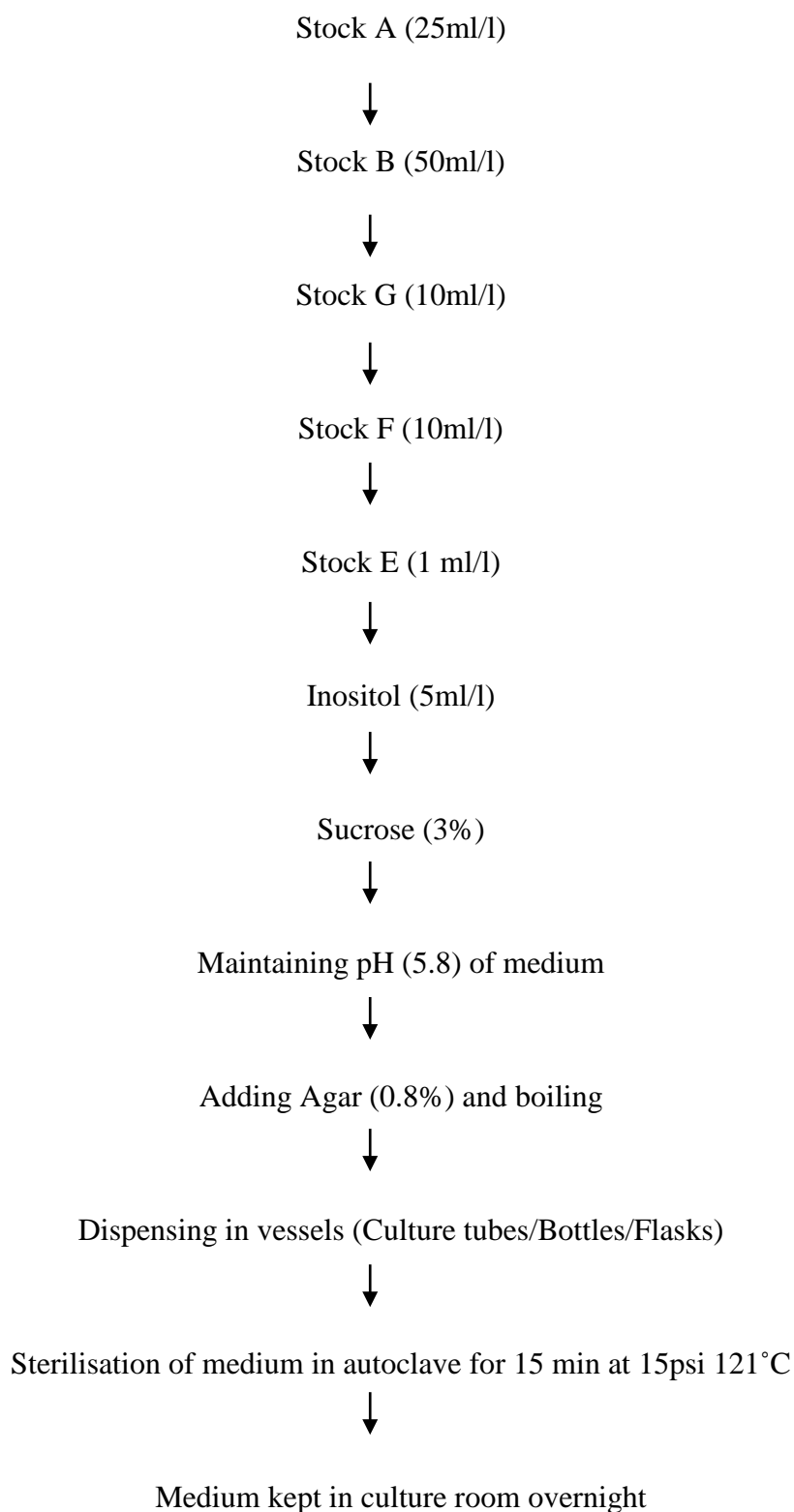
Table2.2:Standard Basal Media Formulations of MS, Gamborg B5 and WPM medium

Constituents	Murashige and Skoog's	Gamborg at al B5	WPM
Inorganic constituents			
Ammonium nitrate (NH ₄ NO ₃)	1650	-	400
Potassium nitrate (KNO ₃)	1900	2527.5	
Calcium chloride dehydrate (CaCl ₂ .2H ₂ O)	440	150	96
Calcium chloride (CaCl ₂)	-	-	
Magnesium sulfate (MgSO ₄ .7H ₂ O)	370	246.5	370
Photassium dihydrogen phosphate (KH ₂ PO ₄)	170	-	170
K ₂ SO ₄	-	-	980
Ammonium sulfate (NH ₄) ₂ SO ₄	-	134	
Calcium nitrate (Ca(NO ₃) ₂ .4H ₂ O)	-	-	556
Sodium nitrate (NaNO ₃)	-	-	-
Sodium sulfate (Na ₂ SO ₄)	-	-	-
Sodium dihydrogen phosphate (NaH ₂ PO ₄ .H ₂ O)	-	150	-
Potassium iodide (KI)	0.83	0.75	-
Boric acid (H ₃ BO ₃)	6.2	3	6.2
Manganese sulfate tetrahydrate (MnSO ₄ .H ₂ O)	22.3	-	22.30
Manganese sulfate monohydrate (MnSO ₄ .H ₂ O)	-	10	-
Zinc sulfate heptahydrate (ZnSO ₄ .7H ₂ O)	8.6	2	8.8
Na ₂ MoO ₄ .2H ₂ O	0.25	0.25	0.25
MoO ₃	-	-	-
Copper sulfate (CuSO ₄ .5H ₂ O)	0.025	0.025	0.25
Cobalt chloride (CoCl ₂ .6H ₂ O)	0.025	0.025	-
Cobalt sulfate (CoSO ₄ .7H ₂ O)	-	-	-
Aluminum chloride (AlCl ₃)	-	-	-
Nickel chloride (NiCl ₂ .6H ₂ O)	-	-	-
Ferric chloride (FeCl ₃ .6H ₂ O)	-	-	-
Ferric sulfate (Fe ₂ (SO ₄) ₃)	-	-	-
Ferrous sulfate (FeSO ₄ 7H ₂ O)	27.8	-	27.8
Sodium salt of EDTA (Na ₂ EDTA.2H ₂ O)	37.3	-	37.3
Sequestrene 330Fe	-	28	-
Organic constituents			
Inositol	100	100	100
Nicotinic acid	0.5	1	0.5
Pyridoxine.HCl	0.5	1	0.5
Thiamine.HCl	0.1	10	0.1

Glycine	2	-	-
Sucrose	3%	2%	2%

2.1J: Preparation of culture medium

Flow chart of medium preparation for (1 litre)



2.2: Plant Materials

Plants: *Citrus aurentifolia* and *Ficus carica*

Explant source: Explants of Fig used were collected from:

1. Sivuri village, Taluka Saswad, Pune, Maharashtra
2. Govind farm, Ghadkan village near Chilloda Chokdi, Ahmedabad

Explant source: Explants of Lemon used were collected from:

1. Sun Agrigenetics farmhouse, Hatipura, Vadodara
2. Govind farm, Ghadkan village near Chilloda Chokdi, Ahmedabad

2.2A: Explant Preparation

2.2A1: Fig explant preparation

Explant washed with running tap water for 45 min.

↓

Add 2-3 drops of Axalin with 0.1 % NaOCl, shake (agitate) for 25 Min.

↓

Clean with sterile water in laminar air flow chamber (LAF)

↓

70% IPA wash (Iso Propyl Alcohol) for 1 min (Rinse with sterile water twice)

↓

Finally treat with 0.1% HgCl₂ for 5 min.

↓

5-7 times wash with sterile water and kept all ex-plant in Antioxidant Solution
(L-ascorbic acid and Citric acid)

2.2A2: Lemon explants preparation

The ex-plants collected from elite tree were washed under running tap water



Add few drops of detergent (Teepol) for removing the dust particles



Rinsed with demineralized water



After a brief 30 second rinse with 70% Iso propyl alcohol



Apical buds, axillary buds from young branches and axillary buds from mature branches were separately surface sterilized with HgCl_2 (0.1%) solution for 5-7 minutes depending upon the tenderness of the tissue



Followed by several rinses with sterile water



The nodal and apical stem sections were trimmed to 0.5 cm size under aseptic conditions and were inoculated into the culture tube

The basal culture medium consisted of MS salts (Murashige and Skoog's 1962), vitamins, inositol, and sucrose 3% and it was gelled with 0.8 % Agar-agar.

2.2B: Culture condition:

All sterilized cultures inoculated in respective media in laminar air flow and kept in growth room under 25 ± 2 °C with 8 hours in light period (2500 lux).

2.3: Establishment of cultures

Fig

MS/WPM+ BAP(0,0.5,1 mg/l)

MS/WPM+ Kn(0,0.5,1 mg/l)

MS+ BAP(1,2,3,4,5 mg/l)+ NAA(0.1 mg/l)

MS+ TDZ(0.1,1,1.5 mg/l)+IBA(1,1.5,2 mg/l)

MS+BAP(1,2,2.5,5,7.5,10 mg/l)+GA(0.1,0.2,0.25,0.3,0.3,0.3mg/l)

MS+BAP(0.2)+NAA(0.2)+GA(0.1mg/l)

MS+BAP(0.2)+NAA(0.2)+GA(0.2mg/l)

Lemon

MS+BAP(0,0.1,0.3,0.5,0.8,1.0,1.5,2.0 mg/l)+NAA(0.1mg/l)

MS+BAP (0.5,1,2.0 mg/l)+GA(0.2 mg/l)

MS+ BAP (0.1,0.5,2.0 mg/l)+NAA(0.1 mg/l) + GA(0.2mg/l)

2.3A: *In vitro* rooting

Fig

1/2 MS + BA and IBA,NAA, IAA (5,10,15μM)

½ MS + IAA 1 μM+ Activated charcoal/Without AC

½ MS + BAP (0.1 mg/l)+ IBA(0,0.1,0.5,1.0,1.5,2.0 mg/l)

Lemon

MS+ BAP (0.1) + IBA (0,0.5,1,1.5,2.0 mg/l)

2.3B: Hardening

In Lemon

Plantlets with well developed root system in following potting mixtures:

Soil: vermiculite (1:1)

Soil: farmyard manure mixture (2:1)

In Fig

Plantlets were transferred to following potting mixtures:

Cocopeat

Cocopeat + 25% Vermiculite

Cocopeat +25% Vermicompost

Cocopeat+25% Sand

2.3C: AMFungi Isolation

Isolation of AM spores

1. Soil Sample collection :-

Soil Samples were collected from Kutch, Gujarat. Random sampling was done. Their

Physico-chemical characteristics studied were as follows:

- 1) Texture
- 2) % Moisture
- 3) Colour
- 4) pH

2. Isolation of AM Fungi from Soil :- (Rhizosphere & Nonrhizosphere)

AM spores were isolated by “Wet Sieving & Decanting method” (Gerdeemann & Nicolson 1963). For identification of AM fungi manual for the isolation of AM Fungi by Sheneck & Perez (1990) was used.

- The residues containing AM spores & soil particles was collected after passing the sample through 60, 90, 150, 201 & 500 μm sieves.
- The residues collected from 210 μm & 150 sieves was examined for sporocarps & large spores.
- The residues from 90 μm and 60 μm sieves were observed for small and detached AM spores.
- Most of the spores were trapped in the sieves.
- The spores were isolated.
- After the spore count, selected spores represent each type was isolated.

3. To find out the association of AM Fungi in the roots of fruit trees (lemon and fig).

- The root system was carefully excavated taking care to ensure the fine roots pre-dominate in sample and exclude entangled roots of other species.
- Roots were kept in moist in condition in clean Polyethylene bags and were brought to lab.
- Roots were properly washed in running tap water and cut into 1 cm segments and 100 such segments were picked up and cleaned in a 10% aq. KOH solution for 24 hrs. Then washed in D. W. to remove traces of KOH.
- Roots were stained in Trypan blue (Phillips and Hayman 1970).
- Stained roots were picked up and were examined under microscope.

A. Preparation of Inoculum

- (1) 200 spores of 15 different AM Fungi were separated from fruit crop field (lemon and fig) by wet sieving & decanting process.
- (2) These spores were sterilized by keeping in 200 PPM Streptomycin sulphate for 5 minute.
- (3) After disinfection, thoroughly washed with D. W. and were used as inoculants for initiating mycorrhization in Maize Plant.

B. Mycorrhization in Maize Plant

1. 5 seeds of Maize (*Zea mays*) were sown in black polybag (PP) filled with sterilized soil and sand mixture (1:1) along with 100 spores of the AM Fungi. The pots were kept in the Green house under constant observation.

[Soil sterilization method Autoclaved]

C. Colonization Studies

After two months, percentage colonization was studied in maize seedlings and 5 best colonizing AM Fungal isolates were selected.

1. AMF colonization was studied in following 2 target plants : lemon and fig
2. The target plants (100 each) were grown in 30 cm diameter pots.
3. The inoculums (5 root pieces, 50 Spores / 100 gm air dried soil) was placed 3 cm deep in 10 cm diameter area in the centre of the pots prior to sowing the seeds.
4. Control treatments without any AMF inoculums was maintained (Method suggested by Al-Karaki, 1998 & Multi spore culture suggested by Walkes (1999).

5. The sapling of 2 target fruit crop were raised after inoculation with 5 AM Fungi for 3 months:
 - i. % root colonization
 - ii. Root length & branching patterns.
 - iii. Fresh and dry wt of Root & shoot biomass was estimated following standard procedure.
6. The AM fungal isolates (same or different isolates) showing better growth and colonization in each of the 2 fruit crops were selected for further studies.

2.3D: AM

Plant Growth Parameters:-

- i) Plant Height
- ii) Number of leaves
- iii) Root Length
- iv) Root Dry Weight or Fresh Weight
- v) Shoot Dry Weight or Fresh Weight

Physico-chemical properties of experimental field Soil:-

- i) pH
- ii) Texture
- iii) Colour

A. Wet Sieving and Decanting Method

1. Weighed 100 gm soil and take 500 ml distilled water.
2. Added 100 gm soil into 500 ml D.W., stir it and keep it to settle for 20 minutes.
3. All the sieves were set from highest to lowest (500 μm \rightarrow 210 μm \rightarrow 150 μm \rightarrow 90 μm \rightarrow 60 μm).
4. Decanted the upper layer of water in it.
5. Various size particles were obtained from 90 μm and 63 μm sieve by adding water to it and observed under binocular microscope.
6. Then transferred the spores to slide and observe under microscope are yellowish in colour and round in shape.
7. Lastly counted the number of spores per 100 gm soil.

B. Determination of the pH of Soil :

- i) Taken 20 gm soil and take 5 times of D.W. (i.e. 100 ml.).
- ii) Then mixed it thoroughly and allowed it to settle and supernatant was used for determination of the pH of soil.

C. Determination of biomass

- Root / Shoot samples were washed blot dried, placed in an oven at 80 degree centigrade for 48 hr. and dry weight was determined.

Requirements: -

1. Polythene bags
2. Pots

3. Distilled water (D.W.)
4. Sieves (Different size μm)
5. Slide & coverslip box
6. Microscope (Stereobionocular)
7. 10 % KOH
8. Trypan Blue
9. Glycerine
10. Lactophenol
11. Blotting Paper
12. Match Box
13. Field Notebook/ Experiment notebook.

During past few years, there has been an increasing interest for *in vitro* multiplications and germplasm conservation of rare, endangered, aromatic and medicinal plants. Mass propagation of plant species through *in vitro* culture is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently, there has been much progress in this technology for some plants which have multiple economic values and become very attractive due to their energy resources.

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. It is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction. Micropropagation involves a set of procedures that multiply plants in tissue culture with minimal genetic and epigenetic variability. Micropropagation is one of the innovative methods of asexual

propagation, which proved to be effective for in vitro propagation of medicinal and endangered plants (Pattnaik and Chand, 1996).