Online Training Course on Horticulture Seed Propagation with Tissue Culture for Caribbean and Latin American Countries

TISSUE CULTURE MEDIA

AGENCY FOR AGRICULTURE EXTENSION
AND HUMAN RESOURCES DEVELOPMENT
AGRICULTURE MINISTRY

What’s in Tissue Culture Medium?

Excised plant tissues and organs will only grow in vitro on a suitable artificially prepared nutrient medium which is known as a **culture medium**.

Media used in plant tissue culture contain nutritional components, which are essential for the growth and development of the cultured tissue. The success of the tissue culture depends very much on the types of culture media used.

Example of some already established Plant Tissue Culture Medium

- Murashige and Skoog (1962): **MS** – media contain desired salt concentration and widely used
- Linsmaier and Skoog (1965): **LS** – media contain desired salt concentration and widely used
- **White's medium** (1963): media contain low salt concentration and used for root culture.

Example of some already established Plant Tissue Culture Medium

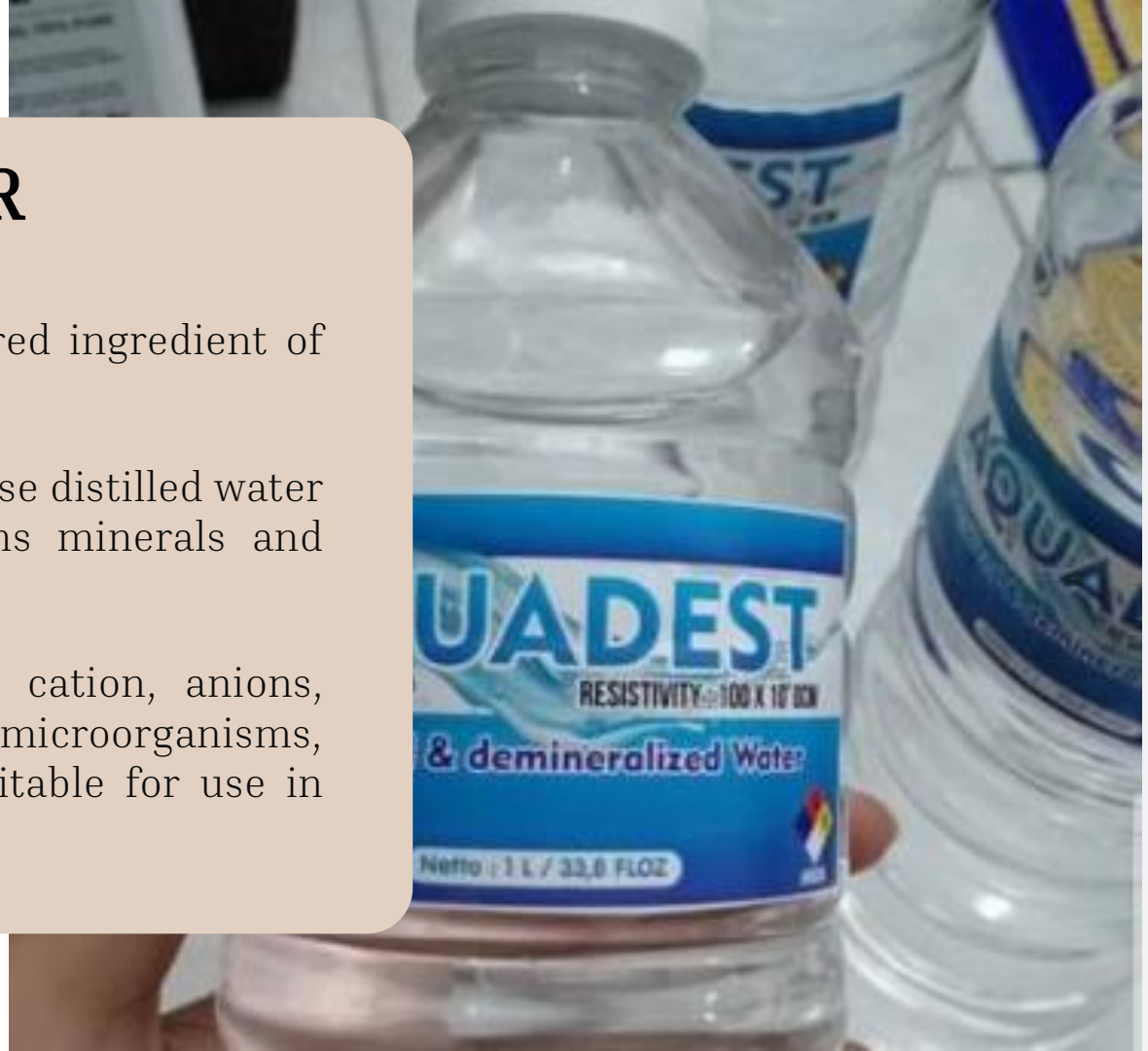
- Gamborg et al.(1968): **B5** - media contain much greater proportion of Ammonium and Nitrate ions and used for cell suspention or callus culture.
- Nitsch and Nitsch (1969): **N6** - media contain low salt concentration and used for anther culture.
- Lloyd & McCown (1981): **WPM** - media contain very low salt concentration and used for tree sp.

Composition of Culture Medium

1. DISTILLED WATER
2. MACRO AND MICRO NUTRIENTS
3. VITAMINS
4. SUGAR
5. AGAR
6. ORGANIC COMPOUNDS
7. PLANT GROWTH REGULATORS

1. DISTILLED WATER

- High quality water is a required ingredient of plant tissue culture media.
- Making stock solutions must use distilled water because it no longer contains minerals and organic compounds.
- Ordinary tap water contains cation, anions, particulates of various kind, microorganisms, and gases that make it unsuitable for use in tissue culture media.



2. MACRO AND MICRO NUTRIENTS

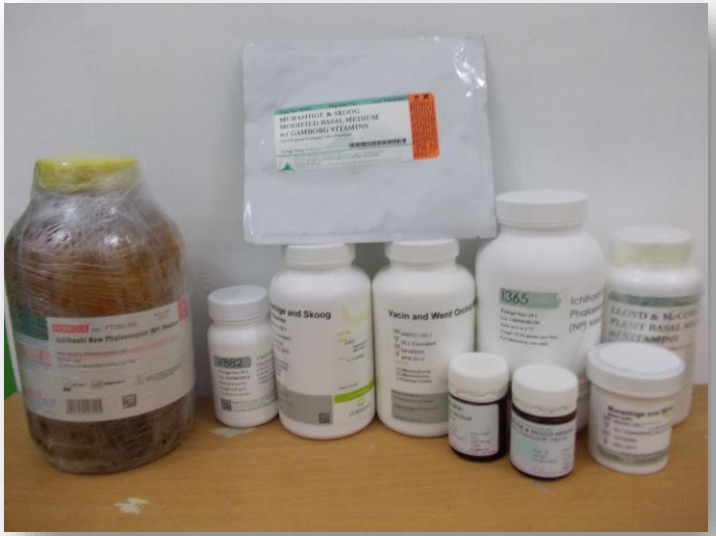
A. Major Mineral Nutrients or Macronutrients

- Nitrogen as either Nitrate (NO_3) and Ammonium (NH_4)
- Calcium as $CaCl_2$ or $Ca(NO_3)_2$
- Magnesium as $MgSO_4$
- Potassium as KCl or K_2HPO_4
- Phosphorus as K_2HPO or KH_2PO_4 or Na Salts
- Sulfur as many SO_4

These elements have both structural and functional roles in protein synthesis (N & S), nucleotide synthesis (P, N & S), cell wall synthesis (Ca), enzyme co-factors (Mg) and membrane integrity (Mg).

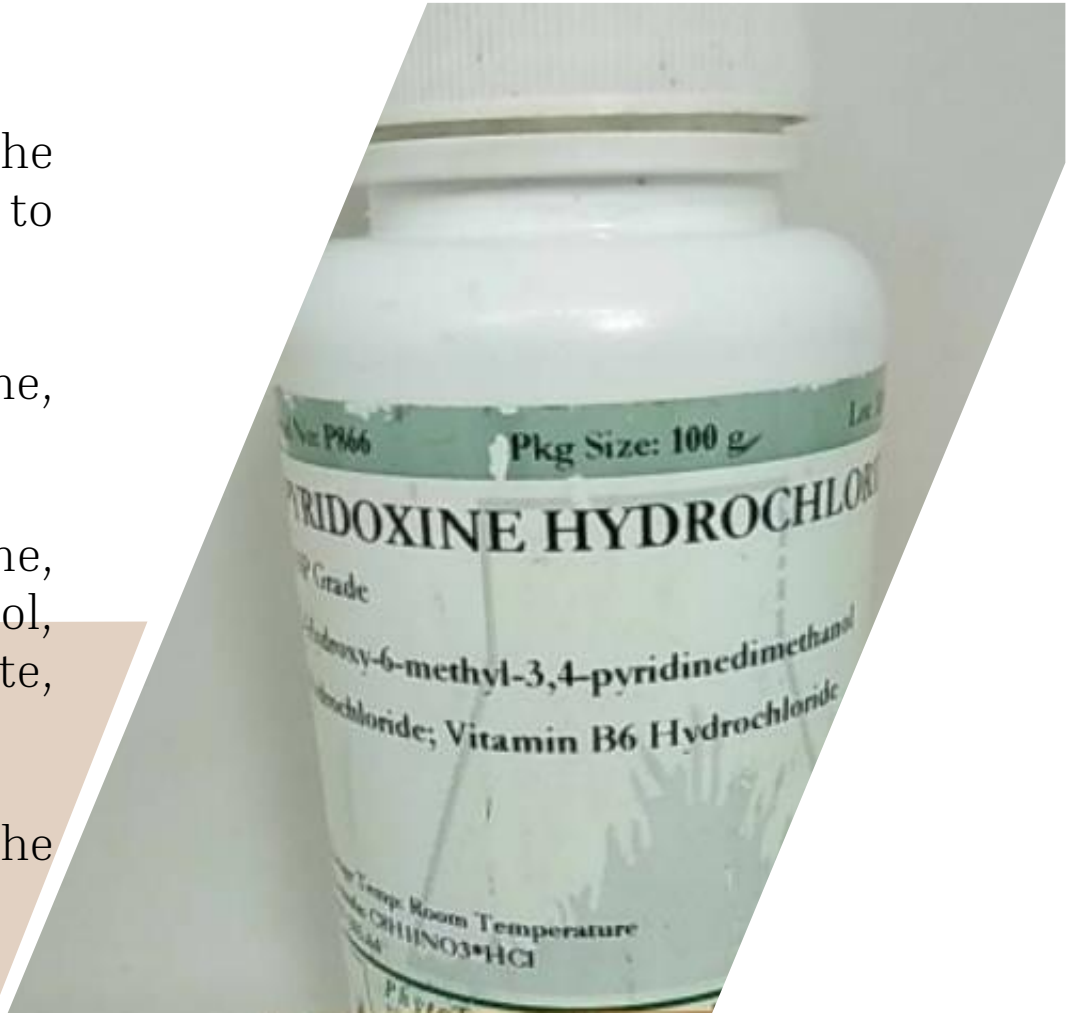
B. Minor Mineral Nutrients or Micronutrients

- Boron (B)
- Cobalt (Co)
- Iron (Fe – usually Chelated with NaEDTA)
- Manganese (Mn)
- Molybdenum (Mo)
- Copper (Cu)
- Zinc (Zn)
- Iodine (I)



3. VITAMINS

- In vitro plant culture requires the addition of vitamins that function to increase plant cell growth.
- Vitamins use are thiamin, pyridoxine, nicotinic acid, riboflavin.
- Amino acid use are glutamine, aspartic acid, arginine, myoinositol, adenine sulfate, casein hydrolysate, glycine, etc.
- Myoinositol use to stimulate the growth of cultured tissues.



4. SUGAR

- Cane Sugar = Sucrose (Fructose and Glucose)
- Corn Sugar = Fructose
- Use of autoclaved fructose is not recommended as it could be detrimental to the growth of tissue
- Typically Added Between 20 and 40 g/l

Why Sucrose is most preferable Carbon Sources for plant tissue culture?

- Cane Sugar = Sucrose (Fructose and Glucose)
- Sucrose is the cheaper source of carbon
- A partial hydrolysis of sucrose occurs when media are autoclaved and formed glucose and fructose
- Its act a good osmotic stabilizer

5. ORGANIC COMPOUNDS

- Natural supplement ingredients, such as tomato juice, orange juice, coconut water, malt extract, yeast extract, are sometimes added to the optimal culture media.

- These ingredients are trusted sources of amino acids, peptides, vitamins, and natural growth regulator.

Why we use the activated charcoal in plant tissue culture medium?

Activated charcoal is mainly used for its irreversible adsorption of inhibitory compounds in the culture medium i.e. **the toxic metabolites**, **phenolic exudation** and **brown exudate**. In addition to this AC is involved adsorption of vitamins, metal ions and plant growth regulators, including **abscisic acid** and gaseous **ethylene**.

6. AGAR (SOLIDIFYING AGENTS)

•**Agar** is a natural product of seaweeds and is obtained from red algae (*Gelidium, Gracilaria*), which consists of **Agarose** and **Agropectine**

•The tertiary structure of a Agarose is a double helix with a central cavity, which can accumulate water molecules (up to 99.5%)

•Agar does not gel well under acidic condition (pH<4.5)

•Crude Agar contains lots of impurities – minerals, organic compounds, which may interfere with tissue culture

•Phytoagar is purified (lacking most impurities) and has a melting point of about 65°C and a gelling point between 40-50°C

•**Agarose** is a purified fraction of **Agar.** Agarose is extracted from agar leaving behind **agropectin** and Typically has low melting and gelling points. It is more expensive and use for protoplasts

- **Gelrite or phytagel:** Gelrite is a naturally derived gelling polymer, produced by the microbial fermentation of a bacterium ***Psudomonous elodae***. It is an attractive alternative to agar for plant tissue culture because of its remarkably clear in appearance than that of agar. One limitation of Gelrite is that concentration such as **calcium and megnisium ions must be within range of 4- 8mM/L**. Higher concentration of this ions do not allow the gelling of the media.

7. PLANT GROWTH REGULATORS

- Organic compounds but not plant nutrients.
- Active in small amounts.
- Synthezised in certain parts of the plant and generally translocated to other parts of the plant where these substances cause biochemical, physiological and morphological responses

- Growth regulators have an important role in tissue culture activities, such as for regulating the development of explants, for example, organogenesis or embryogenesis, by adjusting the type and concentration of certain grow regulators in optimal combinations.

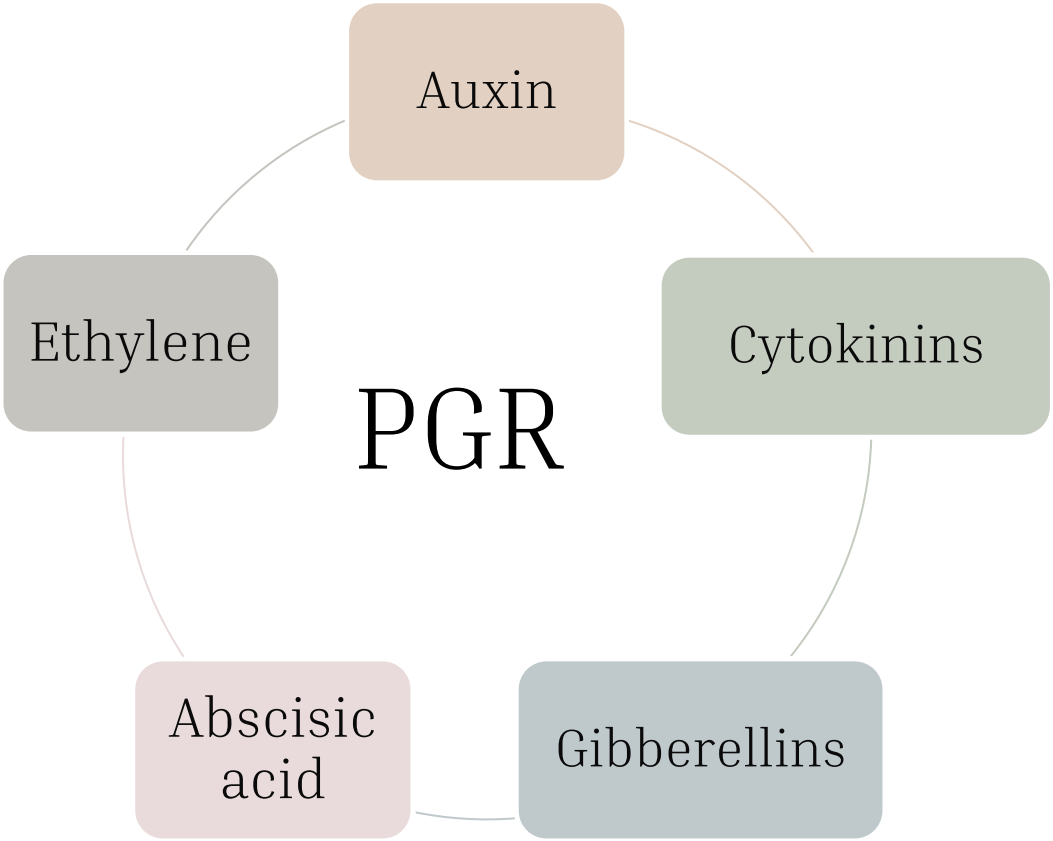
In research/ Commercial applications of plant tissue culture techniques

the investigator must understand the difference between the terms:

-Plant hormone
-Plant growth regulator

- In the strictest sense only those substances that naturally occur in plants and fit the previous definition can be considered plant hormones.

- However, the agrochemical industry has coined a second term : Plant growth Regulator – used to denote only synthetic compounds that exhibit hormonal activity.


Auxin
Cytokinins
Gibberellins
Abscisic acid
Ethylene
PGR

Auxin

- Auxin are required by most plant cells for cell division, cell elongation, cell differentiation, organogenesis and embryogenesis.
- Miller and Skoog (1957) noted that a hifh amount of a Cytokinin and amount low by Auxin forms shoots.
- Roots are induced by a low concentration of a Cytokinin and high Auxin and callus is formed at an intermediate concentration of Auxin and Cytokinin

Cytokinins

Cytokinins are adenine derivatives which are mainly concerned with cell division, modification of apical dominance and shoot differentiation in the tissue culture.

Naturally available cytokinins are BA (6-benzyladenine), Kinetin, Zeatin and **synthetic cytokinins** are BAP (6-benzylaminopurine), *2-ip* (6-γ-γ-dimethyl aminopurine) etc.

Gibberellin

- Gibberellic Acids (GA): More Than 60 Forms GA 4 & 7 Most Commonly Used.
- GA is not frequently used in general culture media.
- However it promotes stem elongation, bulb & corm formation and embryo maturation but can inhibit callus growth and root induction.

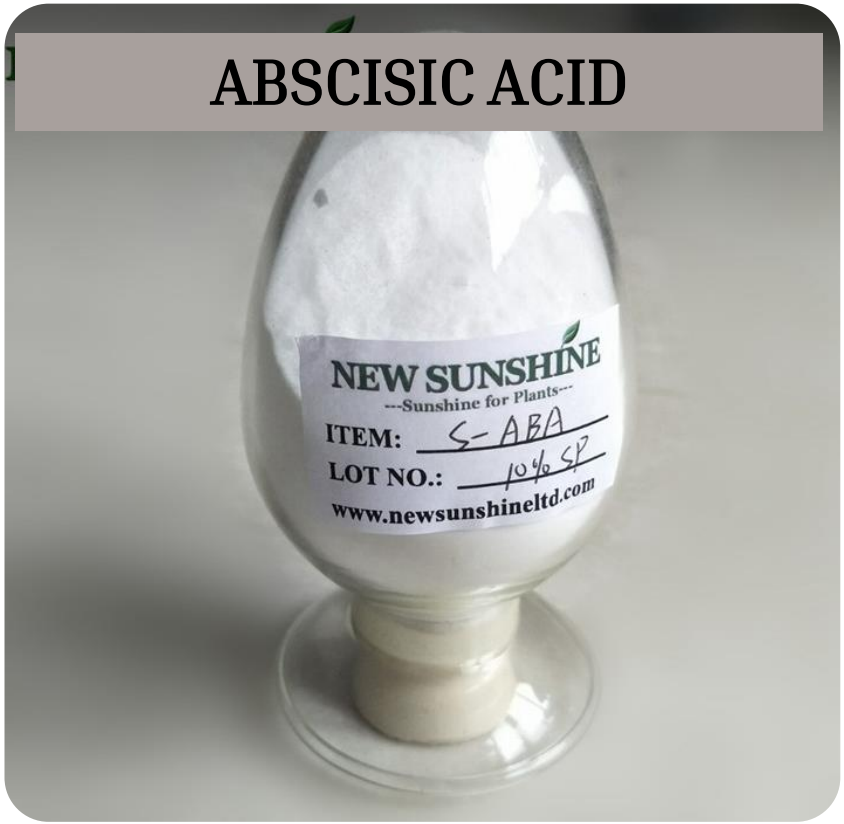
ABSCISIC ACID

ABA is a plant growth regulator that is naturally synthesized in chloroplasts and found in various types of plants

ABA is produced by plants when under stress

ABA is classified as a plant inhibitor because it works opposite to auxins, cytokinins, or gibberellins

In tissue culture, it inhibits shoot growth and germination of embryos but is helpful in embryo culture

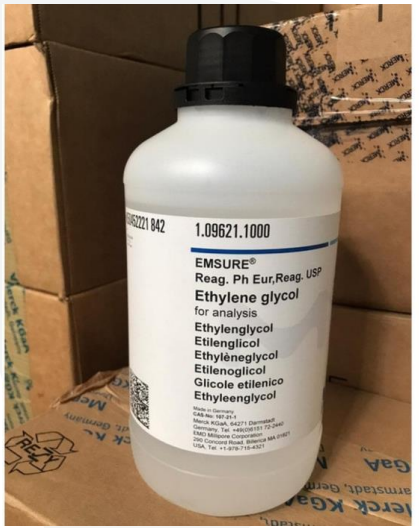


ETHYLENE

The role of ethylene:

- Induce callus growth
- Inhibits apical dominance
- Stimulates the formation of adventitious and axillary shoots
- Stimulates root formation
- Stimulates the formation of flowers

The root formation is not very specific, meaning that there is something that encourages some that inhibits it, this depends on the type of plant and the concentration of ethylene



Plant Growth Regulators

- Used in concentrations of 0.001 – 10 uM
- Many can be autoclaved (especially synthetic), but others degrade with heat and should be filter-sterilized
- Most have interactions with each other – can cause a multitude of effects
- Can be prepared in water, KOH, ethanol, DMSO (Dimethyl sulfoxide)

Preparation of Plant Growth Regulators

Example : Benzyladenine or BA

- Dissolve 20 mg of Either BA (Benzyladenine) in 5 ml of 95% Ethanol or 1.0N KOH
- Bring Volume to 20 ml with Water
- Store at 4°C

Antibiotics

- Antibiotics are not used routinely against contaminants in micropopagation because often they are in effective, kill the culture, or induce chromosomal instability. However, some have been useful on occasion.
  - Cefotaxime (25mg/L)
  - Carbenicillin (500mg/L)
  - Ampicillin (100-400mg/L)

Murashige and Skoog (MS) Media		Woody plant media (WPM)	
Components	mg/l	Components	mg/l
Macro Salt		**Macro Salt**	
KNO_3	1900	NH_4NO_3	400
NH_4NO_3	1650	$MgSO_4.7H_2O$	180.54
KH_2PO_4	170	$CaCl_2.2H_2O$	72.5
$CaCl_2.2H_2O$	440	$Ca(NO_3)_2$	386.8
$MgSO_4.7H_2O$	370	KH_2PO_4	170.00
		K_2SO_4	990.00
Micro Salts		**Micro Salts**	
H_3BO_3	6.2	H_3BO_3	6.25
$CuSO_4.5H_2O$	0.025	$MnSO_4.4H_2O$	22.3
$MnSO_4.4H_2O$	22.3	$ZnSO_4.7H_2O$	8.6
$ZnSO_4.7H_2O$	8.6	$CuSO_4.5H_2O$	0.25
$Na_2MoO_4.2H_2O$	0.25	$Na_2MoO_4.2H_2O$	0.25
KI	0.83		
$CaCl_2.6H_2O$	0.025		
Vitamins		**Vitamins**	
Nicotinic acid	0.5	Nicotinic acid	0.5
Pyridoxine HCl	0.5	Pyridoxine HCl	0.5
Thiamine HCl	0.1	Thiamine HCl	1
Myoinositol	100	Myoinositol	100
Amino acid – Glycine	2	Amino acid – Glycine	2.0
Iron EDTA		**Iron EDTA**	
Na_2 EDTA	37.3	FeNa EDTA	36.7
$FeSO_4.7H_2O$	27.8		
Sucrose	30 g	Sucrose	30 g
Agar - agar	8 g	Agar - agar	8 g

examples of media composition

The tools needed in the stock solutions procedures :

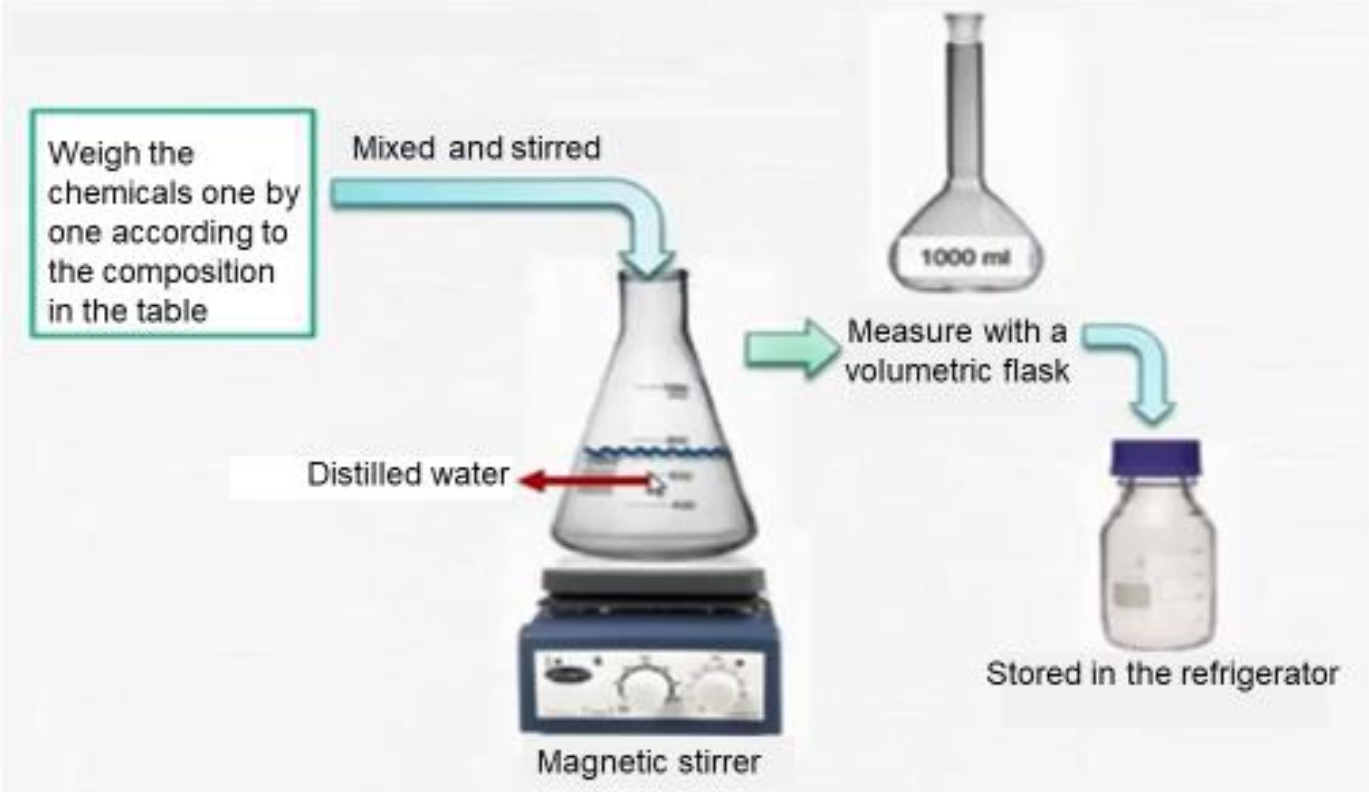
- Analytical balance
- Hotplate Magnetic stirrer
- Spatula
- Watch glass
- Beaker glass
- Measuring cylinder
- Pipette
- Bottles
- Aluminum foil
- Label
- Tissue paper
- pH indicator paper

Designated stock	Compound	Formula	Concentration (mg/L)	Quantity for stock solution (g/20 L)	Volume of solution (ml)	Volume required for 1 L
	Major					
A (Macro 1)	Ammonium nitrate	NH_4NO_3	1650	33	1000	50
	Potassium nitrate	KNO_3	1900	38		
	Magnesium sulfate	$MgSO_4{\cdot}7H_2O$	370	7.4		
	Potassium dihydrogen phosphate	KH_2PO_4	170	3.4		
B (Macro 2)	Calcium chloride	$CaCl_2{\cdot}2H_2O$	440	8.8	1000	50
C	*Minor*					
	Manganese sulfate	$MnSO_4{\cdot}4H_2O$	22.3	0.446	200	10
	Zinc sulfate	$ZnSO_4{\cdot}7H_2O$	8.6	0.172		
	Boric acid	H_3BO_3	6.2	0.124		
	Potassium iodide	KI	0.83	0.0166		
	Sodium molybdate	$Na_2MoO_4{\cdot}2H_2O$	0.25	0.005		
	Copper sulfate	$CuSO_4{\cdot}5H_2O$	0.025	0.0005		
	Cobalt chloride	$CoCl_2{\cdot}6H_2O$	0.025	0.0005		
D	Iron					
	Ferrous sulfate	$FeSO_4{\cdot}7H_2O$	27.8	0.556	200	10
	EDTA disodium	$Na_2EDTA{\cdot}2H_2O$	37.2	0.744		
E	Vitamins					
	Nicotinic acid		0.5	0.01	20	1
	Pyridoxine hydrochloride		0.5	0.01		
	Thiamine hydrochloride		0.1	0.002		
	Glycine		2	0.04		
F	Meso-inositol		100	2	200	10
	Sucrose		30 g/L			
	Agar		8 g/L			

Description :

A. Macro stock 1
B. Macro stock 2
C. Micro stock
D. IronEDTA stock
E. Vitamins stock
F. Plant Growth Regulator stock

Stock Solution Procedures



A flow chart for media preparation

For **1L media** preparation take **750ml of distilled water** and desired amount of **sucrose**

Preparation of Stock Solution

- Stock solution of Macronutrients (X20)
- Stock solution of Micronutrients (X100)
- Stock solution of Vitamins (X100)
- Stock solution of Iron EDTA (X100)
- Stock solution of Hormons (1 mg/ml)

Add minerals, vitamins, hormones and other ingredients from the stock solution

Make the final volume to 1L with distilled water

Adjust the pH of the medium 5.6-5.8 with 0.1N HCl or 0.1N KOH

Add solidifying agent if the desired melt it and distributed into jars.
The medium is finally sterilized by autoclaving

pH

Plant cell and tissues require optimum pH for growth and development in cultures. The pH affects uptake of ions and for most of the culture media, pH 5.0 to 6.0 before sterilization is considered optimal. Higher pH is likely to give a hard medium while a low pH results in unsatisfactory solidification of the agar.

Autoclave


WARNING
Hot surfaces
Heat resistant
safety gloves
required
Danger
High voltage
TRIUP INTERNATIONAL CORP.
PRES
TEMP
TEMP.CONTROL
TIMER
POWER
ON
OFF

Storage of Culture Media

Cold Room for Long-term Storage. Need to be Careful About Condensation of Water and Contamination. Usually About 4°C and in Darkness – Important for some PGRs (IAA).

Room Temperature in the Dark. Should be Wrapped or in Plastic Bag to Prevent Desication. Good for Assessing Contamination of Media Before Use.

Media Storage

Tissue Culture Media in Hot Pepper Seed Production

Hot peppers seed sterilization

↓

Seed initiaton media
MS without plant growth regulator

↓

Callus formation media
MS + 3 mg/L BAP + 1 mg/L NAA

↓

Shoot elongation media
MS + 1 mg/L BAP + 0.5 mg/L TDZ

→

Root induction media
MS + 0.5 mg/L NAA

↓

Plantlet acclimatization

Source : Ifa Manzila et al, 2010

Tissue Culture Media in Tomatoes Seed Production

Tomato seed sterilization

↓

Germination seed media
MS without plant growth regulator

↓

Initiation media
MS + 0.2 mg/L IAA + 2 mg/L BAP

→

Root induction media
MS + 0.5 mg/L IBA

↓

Plantlet acclimatization

Source : Maria Almeida et al, 2020

Thanks!

Any questions?

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