Online Training Course on Horticulture Seed Propagation with Tissue Culture for African Countries

AGENCY FOR AGRICULTURE EXTENSION
AND HUMAN RESOURCES DEVELOPMENT
AGRICULTURE MINISTRY

Introduction

TISSUE CULTURE MEDIA is one of the critical success factors for plant propagation using tissue culture techniques

Various compositions of culture media have been formulating to optimize the growth and development of cultured plants

Example

- Knudson C (1946)
- Heller (1953)
- Gamborg, B5 (1976)
- Murashige and Skoog, MS (1962)
- Woody Plant Medium, WPM (Loyd and McCown, 1980)

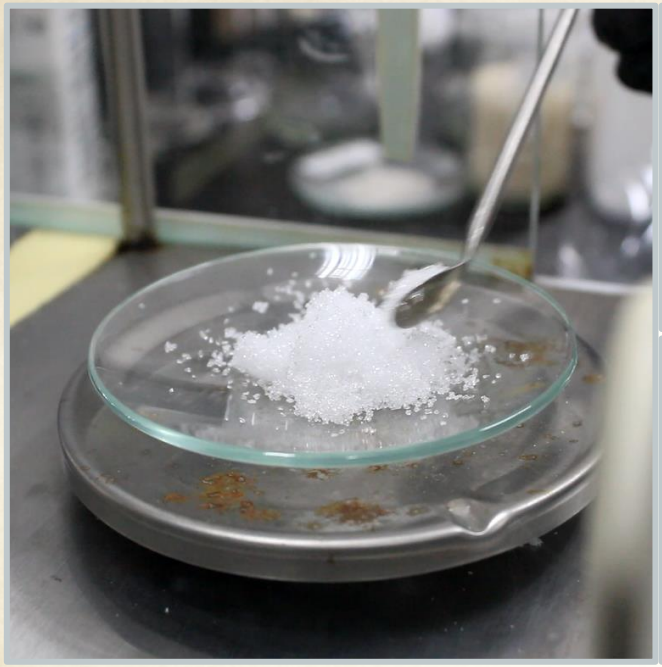
Tissue Culture Media …

Is the media needed so that isolated plant cells or tissues can grow and develop.

Has a nutrient composition that can support the growth of the explants as desired.

Nutritional requirements for optimal growth vary widely between types of plants, even between different plant section.

The Components of Tissue Culture Media



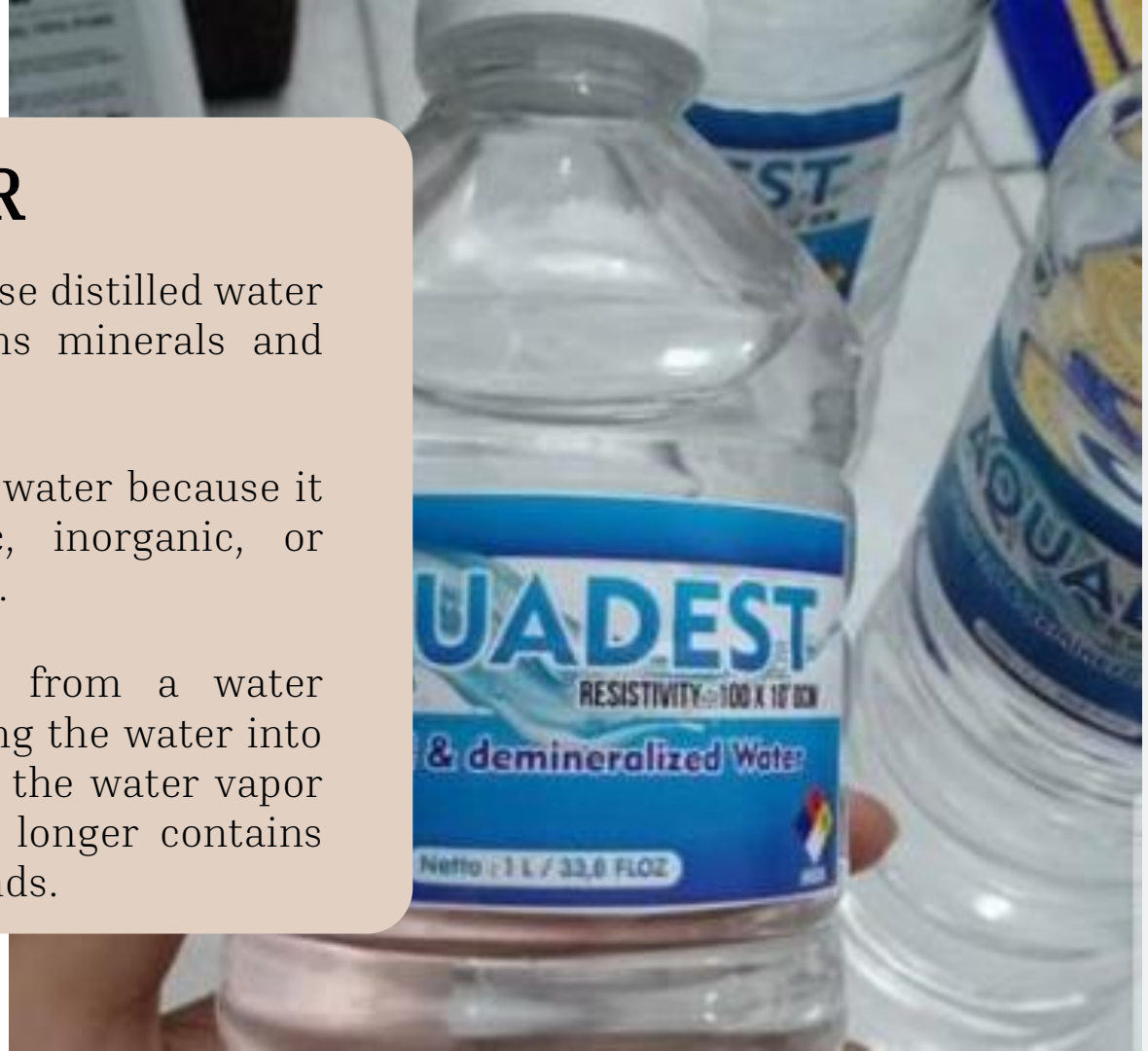
1. DISTILLED WATER
2. MACRO AND MICRO NUTRIENTS
3. VITAMINS
4. SUGAR
5. AGAR
6. NATURAL SUPPLEMENT INGREDIENTS
7. PLANT GROWTH REGULATOR

1. DISTILLED WATER

- Making stock solutions must use distilled water because it no longer contains minerals and organic compounds.

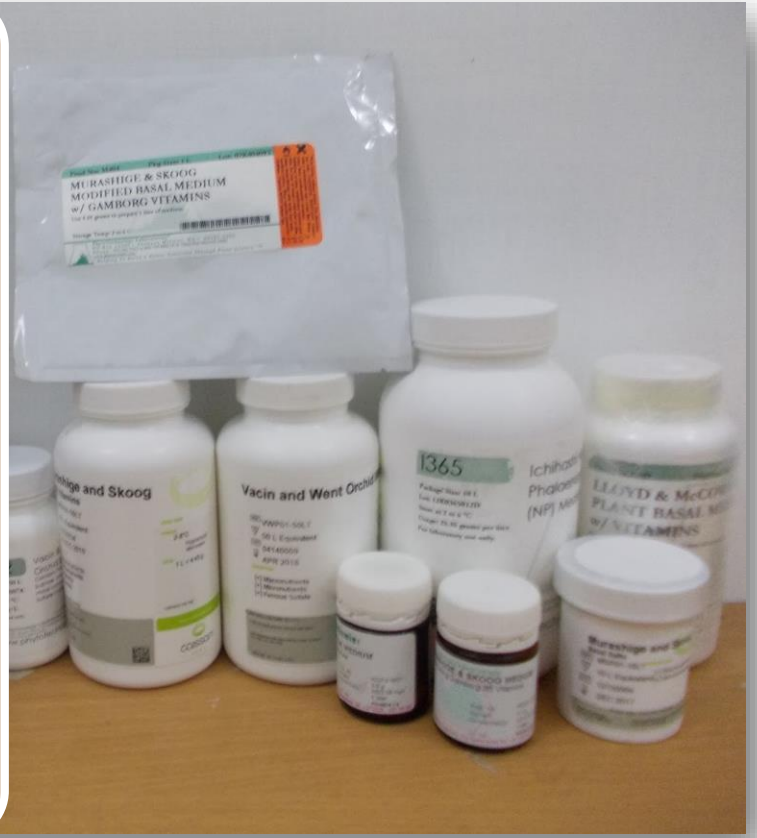
- Making media cannot use tap water because it contains too much organic, inorganic, or microorganism contamination.

- Distilled water is produced from a water distillation device by converting the water into water vapor, then condensing the water vapor into distilled water which no longer contains minerals and organic compounds.



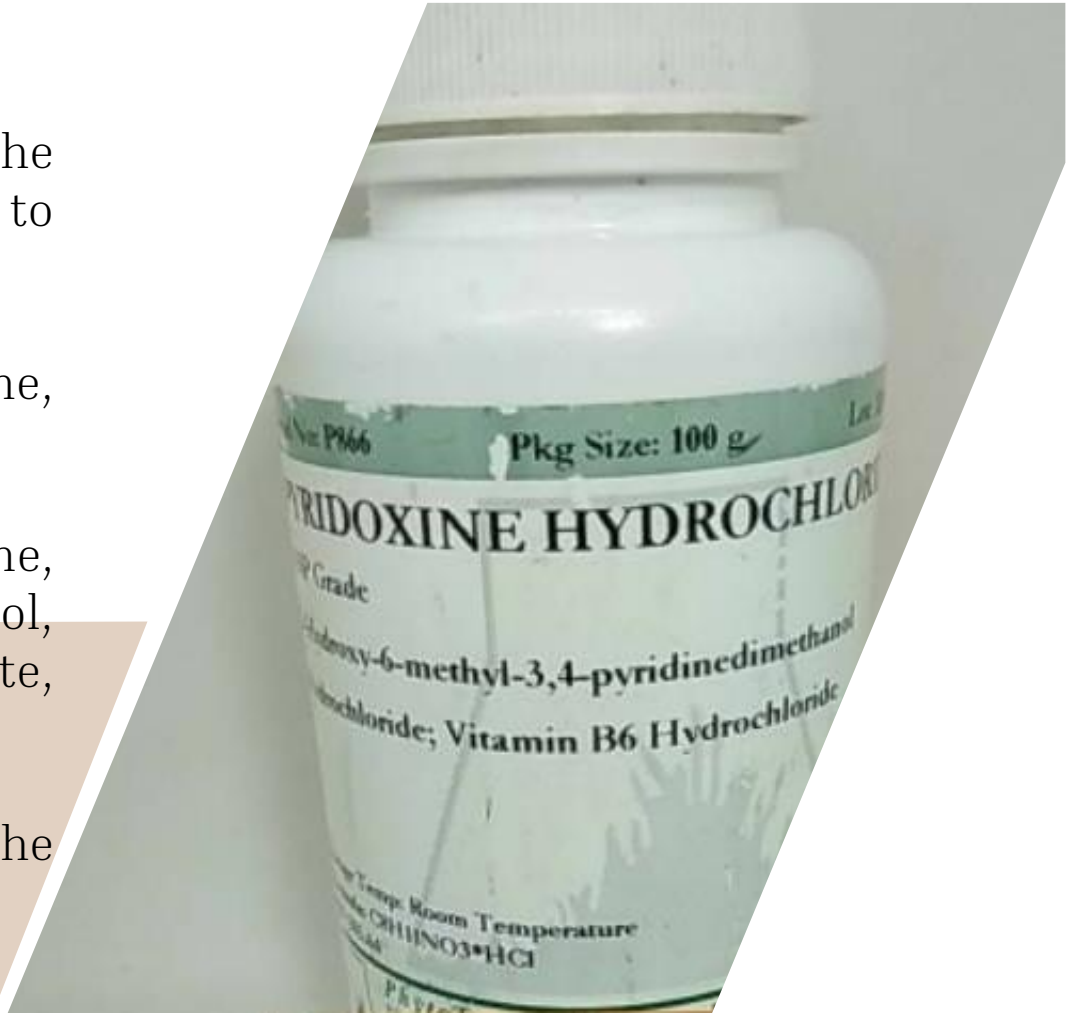
2. MACRO AND MICRO NUTRIENTS

- The inorganic salt requirement for plants cultured in vitro is the same as the inorganic salt requirement for plants are normally grown in their natural environment.
- Inorganic salt required in large quantities are known as macronutrients included : elements N, P, K, Ca, Mg, and S.
- Inorganic salts needed by plants in small amounts are known as micronutrients included : Fe, Cu, Mn, Zn, B, Mo, and Co elements.



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

- Sugar is used as an energy source in culture media because generally, plants cultured in vitro do not carry out perfect photosynthesis, so they require ready-made carbohydrates.

- The most commonly used sugar is sucrose. For this reason, we can use daily sugar because it contains 99.9% sucrose.

- Glucose and fructose can be used but not always better than sucrose.

- Concentration of sucrose used at a concentration of 2-3%.

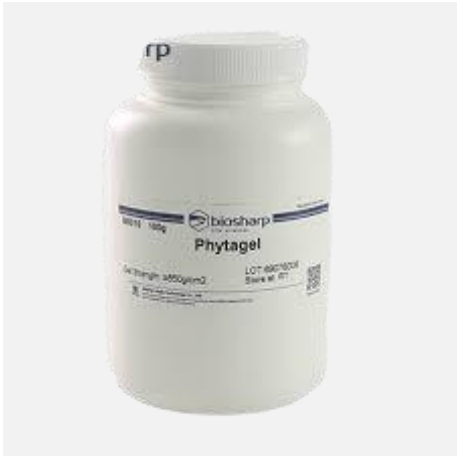
5. NATURAL SUPPLEMENT INGREDIENTS

❖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.

6. AGAR (MEDIA COMPACTOR)

- Make the media solution compact so that explants can be planted on the media and absorb the nutrients properly.



7. PLANT GROWTH REGULATOR

- 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 growth regulators in optimal combinations.

One way to make it easier to determine the type and concentration of growth regulators in certain cultures is :

1. Get to know more about the growth regulators used
2. See and study examples of its use and the results obtained

There are five groups of growth regulators used in tissue culture activities :

- Auxin
- Cytokinins
- Gibberellins
- Abscisic Acid
- Ethylene

Auxin

Auxin is a plant body that has a very complex effect and can control growth.

The physiological roles of auxins in plants such as:

- Enlargement of cells and organs,
- Root formation,
- Maintains apical dominance,
- Stimulate vascular tissue differentiation,
- Triggers ethylene synthesis

Types of auxins used in tissue culture techniques:

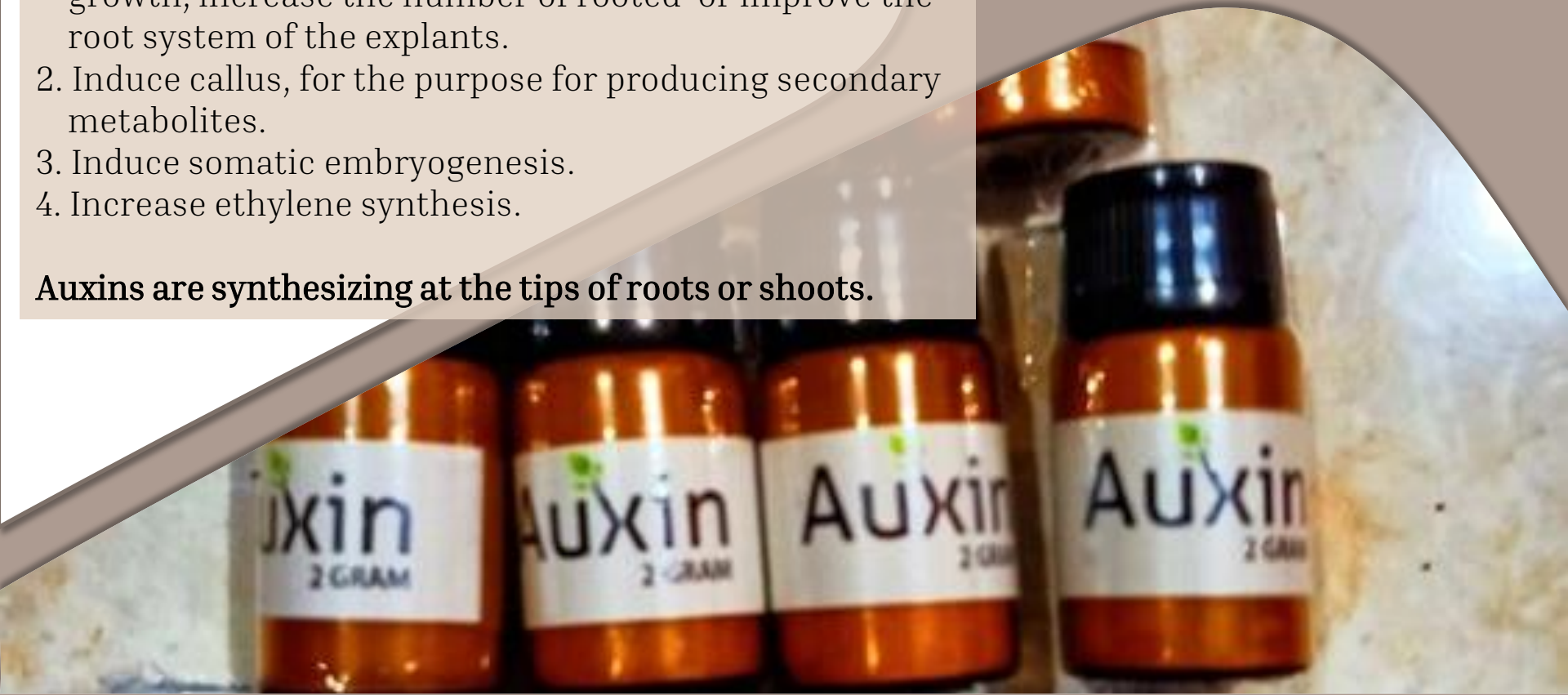
- Natural auxins, for example, **IAA (Indol Acetic Acid)**
- Synthetic auxins, for example, **IBA (Indolebutyric acid), NAA (Naphthalene acetic acid) and 2,4-D (2,4-dichloro phenoxy acetic acid)**

Auxin

The roles of auxins in tissue culture techniques are:

1. Inducing roots in these cases auxin can accelerate root growth, increase the number of rooted or improve the root system of the explants.
2. Induce callus, for the purpose for producing secondary metabolites.
3. Induce somatic embryogenesis.
4. Increase ethylene synthesis.

Auxins are synthesizing at the tips of roots or shoots.



Cytokinins

Cytokinins are the name of a group of growth regulators that are very important as **a stimulant for cell division and morphogenesis in tissue culture**.

More than 30 types of natural cytokinins are found, but two are widely used in tissue culture, such as:

- Zeatin
- 2-iP [N6-(2-isopentyl) adenine]

Synthetic cytokinins that are commonly used in tissue culture activities are:

- Kinetin
- BAP (6-benzylaminopurine)
- Thidiazuron

In tissue culture activities, cytokinins have been showing to stimulate the occurrence of :

- Cell division,
- Callus proliferation,
- Formation of shoots,
- Flowering,
- Chloroplast formation and,
- Inhibits root formation.

Gibberellin

In whole plants, gibberellins:

- Can affect the elongation of the stem or stem segment,
- Encourage flowering,
- Fruit induction, and
- Breaker of bud dormancy.

The growth regulator often used in tissue culture is Gibberellic acid (GA3).

ABSCISIC ACID

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

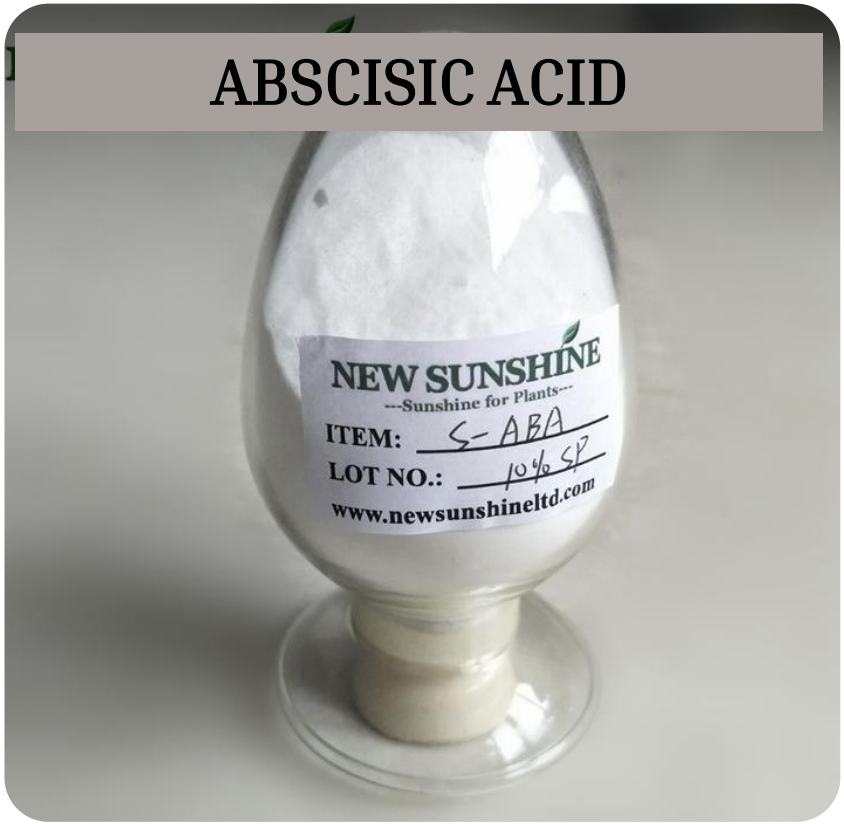
ABA is produced by plants when under stress

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

In plants, ABA affects stomata regulation, bud dormancy, seed dormancy

In the somatic embryogenesis method, it can encourage somatic embryo maturation and in seeds it can induce dormancy

ABA is also recognized to be able to increase tissue resistance in germplasm preservation



ETHYLENE

Ethylene is the only growth regulator that is gaseous and will form in any tissue that undergoes aging or stres

Ethylene is synthesized in all parts or organs of plants, especially tissues that are age, injured, or stressed plants

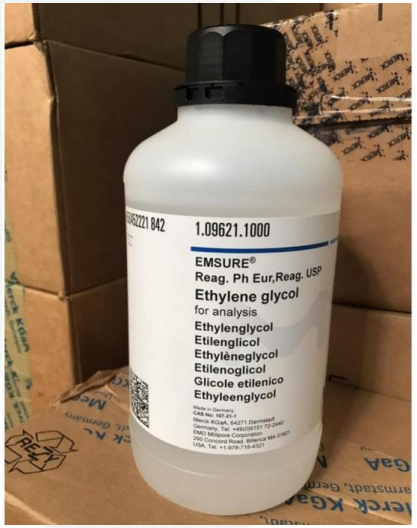
Cells, tissues, or organs in vitro culture always produce ethylene

Ethylene then accumulates in the culture vessel and can diffuse through the bottle cap. The amount will vary depending on the size of the container, the type of tissue being grown, the weight of the tissue, and the media

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



MS Stock Solution Procedures

MAKING OF MS STOCK SOLUTION (Murashige and Skoog, 1962)

The preparation of the media in principle by dissolving all components in water, according to their concentration in the desired formulate

However, weighing the components of the media one by one for each preparation of culture media is not practical and can only be done if the amount of substance is large enough to be weight

Making stock solutions can solve the problem

The stock solution is a solution containing one or more media components of media can be done by taking several stock solutions so that the concentration becomes, by that contained in the desired media formulation

examples of media composition

	Concentration in the media (mg/l)		
	MS	WPM	B5
Hara Makro			
NH_4NO_3	1.650	400	-
KNO_3	1.900	-	2.500
$(NH_4)SO_4$	-	-	134
$CaCl_2.2H_2O$	440	96	150
$MgSO_4.7H_2O$	370	370	250
KH_2PO_4	170	170	-
K_2SO_4	-	990	-
$NaH_2PO_4.2H_2O$	-	-	130,5
Hara Mikro			
$MnSO_4.4H_2O$	22,3	22,3	10
$ZnSO_4.7H_2O$	8,6	8,6	2
H_3BO_3	6,2	6,2	3
KI	0,83	-	0,75
$Na_2M_oO_4.2H_2O$	0,25	0,25	0,25
$CuSO_4.5H_2O$	0,025	0,25	0,025
$CoCl.6H_2O$	0,025	-	-
$Ca(NO_3)_2.4H_2O$	-	556	-
Iron			
Na_2EDTA	37,3	37,2	37,3
$FeSO_4.7H_2O$	27,8	27,8	27,8
Vitamin			
Glycine	2	2	-
Nicotine Acid	0,5	0,5	1
Pyrodoxin HCl	0,5	0,5	1
Thyamine HCl	0,1	1	10
Myo Inositol	100	100	100
Sukrosa/gula putih	30.000	30.000	30.000
Agar	7.000 – 8.000	7.000 – 8.000	7.000 – 8.000
pH	5,6 – 5,8	5,6 – 5,8	5,6 – 5,8

Source : Murashige and Skoog 1962; Lloyd and McCown 1981; Gamborg *et al*. 1968.

For the making of MS media, the component is group into several stock solutions:

A. Macrostock solution
B. Microstock solution
C. Stock solution of iron (Fe)
D. Vitamin stock solution
E. Growth Regulator Stock Solution

Preparation of stock solutions must be carried out carefully, especially in weighing the ingredients carefully and as carefully as possible, because it will affect the results of plant grow

Another thing to consider is the storage of stock solutions, as some materials are not resistant to high temperatures and light

Avoid any precipitated, because if the solution has precipitated, you should not use it

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

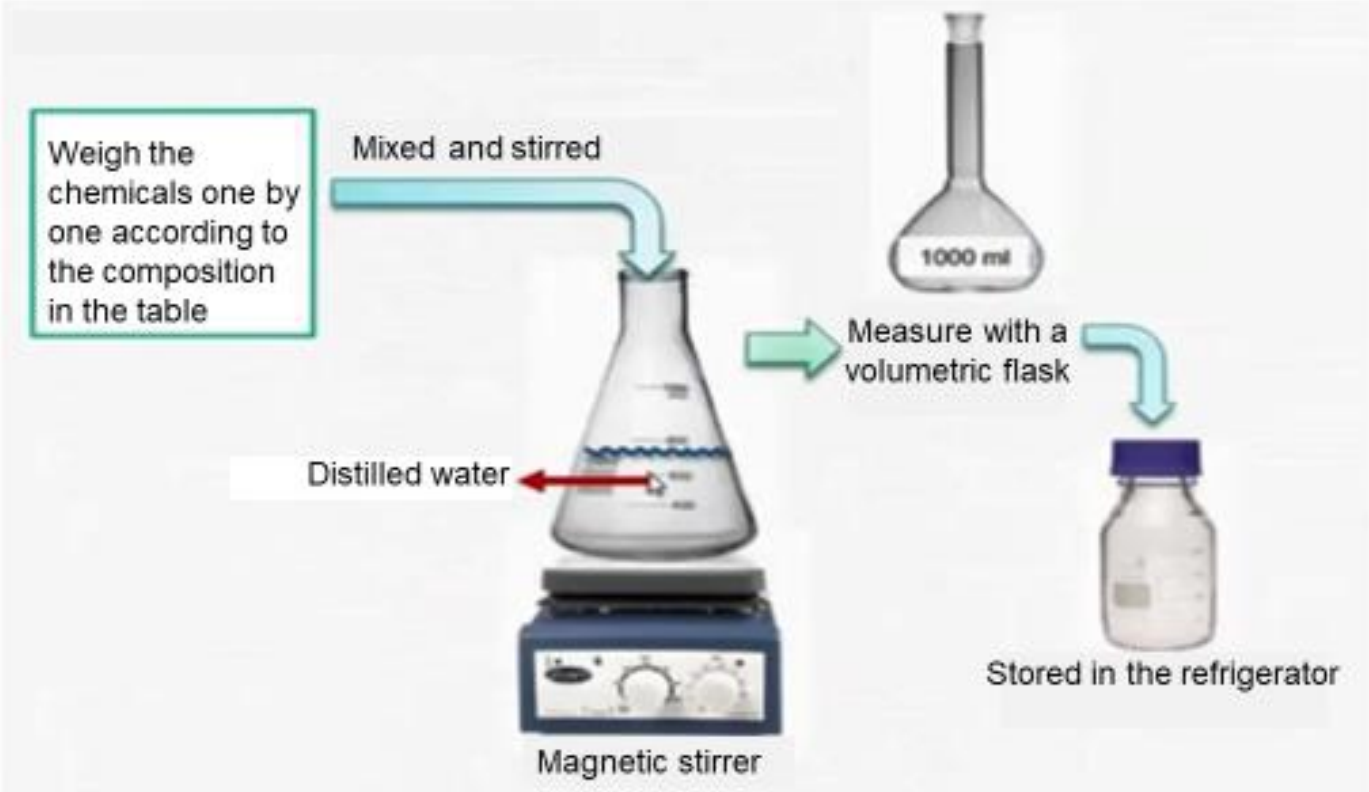
A. Macro MS stock solution (concentration for 10 liters of media)

No	Chemical Material	Quantity (gr)
1	NH_4NO_3	16.5
2	KNO_3	19.0
3	$CaCl_2$.H2O	4.4
4	$MgSO_4.7H_2O$	3.7
5	KH_2PO_4	1.7

How to make MS macro stock solution:

- Prepare 500 ml of distilled water while stirring, pour in the chemistries that have been weighed one by one sequentially from numbers 1, 2, 3, 4, and 5.
- It should be noted not to enter the chemical together because it will be difficult to dissolve.
- After stirring homogeneously, then add distilled water up to 1000 ml. Then store in a bottle.
- The use of this macro stock solution is 100 ml for one liter of MS media.

Stock Solution Procedures



B. Micro MS stock solution (concentration for 100 liters of media)

No	Chemical Material	Quantity (mg)
1	H_3BO_3	620
2	$MnSO_4.H2O$	1.690
3	$ZnSO_4.7H2O$	860
4	KI	83
5	$Na_2MoO4.7H_2O$	25
6	$CuSO_4.5H_2O$	2.5
7	$CoCl_2.6H_2O$	2.5

How to make MS Micro Stock Solution:

- The process is the same as above (how to make macros) and what needs to be considered is that the weighing method must be careful, thorough and careful, because weighing is in micro quantities.
- After stirring homogeneously, add distilled water up to 1000 ml.
- The use of this micro stock solution is 10 ml for one liter of MS media.

C. FeEDTA stock solution (concentration for 100 liters of media)

No	Chemical Material	Quantity (gr)
1	NaEDTA	3.73
2	$FeSO_4.7H_2O$	2.78

How to make a FeEDTA stock solution:

- Prepare 500 ml of distilled water, then heat it until it is lukewarm to accelerate the dissolution of Fe while being shaken with a magnetic stirrer.
- Then add the chemicals that have been weighed one by one in sequence and stirring until evenly distributed (homogeneous). Then add distilled water up to 1000 ml.
- Put it in bottle wrap with aluminum foil because this solution will react with light.
- The use of this FeEDTA stock solution is 10 ml for one liter of MS media.

D. Vitamin stock solution (concentration for 100 liters of media)

No	Chemical Material	Quantity (mg)
1	Glycine	200
2	Pyridoxine HCl	50
3	Nicotinic acid	50
4	Thyamine HCl	40

How to make Vitamin MS stock solution:

- Prepare 50 ml of distilled water while shaking, pour the vitamin ingredients that have been weighed one by one sequentially, then stir until evenly distributed (homogeneous) and add up to 100 ml of distilled water using a 100 ml volumetric flask.
- Pour in a bottle and store it in the refrigerator with a temperature of 4^0C, and the expiration date is about eight weeks.
- The use of vitamin stock solution is 1 ml for one liter of MS media.

Growth Regulator Solution Procedures

MAKING OF GROWTH REGULATOR SOLUTION

1. **Cytokinin stock solution BAP (Benzylamino Purine) (concentration 10^{-3})**

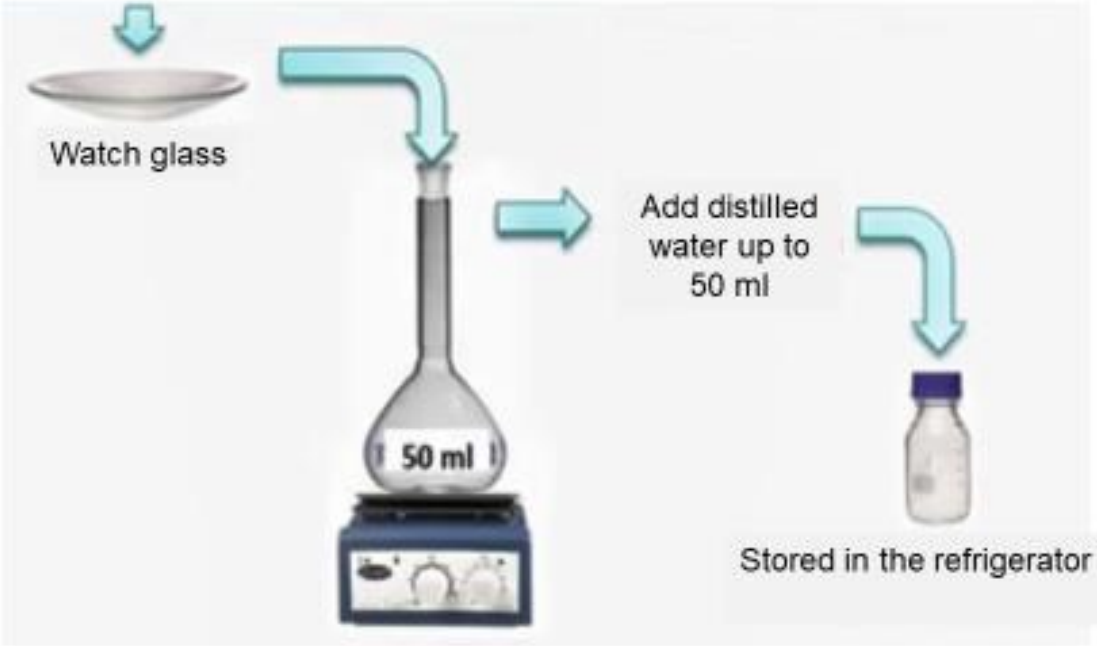
- Weigh the 50 mg BAP hormone, then add 20 drops of acid solution or 1N HCl solution until dissolved while shaking, then add aquadest up to 50 ml. Because cytokinins are alkaline, acid solutions are used as solvents.
- Pour into a bottle and store in the refrigerator with a temperature of 4^0C because hormones are labile materials.

2. Auxin stock solution NAA (Naphthalene Acetic Acid) (concentration 10^{-3})

- Weigh the 50 mg NAA hormone, then add 20 drops of 1N NaOH or KOH solution until dissolved while shaking, then add aquadest up to 50 ml. Because cytokinins are acidic, alkaline solutions are used as solvents.
- Pour into a bottle and store in the refrigerator with a temperature of 4^0C because hormones are labile materials.

CYTOKININ/AUXIN SOLUTION PROCEDURES

- Weigh Cytokinin 50 mg BAP/Auxin 50 mg NAA
- Drop with 1 N HCl/KOH 1 N in a watch glass gelas
- Stir until dissolved



Making Tissue Culture Media

Preparation of 1 liter of shoot induction media with basic media composition MS + BAP 5 mg/l + NAA 0.5 mg/l is as follows:

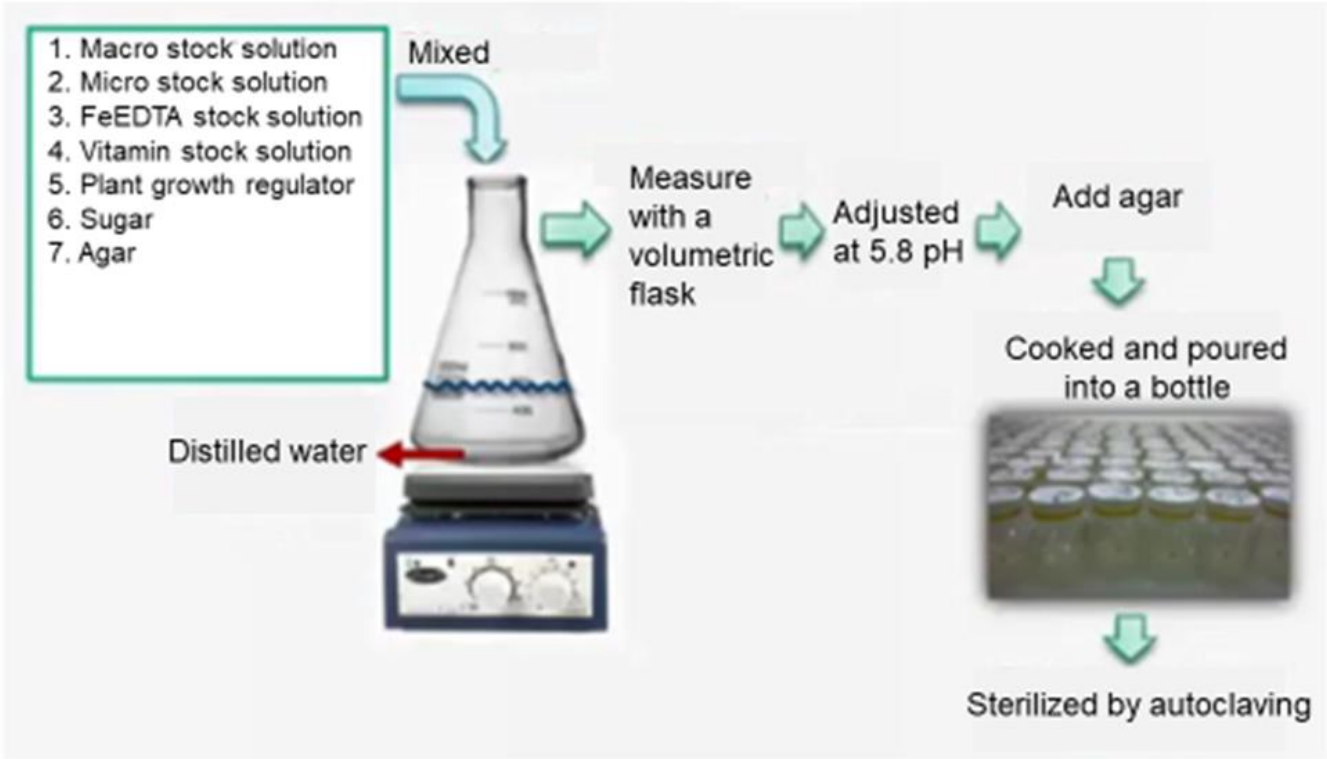
No	Media component	Quantity for 1 liter of media
1	Makro	100 ml
2	Mikro	10 ml
3	FeEDTA	10 ml
4	Myo inositol	100 mg
5	Vitamin	1 ml
6	BAP	5 ml
7	NAA	0.5 ml
8	Sugar	30 gr
9	pH measurement	5.7
10	Agar	7 gr

Tissue culture media procedures:

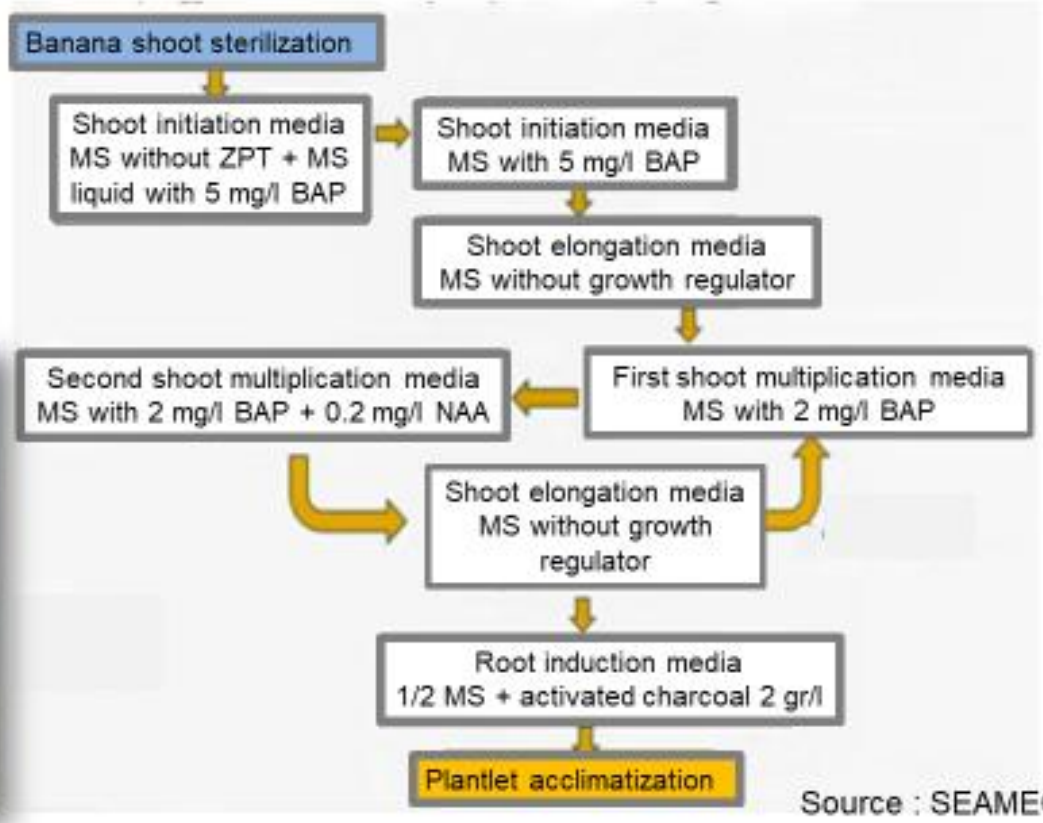
1. All media components (except agar), both in the form of stock solution and weighed material, were put one by one into a glass beaker.
2. The final volume is made to 1 liter by adding distilled water.
3. The solution was stirred using a magnetic stirrer until homogeneous.
4. pH was adjusted to 5.7 using a pH indicator paper. If the pH is too high drop HCl, then the pH is lower add KOH.
5. Add agar before the medium cooks.
6. After boiling the media was poured into a culture bottle, then the bottle was closed.
7. The media was sterilized by autoclaving for 15 minutes.

SCHEME FOR MAKING BUDS INDUCTION MEDIA

Solid MS Media + Growth Regulator (BAP + NAA)



Tissue Culture Media in Banana Seed Production



Source : SEAMEO BIOTROP, 2020

Tissue Culture Media in Potato Seed Production

Source : Munggarani et al, 2018

Potato meristem sterilization

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Meristem initiaton media
MS without plant growth regulator

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Multiplication media
MS + BAP

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Plantlet acclimatization

Thanks!

Any questions?

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