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College of Sciences
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Practical Labs of Plant Tissue Culture

Fourth Stage

The First Semester (2020-2021)

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Plant tissue culture syllabus

For fourth year students

2020-2021

Labs No.	subject
Lab.1	Introduction to plant tissue culture (P.T.C) science
Lab. 2	Tools and Techniques for P. T. C.
Lab. 3	Types of media and its components (1)
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Introduction

Plant tissue culture is communally used to describe the in vitro aseptic growth for any plant part on a nutrient media. This technology is depend on three fundamental objectives:

1. The part or explants must be isolated from the rest of the plant body.

2. The explant must be maintained in controlled physically (environmental) and chemically defined (nutrient medium) conditions.

3. Asepsis must be maintained.

There a number of basic facilities and minimum level of organization that should be available to the people working the lab. **And they are:**

1- Washing area:

This area contain large sinks, D.W and double D.W unit, oven for drying and sterilization and glass wares they should be heat resisting (Pyrex) and must be in different shapes and large quintets.

2- Media preparation , sterilization and storage area:

A media preparation room should have ample beach (working) and storage apace, glassware, culture vessel, cabinet, balance, pH meter, refrigerators, deepfreeze, autoclaves, and magnetic stirrer etc. all are required to store stock sol., measure pH, sterilize the media. D.W is essential and used in washing and media preparation should be well stored. Proper distillation will effectively remove large organic and molecules, M.O.s and other ionic and nonionic contaminates.

3- Transfer area:

A sterile transfer room or a laminar air flow bench should be available in all manipulations. The maintenance of asepsis is essential in all manipulation, a small room can be fitted with overhead ultra violet light and positive pressure ventilation unit with a bacteria proof high efficiency particulate air (HEPA) filter. This transfer chamber should be

provided with gas, electricity and compressed air out lets for used during transfer. The area should be cleaned without creating dusty atmosphere.

4- Culture Facility:

P.T.C., cell suspension and liquid or static cultures on solid media, should be incubated under environmentally controlled conditions of temperature, humidity, illumination, photoperiod and air circulation. All of these factors can effects the growth and differentiation response of tissues during culture.

Advantages of plant tissue culture (P. T. C.)

The practical and scientific applications for plant production using P. T. C. techniques are so many here are some of them:

1. Rapid propagation for new hybrid plants.
2. Production of disease free plants.
3. Continuous production of plants using P.T.C. techniques all through the year.
4. Production of plants in large amounts and also maintaining the original genetic features of the mother plant.
5. Propagation of some plant species that are hard to be propagate using conventional methods.
6. Production of haploid plants using anther and pollen cultures.
7. Initiating DNA changes(mutations) in the plant cell using protoplast cultures to produce new plants that were incompatible with each other.
8. Induction and selection of mutants using somatic culture to produce disease free plants.
9. Clonal propagation or rapid multiplication of specific genotype, this way is commercially important due to the use of small explants piece, the ability to prepare controlled environment for production all through the year and a massive production of plants in a large amount in short period of time.
10. Increase the production of secondary plant products which are produces in small amount naturally and pharmacological importance like alkaloids and coumarins.

Culture conditions:

The environment of the cultures consists of the nutrient sol. (medium) and physical culture conditions in which cultures grow. The culture media used communally in P. T.C. lab. are Murashage and Skoog 1962.

The physical environment (culture conditions) have direct bearing on the growth of the tissues following conditions are generally used:

1. Temp. of incubation is 22˚C-28˚C.
2. Illumination 0-5000 lux, photoperiod 8-16 hrs.
3. Relative humidity about 60% RH.
4. Subcultures of tissues: 2-8 weeks for static culture and 1-2 weeks for cell suspensions. Any modification in these (singly or in combination) may cause variation in growth and metabolism of the cultured tissues during different experiments.

Callus: A group of undifferentiated cells that could be differentiated according to the experiment aim.

Explant: Any plant part taking for culturing on a proper nutrient media.

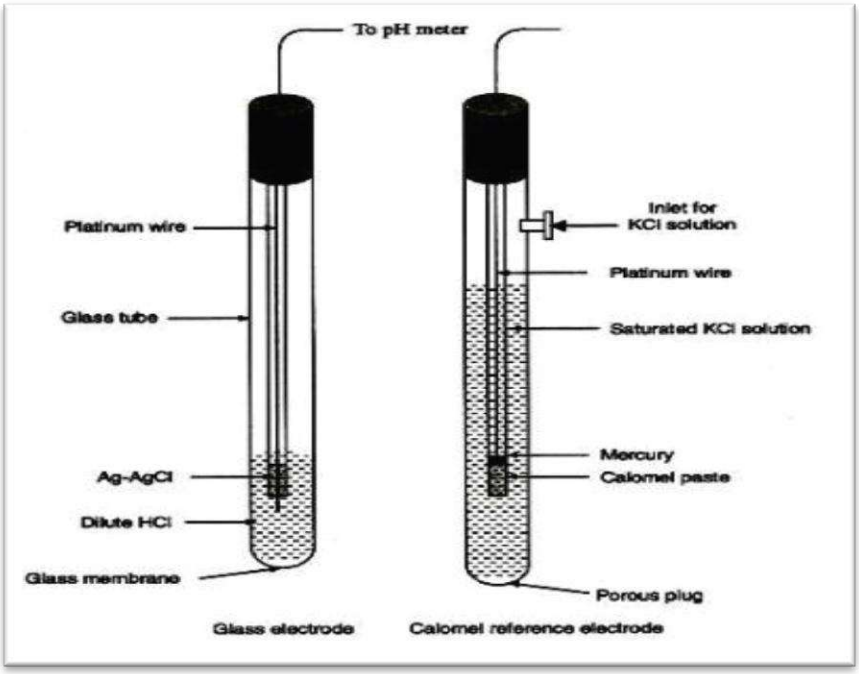
Totipotency :- The ability of one plant cell to produce to regenerate or form a while plant if provided with all the proper environmental condition.

Tools and Techniques for P. T. C.

1) pH meter:

It is used to measure the hydrogen ion concentration in a solution. The hydrogen ion is concentration of most sol. is extremely low; the pH of pure water is 7 at 25˚C. Generally distal water is used for the preparation of medium culture but sometimes sol. is used to keep the pH of the medium constant.

A stander pH meter has two electrodes, one glass electrode for measuring pH and other calomel reference electrode. One of the most important uses of the pH meter is to adjust pH of different solutions, buffers and culture medium.



2) Autoclave:

Autoclave is used to sterilize medium, glassware and tools for the purpose of P.T.C. The same equipment is used in hospital to sterilize gauge, cotton and tools. Sterilization of material is carried out by increasing moist heat (121˚C) due to increased pressure inside the vessel (15-22 psi, pound square inch or 1.02-1.5 Kg/cm^2) for 15 min. for routine sterilization. Moist heat kills the M.O.s and makes the materials free of microbes. Autoclaves for different sizes of 5L to several hundred liters capacity are available in

horizontal or vertical designs. Autoclaves may be constructed of aluminum, mild steel or stainless steel.

It is operated as follows, place the materials wrapped in AL. foil or paper or in metal box and glassware's containing medium (plugged in non-absorbent cotton and covered with AL. foil) in the bucket, check water level for appropriate level tighten all the screws and switch on the current. Allow the steam to pass freely from release valve for 5 min. and then close the valve after attaining pressure of 15 psi, count 15 min. for sterilization and then switch of the current. Pressure is maintained by safety valve. Modern autoclaves fitted with temperature and time control units and can automatically control the period for sterilization and then switch off themselves.

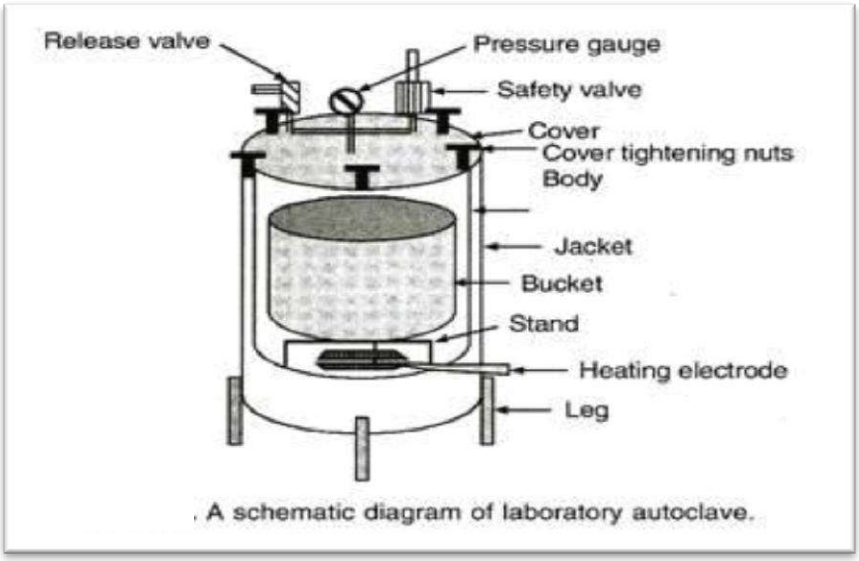
Precautions:

a) Check water levels each time, the heating elements should be immersed in water.

b) Check spring of safety valve frequently and clean opening whenever necessary.

c) Screws of the led should be tightened.

d) Use permanent markers to mark your media.



. A schematic diagram of laboratory autoclave.

3) Plant growth chambers (incubators):

Incubators are used for the incubation of the explants cultured on the growth media, essentially plant growth chambers has three environmental control systems:

a) Light intensity and duration cycle: (luxometer)

Light is fixed in the roof or in the shelves of the chambers, using fluorescent lights usually the intensity of them are 2000-2500 lux, the duration of light and dark cycle is adjusted as per requirement, usually 16h light and 8h darkness.

b) Temperature control and regulation: (thermometer)

Temperature is regulated by good quality platinum temperature sensor, usually of 22-28˚C is or used for growing plant tissue cultures.

c) Humidity control and regulation: (Hygrometer)

Usually 60% RH (Relative Humidity) is used to maintain healthy growth. Low humidity causes early drying of the medium while high humidity may causes fungal growth in the environment.

4) Laminar air flow bench:

Laminar air flow bench (LFC) is the main working table for aseptic manipulation related to P. T. C. this is an equipment fitted with high efficiency particulate air (HEPA) filters which allow air to pass but retain all the particles and microorganism. These HEPA filters have very small pore size (0.3µm) with efficiency to kill 99.97-99.99% M.O. air blow by blower is speed through these filters, thus always a positive air pressure is maintained from inside to outside. This positive pressure does not allow any particles to

inter the working area of LFC bench. Equipment is fitted with UV light and visible light source, UV is switched on for 30 min. before starting the work to make area free from microbes.

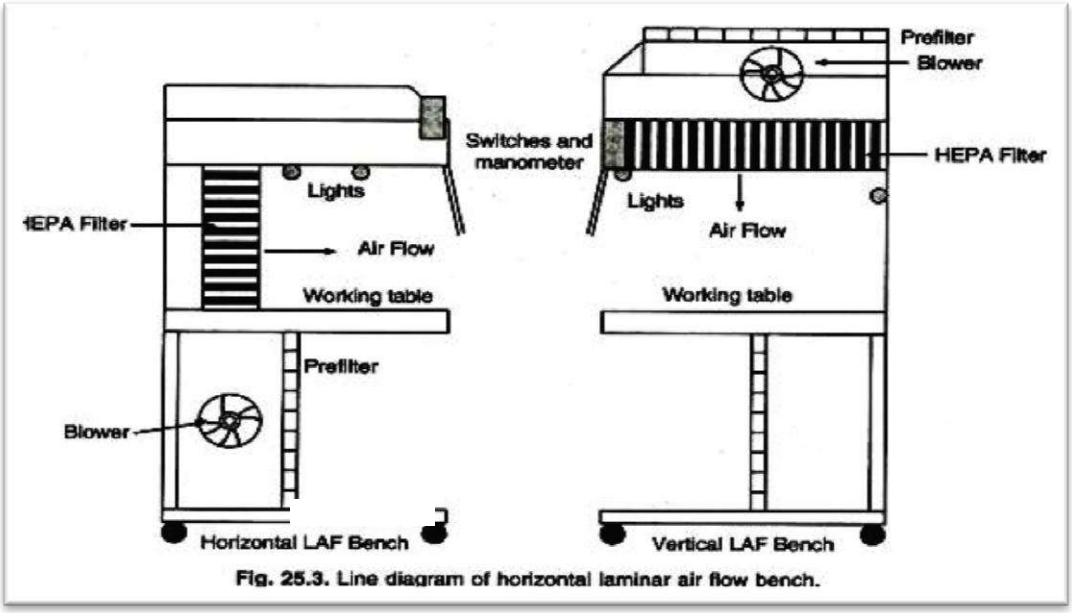


Fig. 25.3. Line diagram of horizontal laminar air flow bench.

5) Microscopy:

a) Electron microscope: permits a detailed study of the sub-cellular organelles as its resolving power in much greater than that of the microscope.

b) SEM microscope: scanning electron microscope (SEM) provides surface views of whole structure of specimen.

c) Light microscopy: bright field microscopy is absolutely indispensable tool for cell biologist. This is required for routine observation of cells, cellular differentiation and pigmentation.

6) Centrifugation:

It's an instrument that introduces centrifugal force by rotating the samples around a central axis with the help of an electrical motor. Centrifuge can be categorized in to the clinical type (5-10000 rpm), refrigerated high speed centrifuges (10000-20000 rpm) and ultra-centrifuges (20000-80000 rpm).

7) Chromatography:

Chromatography meaning (colored-writing) is a technique to separate molecules on the basis of differences in size, shape, mass, charge and adsorption properties. Example of chromatography is TLC (thin layer chromatography).

8) Sensitive balance:

It is used to weight relatively trace amount of chemicals mainly for media stocks preparation.

9) Oven:

It is used in dehydrating explants and callus pieces, usually at 70˚C. Oven comes in different sizes, shapes and temperatures according to their use in the lab.

10) Shakers:

They are used to agitate liquid media containing explants and also to mix chemicals with each other well.

11) Distillation Device:

It is one of the important equipment found in the P. T. C. lab. It provides distal water (D.W) for media, buffer and stocks preparations. Sterile D.W is achieved by autoclaving the D.W, this is very important as shown why in the following table:

	Sterile D.W	Tap Water Contains:
1	Uses to maintain asepsis working area.	Cations (Ca, Fe, Mg etc.)
2	To ensure obtaining the needed results of experiment.	Anions(carbonate, fluoride etc)
3	To ensure the growth of the explants without any contamination.	Particulate matter (oils, silica, silt etc.)
4	Prevent media contamination during its preparation and preservation.	M.O. (bacteria, viruses etc.)
5	----------	Gases (Co_2, CL etc.)

Types of media and its components

Introduction:

Optimal growth and morphogenesis of tissues may vary for their nutritional requirements, Moreover; tissues from different parts of plants may also have different requirements for satisfactory growth. Tissue culture media were first developed from nutrient solutions used for culturing whole plants e.g. root culture medium of White and callus culture medium of Gautheret.

Basic media that are frequently used include Murashige and Skoog (MS) medium, Linsmaier and Skoog (LS) medium, Gamborg (BS) medium and Nitsch and Nitsch (NN) medium. Plant tissue culture media should generally contain some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source(s) of carbon, growth regulators and solidifying agents.

NOTE:

The explants used in P.T.C. determine the type of the media needed for its growth depending on explants juice contents (organic and inorganic salt, amino acids and sugars).

Composition of the nutrient media:

1. Organic salts:

a. Carbohydrates

In plant cell culture media, besides the sucrose, frequently used as carbon source at a concentration of 2-5%, other carbohydrates are also used. These include lactose, galactose, maltose and starch and they were reported to be less

effective than either sucrose or glucose, the latter was similarly more effective than fructose considering that glucose is utilized by the cells in the beginning, followed by fructose. It was frequently demonstrated that autoclaved sucrose was better for growth than filter sterilized sucrose. Autoclaving seems to hydrolyze sucrose into more efficiently utilizable sugars such as fructose. Sucrose was reported to act as morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots. Generally separated roots of dicots grows better on media containing sucrose while monocots on glucose.

b. Vitamins

Some plant are able synthesize essential requirements of vitamins for their growth. Some vitamins are required for normal growth and development of plants; they are required by plants as catalysts in various metabolic processes.

The vitamins most used in the cell and tissue culture media include: thiamin (B1), nicotinic acid (B3) and pyridoxine (B6). Thiamin is necessarily required by all cells for growth. Thiamin is used at concentrations ranging from 0.1 to 10 mg/ l. Nicotinic acid is used at a concentration range 0.1-5 mg/l and pyridoxine is used at 0.1-10mg/l. Myo-inositol is added in small quantities to stimulate cell growth of most plant species, myo- inositol is believed to play a role in cell division because of its breakdown to ascorbic acid and pectin. It is generally used in plant cell and tissue culture media at concentrations of 50-5000 mg/l.

c. Amino acids

The required amino acids for optimal growth are usually synthesized by most plants however, the addition of certain amino acids or amino acid mixtures is particularly important for establishing cultures of cells and

protoplasts. Amino acids provide plant cells with a source of nitrogen that is easily assimilated tissues and cells faster than inorganic nitrogen sources. Amino acid mixtures such as casein hydrolysate, L- glutamine, L-asaragine and adenine are frequently used as source of organic nitrogen in culture media. Some media were supplemented with natural substances or extract, ground banana, organic juice and tomato juice.

Coconut milk contains:

1. Natural cytokinins (Zeatin) that stimulates the growth and differentiation of the explants.
2. Contains phenylalanine which is an amino acid that has a stimulating effect in the division of the plant cells (callus formation).
3. Contains myo-instol that stimulates cells growth and inters in the phospholipid layer composition.

2. Inorganic salts

a. Macronutrients(major element)

The essential elements in plant cell or tissue culture media include, besides C, H and O, macroelements: nitrogen (N), phosphorus (P), potassium (K) calcium (Ca), magnesium (Mg) and sulphur (S) for satisfactory growth and morphogenesis.

b. Micronutrients

The essential micronutrients (minor elements) for plant cell and tissue growth include iron (Fe), manganese (Mn), Zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo). Iron is usually the most critical of all micronutrients.

3. Plant growth regulators (PGR):

Plant growth regulators are important in plant tissue culture since they play vital roles in stem elongation, tropism, and apical dominance. They are generally classified into the following groups; Auxins, Cytokinins, Gibberellins and Abscisic Acid. Moreover, proportion of Auxins to Cytokinins determines the type and extent of organogenesis in plant cell cultures.

* Auxins:

The common auxins used in plant tissue culture media include: indole -3-acetic acid tissue (IAA), indole-3- butric acid (IBA),2,4-dichlorophenoxy-acetic acid (2,4-D) and naphthalene-acetic acid (NAA). IAA is the only natural auxin occurring in plant tissue cultures, auxins are usually used to stimulate callus production and cell growth, to initiate roots, to induce somatic embryogenesis, to stimulate growth from shoot apices and shoot stem culture. Generally IAA and 2,4-D are dissolved in a small volume of 95% ethyl alcohol. NAA, 2,4-D and IAA can be dissolved in a small amount of 1N NaOH.

** Cytokinins

Cytokinins commonly used in culture media include BAP (6-benzylaminopurine), 2iP (6-dimethylaminopurine), kinetic (N-2-furanylmethyl-1H-purine-6-amine), Zeatin (6- 4- hydroxy-3-methyl-trans-2-butenylaminopurine) and TDZ (thiazuron-N-phenyl-N- 1,2,3 thiadiazol-5ylurea).

Zeatin and 2iP are naturally occurring cytokinins and zeatin is more effective. In culture media, cytokinins proved to stimulate cell division, induce shoot formation and axillary shoot proliferation and to related root formation. The cytokinins are relatively stable compounds in culture media

and can be stored desiccated at -20˚C. Cytokinins are frequently reported to be difficult to dissolve and sometimes addition of few drops of 1N HCl facilitates their dissolution.

Tissues are divided into 4 types according to their PGR need:

1- Tissues need auxins only like Helianthus tuberosrs (درنات نبات الالمازة) and Cichorium roots (جذورنبات السيكوريا).

2- Tissues that need only cytokinins like white turnip (الفجل)

3- Tissues that responds only to natural extracts like coconut milk like orchid tissues.

Types of media and its components

4- Solidifying agents:

Hardness of the culture medium greatly influences the growth of cultured tissues. There are a number of gelling agents such as agar, agarose and gellan gum. Agar, a polysaccarid obtained from seaweeds, is of universal use as a gelling agent for preparing semi-solid and plant tissue culture media. Agar has several advantages over other gelling agents; mixed with water, it easily melts in a temperature range 60-100˚C and solidifies at approximately 45˚C and it forms a gel stable at all feasible incubation temperatures. Agar gels do not react with media constituents (biologically inert) and are not digested by plant enzymes. It is commonly used in media at concentrations ranging 0.8-1.0%. Pure agar preparation is of great importance especially in experiments dealing with tissue metabolism. These solidifying agents are affected by pH level if the pH is over than 6 the media will be so rigid that no nutrients can reach the explants, and if it is less than 5, the media will be very loss that the explants would be submerged leading to its death due to suffocation.

***Activated charcoal**

The addition of activated charcoal is sometimes added to culture media where it may have either a beneficial or deleterious effect. Growth and differentiations were stimulated in orchids, onions and carrots, tomatoes. On the other hand, an inhibition of cell growth was noticed in soybean. Explanation of the mode of action of activated charcoal was based on adsorption of inhibitory compound from the medium and darkening of the medium, and it's also prevents the oxidation of the phenolic compound in the callus preventing its browning.

*** pH regulation:**

pH measurement is very important in the MS media preparation it should be between (5.8-6) if its more than 6 the media will be solid that the explants can't absorb the nutrients from the media and if its less than 5.8 it will not solidify causing the drowning of the explants. pH level can be adjusted by acidic and alkaloids solutions like NaOH and HCl.

-The difference between the industrial and natural extracts used in P. T. C. media is that the natural extracts are safer, cheaper and give the same effect of the industrial extracts.

- According to the type of the explant and the aim of the experiment the MS media strength can be modified to 1/2 or even 1/4 power. This is accomplished by reducing the amount of the macronutrients and sometimes the sugar too.

*** Media preparation**

1- Weight the media.
2- Weight the sucrose.
3- Complete the volume to the required amount.
4- Add the hormones.
5- Adjust the pH to 5.8.
6- Weight and add the agar to the medium.
7- Give it a boil and dispense in universal jars.

***Calculations**

The standard amount of media is 5 g/L. The standard amount of sugar is 30 g/L, and the standard amount of agar is 7 g/L.

The addition of hormones depends on the law:

$$C_1V_1=C_2V_2$$

Examples:

Q/ Prepare 350 ml of semisolid MS media. Media 5g/1000 ml = X/35 ml

=1.75 g/350 ml

Sugar 30g/ 1000 ml= X/350 ml

= 10.5 g / 350 ml

Agar 7 g / 1000 ml= X/ 350 ml

=2.45 g /350 ml.

Procedure:

1- Weight 1.75 g of the media

2- Weight 10.5 g of the sugar and added to the media.

3- Complete the volume to 350 ml with D.W.

4- Adjust the pH to (5.8-6).

5- Weight 2.45 g of the agar and add it to the media.

6- Give it a boil with continuous stirring the dispense in universal jars.

Quizzes:

Q1/ Prepare 350 ml of half power MS media, give a complete procedure?

Q2/ You have500 ml of MS media (sugar +MS media) containing 1 mg/ L of TDZ (stock solution conc. 100 mg/ 100 ml) give a complete procedure.

Q3/ Prepare one litter of liquid MS media containing 0.5 mg/L of Zeatin (stock sol. conc. is 100 mg/ 100 ml) and 1 mg/L of TAA (stock sol. conc. is 200 mg/L) give a full procedure.

Q4/ Prepare (450 ml) of (1/2) p. MS media containing (0.7 mg/L) of KIN (stock sol. conc. is 10 mg /100 ml) and 4 mg/L of NAA (stock sol. conc. is 2 mg/10 ml) give a full procedure.

Sterilization Techniques

The media used for plant tissue culture contain sugar as a carbon source there by attracting a variety of microorganisms including bacteria and fungi. These organisms grow much faster than the cultured tissues and produce metabolic substances which are toxic to plant tissues.

There are a number of sources through which the cultures may get contaminated which include the culture vessels, instruments, media, explant, transfer area and growth room. Therefore, sterilization is essential to provide and maintain a completely aseptic environment during in vitro cultivation of plant cells or organs. Sterilization is a procedure used for elimination of microorganisms and maintaining aseptic or sterile conditions for successful culture of plant tissues or organs.

The different techniques used for sterilization are mentioned below:

1. Dry heat sterilization (Oven)

2. Wet heat sterilization (Autoclave)

3. Filter sterilization (using filter paper or milli-pore filters)

4. UV sterilization

5. Flame sterilization

6. Wiping with 70 % alcohol

Explants sterilization and culture:

Generally all plants have microbes on their surfaces, so to obtain aseptic plant parts are essential in the success of plant tissue culturing. Antibiotics were used in the nutrient media but they had bounded activity in addition they might affect the growth of the explant and if the explant was taken from virus infected tree it will automatically produce infected callus.

Many chemicals were used for explant sterilization; the disinfectant used for sterilization depends on:

1. The type of chemical used.
2. Period of sterilization.
3. The explant type.

Sever sterilization could hot just kill microbes but also could kill part of the explant (burn it giving it brown or colorless color), therefore it is important to carefully and specifically chose the disinfectant used for sterilizing the explants. The disinfectant or sterilizing agent which used should be easily and totally removed by sterile distilled water because any harm to the explant will effect on its growth and the formation of callus.

Enhancing the sterilizing agent performance can be achieved by adding few drops of tween 20 teepol and trigetol (wetting agents) in the concentration **0.05 %** this will remove the surface tension making these parts highly susceptible for the sterilizing agent that penetrate the outer surface of the explant and killing all the microbes. There are other methods used for surface sterilizing like dipping the explant in pure alcohol quickly before putting it in the sterilizing substance. In addition to the mentioned above, there are other disinfectants used like:

1- Bromine water (1%):

Used for sterilizing plant **roots** and **seeds** but it was discovered that this substance could damage the embryos therefore it was replaced by chlorine solution (1%) in concentration.

2- Chlorine solution

Also known as hypochlorine which is widely used in sterilization **due to** its availability, low toxicity and easily to be removed by D.W. The most used types of chlorine sol. are NaOCl and CaOCl.

3- Alcoholic mercuric chloride (1%)

It was used for explant sterilization but not any more **due to** its toxicity and difficulty of removal.

4- Ethanol and detergents

Using these two chemicals together or each a side considered a successful way of sterilizing the explant surface (with cut in surfaces or the uneven surfaces).

Procedure of seed culture:

1- Wash the seeds well with tap water.

2- Sterilize the seeds for a 15 min using a sterilizing solution (NaOCl) at a concentration of (3%).

3- Rinse the sterilized seeds at least 3 times using S.D.W. **(why)**?

4- Seeds are planted upside down **(why)**? On the hormone free media.

Procedure of leaves culture:

1- Select the explant (leaves).

2- Wash them with tap water (to remove dirt and soil).

3- Prepare the sterilizing agent NaOCl at conc. (3%) (origin conc. 6.2%, take an equal vol. of the detergent to the D.W. For example: 3ml of NaOCl and add to it 3 ml of D.W.) with few drops of cleaning detergent (as wetting agent), shake well and submerge the explant in it for 10 min.

4- Wash the explants with S.D.W. for three times at least (for the complete removal of the sterilizing agent)

5- Dissect the explant to equal pieces in size using sterile scalp and forceps.

6- Injure these pieces slightly **(why)?** and then put the upper surface of the leaf facing the media surface (for callus formation but if we culture the leaf on its lower surface it will give us hiary roots).

* Important Notes:

* The sterilizing agent type, concentration and time of sterilization differs according to the explant used, for example: seeds requires longer and higher concentration of the disinfectant than leaves do (depending on the type of explant tissues and surrounding layers).

* Explant size should be approximately **5mm** in diameter bigger than that will increase the risk of contamination and smaller ones will increase the ratio of wounded to intact cells. A high surface volume is required to facilitate gas exchange and nutrient uptake**.**

* Explant may suffer from oxidation and will turn brown due to wounding it, this situation may be remedial by increasing hormones levels and adding coconut milk to the media to enhance callus growth or by

supplementing the media with antioxidants like 50-100mg of ascorbate (Vitamin C).

* The alcohol used for LFC (Laminar flow cabinet) sterilization is 70-75% in conc. not 95-99% in conc. Because the last conc. will rapidly evaporate so it will not kill all microbes especially will not kill any fungi spores (because they will form cysts protecting themselves from the disinfectant). In the 70-75% the spores will attempt to adjust themselves with this disinfectant but they cannot because the alcohol will kill them.

Shoot-Tip And Meristem Culture

The Most of the horticultural and forest crops are infected by systemic disease caused by viruses, bacteria, mycoplasma and nematodes. While plant infected with bacteria and may respond to treatments with bactericidal and fungicidal compounds, there is no commercially available treatment to cure virus-infected plants. It is possible to produce disease-free plants through tissue culture. Apical meristems in the infected plants are generally either free or carry very low concentration of the viruses. The various reasons attributed to the escape of the meristems by virus invasion are:

a) Viruses move readily in a plant body through the vascular system which in meristems is absent.

b) A high metabolite activity in the actively dividing meristematic cells does not allow virus replication.

c) A high endogenous auxin level in the shoot apices may inhibit virus multiplication.

Meristem tip cultures have also enabled plants to be free from other pathogens including: Viroids, Mycoplasmas, Bacteria and Fungi. Therefore, main objective of shoot-tip and meristem-tip cultures is the production of disease-free plants through micro propagation.

Shoot-Tip Culture:

It may be described as the culture of terminal (0.1-1.0 mm) portion of a shoot comprising the meristem (0.05-0.1) together with primordial and developing leaves and adjacent stem tissue.

Meristem Culture:

Meristem culture is the in vitro culture of a generally shiny special dome like structure measuring less than 0.1 mm in length and only one or two pairs of youngest leaf primordial (buds), most excised from the shoot apex.

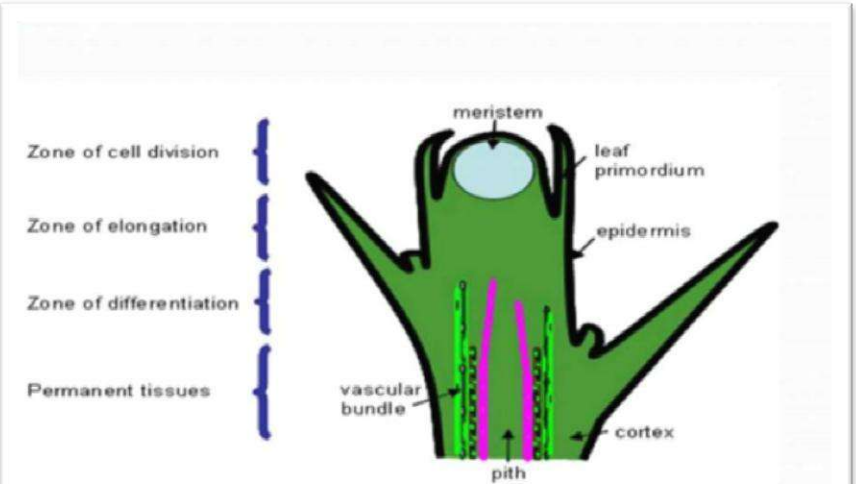


Fig. 1 : Apical Meristem

Note:

Plant tissues with high phenolic content are difficult to culture. Due to injury polyphenol to a dark brown colored growth inhibiting substance. This has been observed in *Eucalyptus grandis*. *So*, **<u>This difficulty can be removed by:</u>**

a) Adding antioxidants to the medium.
b) Pre-soaking the explants in antioxidants.
c) Initial culture is grown in absence of light.

Protocol (Procedure):

1- Remove the young twigs (Branches) from the healthy plant. Cut the tip portion of the twig.

2- Surfaces sterilize the shoot apices by incubation in a sodium hypochlorite solution (1% available chlorine) for 10 minutes. The explants are thoroughly rinsed 4 times in sterile distilled water.

3- Transfer each explant to a sterile petri dish.

4- Remove the outer leaves from each shoot apices with pair of forceps. This lessons (reduce or decrease) the possibility of cutting into the softer underlying tissues.

5- After the removal of all the outer leaves, the apex is exposed. Cut of the ultimate apex with the help of scalpel (cutter or lancet) and transfer only those less than 1 mm in length to the surface of the agar medium or to the surface of Filter Paper Bridge. Flame the neck of the culture tube before and after the transfer of excised tips. Binocular dissecting microscope can be used for cutting the true meristem or shoot tip perfectly.

6- Incubate the culture under 16 hrs light at 25 0C.

7- As soon as the growing single leafy shoot or multiple shoots obtained from single shoot tip or meristem, transfer them to hormone free medium to develop roots.

8- The plants form by this way are later transferred to pots containing compost and kept under greenhouse condition for hardening (rigidity).

Note:

Addition of a malachite green or 2-4-D or thiouracil helps the growth of shoot tips. For chlorophyll formation 1000 lux fluorescent lamp is used during initial stages and 3000-10.000 lux lamp is used in later stages. The photoperiod is 16 hours.

Principle:

The excised shoot tip and meristem can be cultured aseptically on agar solidified simple nutrient medium or on paper bridges dipping into liquid

medium and under appropriate conditions will grow out directly into a small leafy shoot or multiple shoots. Alternatively, the meristem may form a small callus as its cut base on which a large number of shoot primordial will develop. These shoot primordial grow out into multiple shoots. Once the shoot has been grown directly from the excised shoot tip or meristem, they can be propagated further by nodal cuttings. This process involves separating the shoot into small segment each containing one node. The axillary bud on each segment will grow out in culture to form a yet another shoot. The excised stem tips of orchids in culture proliferate to form callus from which some organized juvenile structures known as protocorm develop. When the protocorm are separated and cultured on fresh medium, they develop into normal plants. The stem tips of *Cuscuta reflexa* in culture can be induced to flower when they are maintained in the dark.

Protocorm:

A tuber-shaped body with rhizoids that is produced by the young seedlings of various orchids and some other plants having associated mycorrhizal fungi.

Exogenously supplied cytokines in the nutrient medium plays a major role of the development of a leaf shoot or multiple shoots from the meristem or shoot tip. Generally high cytokines and low auxin are used in combination for the culture of shoot tip or meristem. Addition of adenine sulfate in the nutrient

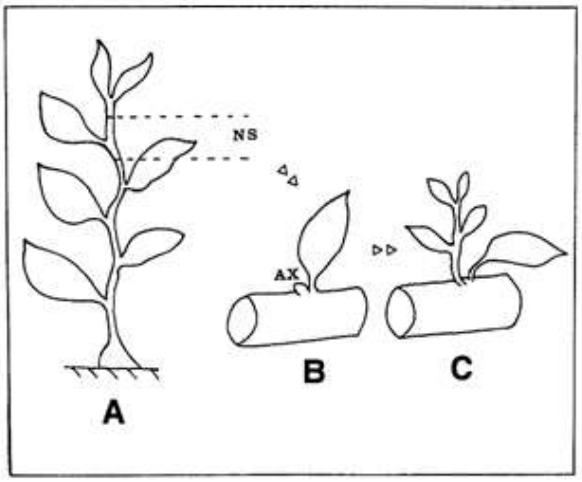


Fig 2 Propagation from meristem-tip derived plantlets by the technique of nodal culture
A: A plantlet showing extension growth in vitro. The nodal segment to be excised is indicated (NS). B: The excised nodal segment as it is transferred onto fresh culture medium, showing the axillary bud (AX) that will be responsible for subsequent growth. (C) The pattern of development of a successful nodal segment culture, showing extension growth of the new plantlet

medium also induces shoot tip multiplication in some areas.

BAP (Benzyll-aminopurine Synthetic purine cytokines) is the most effective cytokine commonly used in shoot tip or meristem culture. Similarly, NAA (Naphthalene acetic acid- Synthetic auxin) is the most effective auxin used in shoot tip culture. Coconut milk and gibberlic acid (GA) are also equally effective of the growth of shoot apices in some cases.

Embryo Culture and Initiation

The culture of embryos for different plants was successful while they were in the zygotic phase. Culture of embryo like the orchids embryos which is approximately 100µm should be before embryo differentiation protocrom which is undifferentiated tissue like callus have one or more cell wall with few buds and have no other embryonic feature. Many plants have the ability to form protocrom and the smallest one is 60 µm of sugar cane plant.

Nutrient compound necessary for embryonic growth:

The coconut milk was used in culturing somatic and zygotic embryos (due to its highly nutrient contents), different media were used for culturing these embryos in vitro like MS, B5 and others. The addition of ammonium malate helps the embryo grow better in the nutrient media while adding sodium malate inhibit its growth.

Note:

That embryos grow better on a media containing high concentrations of nitrogen and carbohydrates as energy source, they also grow better on a half power MS media because the full power can be considered as a stress effecting the embryos growth.

The effect of hormone on embryo culture:

Plant hormones are important in the growth of culture embryos were the increase of auxin concentration in the media results in activating somatic embryos, while low concentration of it helps embryos to differentiate. The addition of (Gebrillic acid hormone) GA hormone helps in the early growth of embryos but if it was added in the concentration of 0.01 mg/L to the culture media it will cause embryos differentiation without going through

the germination phase.

The addition of cytokinins is not effective on the growth of embryos and their differentiation, but when kinetin is combined with IAA hormone adventitious embryos.

Why do we use embryos in P.T.C?

We use them because they are undifferentiated tissue like callus, easily handled and manipulated to give any required results according to the experiment aim.

Procedure for embryo culture and initiation:

1- Wash the selected seeds with tap water to remove dirt and soil residues.

2- Put these seeds in D.W. for 24hr (This will enlarged the seeds size for easy removal of the embryo).

3- Sterilize it with sterilizing agent (Sodium hypochlorite NaOCl) in the conc. 3% and add to it some drops of wetting agent like tween 20. Sterilization process take about 15-30 min. (**Why?**).

4- Wash the seeds with S.D.W. for three times at least. (**Why?**).

5- Dry the seeds with sterile filter paper and remove the embryo from them.

6- Culture the embryo in half power MS media in an upside down (**Why?**), then incubate them at 25℃ in total darkness(**Why?**).

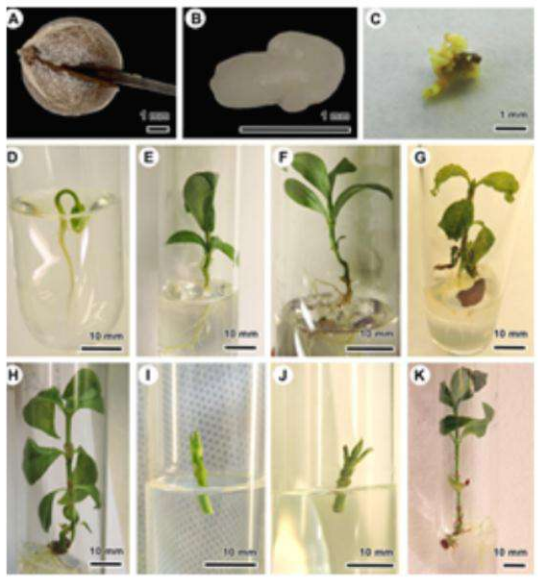


Fig. Illustration of the technique embryo culture: **(A)** Expose the embryo, **(B)** The isolated embryo, **(C)** Adventive embryo, **(D)** Two-week-old seedling obtained via embryo culture. **(E)** Three-month-old, **(F)** Shoot obtained via embryo culture. **(G)** Three-month-old, **(H)** Shoot obtained via embryo culture. **(I-K)** Illustration of the technique of shoot culture. **(I)** Example of a sterilized cutting transferred to in vitro culture medium, **(J)** Two week-old obtained via shoot culture. **(K)** Eight-month-old with nodules obtained via shoot culture.

SOME CLEVER QUESTIONS:

1- Why do we sterilize the seeds if the embryos inside them are aseptic?

To remove any microbes that can be found on its surface leading to embryos contamination and this will in turn gives contamination callus.

2- What type of embryo did we use in our experiment?

Zygotic embryo

3- How many types of embryo is there?

1- Somatic Embryo → Any part of the plant.

2- Zygotic embryo → Sexual fertilization.

4- Can we culture embryos on hormone free culture? Why?

Yes, because some embryo can produce hormones.

Note:

Some embryos are cultured in the darkness, because light could be considered as a stress agent that could affect the embryos growth and kill them or may stimulate organogenesis.

Subculture and Reculture in Plant Tissue Culture

Callus translocation processes from exhausted media to a fresh one according to close period of time, this is done due to:

1- Callus is a stable mass that absorbs only the nutrients found under it in the soled media leading to its exhaustion and because this mass don't have any roots or adventitious roots it can reach the rest of the nutrients found in the media, this require sub-culturing it or re-culturing it.
2- Dryness of the media due to long culturing periods.
3- Accumulation of the explant or callus metabolites that is conceded is toxic to the cultured tissue.
4- Contamination of the culture media with different microorganisms.

Sub-culturing process is important to preserve the culture tissues health and survival. So, when performing it we should take notice to the following:

1- Culture explant on solid media under 25℃ should be sub-cultured in (4-6) weeks according to the size and type of the explant (why)? Because the larger explant and depending on its type the faster it needs subculturing due to its rapid nutrients consumption from the media.
2- When sub-culturing the callus we should choose the healthy parts of it to insure that it will survive and give healthy plant, if not, the week parts will give week plant.
3- Sometimes may notice a browning area that because oxidation of phenolic compounds, so we have to banish this parts.
4- Any delay in this process will lead to callus lose and damage.

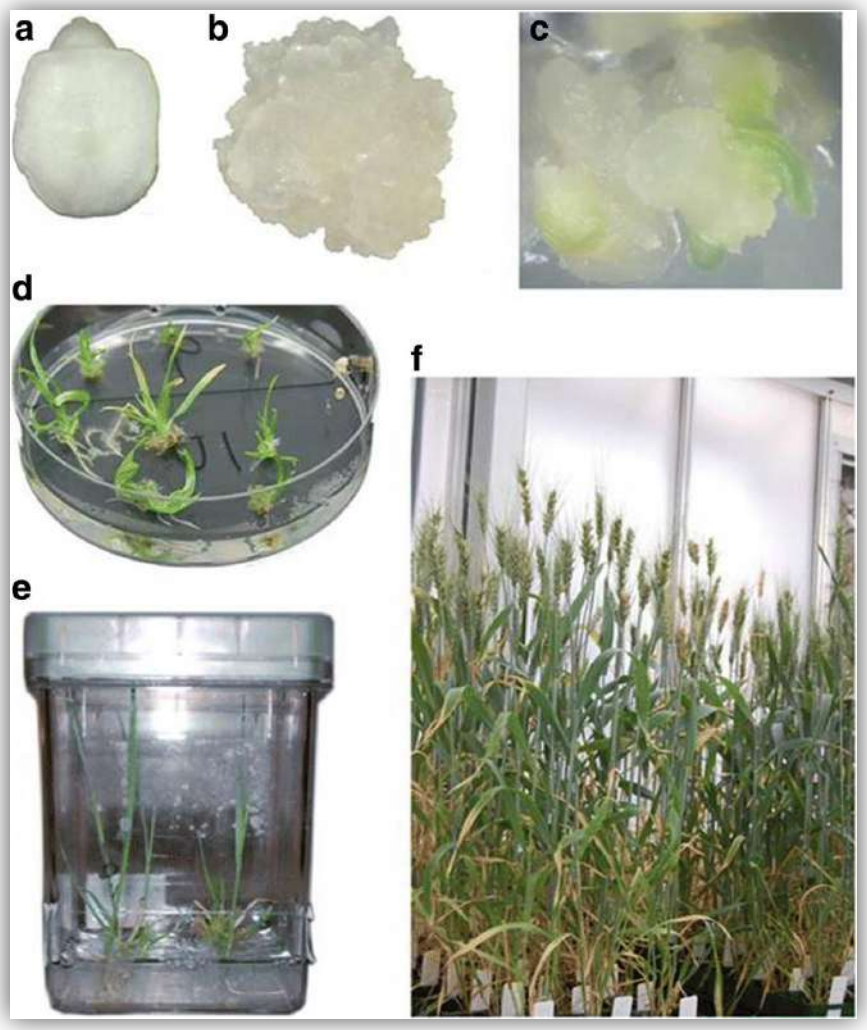


Fig. (1): Transformation process with hygromycin selection. **a** Freshly isolated immature embryo; (**b**) 2 week-old embryogenic callus; (**c**) Shoot regeneration on 50 mg/L hygromycin selection medium; (**d**) Shoot and root regeneration on 50 mg/L hygromycin selection medium; (**e**) Plantlet recovery in 1/2 MS medium; (**f**) T_0 transgenic plants in the greenhouse**.**

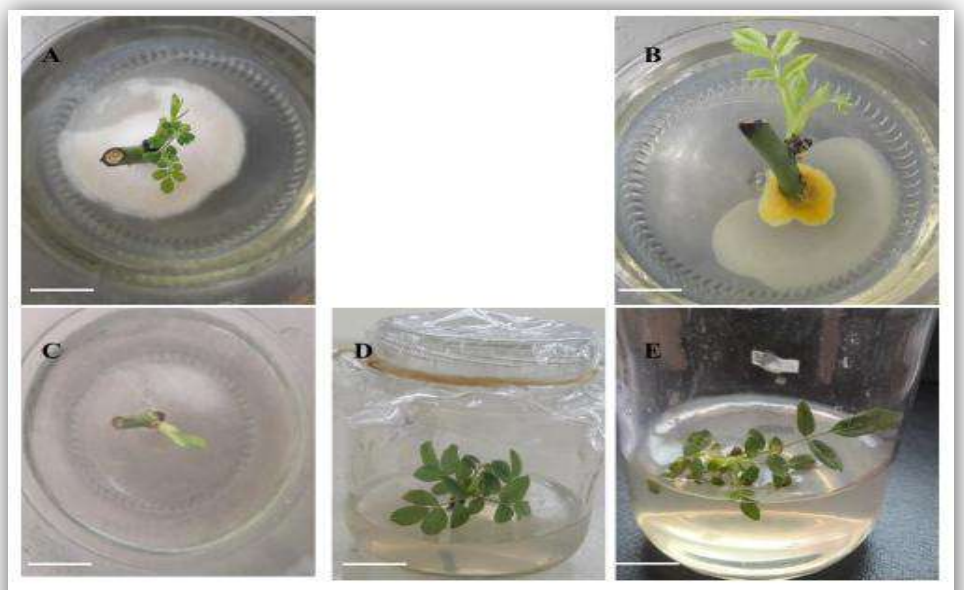


Figure 2 Nodal explants on MS medium supplemented with various concentrations of BAP. Explant contaminated with fungus (**A**) and bacteria (**B**) after 10 days of culture. Shoot response of nodal explants on MS medium supplemented with 2.0 mg·L^{-1} after 6 days (**C**) and four weeks of culture (**D**). Shoots induced on MS medium without BAP (**E**) after four weeks of culture. Bars represent 10 mm.

Transgenic plants

This method caused by (*Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* bacteria), the first one contain tumer inducing plasmid and the other are contains root inducing plasmid.

Ti plasmid: Cause tumor cells like callus in formation.

Ri plasmid: Causes semi roots formation.

T-DNA: It contains the **8** genes responsible for opines formation which is not produced naturally by plant but the bacteria forces the plant to produce it. The **T-DNA** is responsible the irregular cell division inside the plant.

(The bacteria transform the plant cells according to their need)

Agrobacterium bacteria**:** It's a rod bacteria (1-3 micron) in length and (0.4-0.8 micron) diameter. Usually free and single, they grow on media containing a gas in white small colonies but they are sun light sensitive (why? Because their natural habitat is deep in the soil where it is dark) and to dryness so, a certain amount of humidity is required in the soil which help the bacterial growth and causes root infection.

There are many methods for inserting these genes to plant cells:

- **Protoplast culture:** This method depends on the removal of plant cell wall which is considered an opstical agensit the entrance of plasmid DNA, to remove this opstical the explant treating with certain enzymes used for cell wall breakage then expose the extracted protoplast to the bacteria. Then the **T-DNA** inters the cell membrane submerge with the cell DNA, after that take the protoplast and culture it in certain conditions for reconstruction of the plant cell wall by the end of this step, we'll have a **genetically transformed plant cell** containing the **T-DNA.**

**** Disadvantages of protoplast culture:**

One of the problems of this method is the difficulty of gaining some plants protoplast. In addition cell wall build up (recovery), protoplast synthesis and whole plant production is facing many problems especially in crop plants.

- **Disk culture:** This method is a **replacement** of the first method due to its capability of overcoming the previous method difficulties and can be summarized in dissecting plant leave to small pieces after sterilizing them. Infect these pieces with *Agrobacterium* Bacteria and remove them to culture media that help the growth of the transformed cells into whole hybrid cells is more sensitive for agrobacterium infection. This method proved high efficiency in many explants.

Procedure

1- Inoculating the explants with the bacteria. After dissecting it, the explants then culture on nutrient media appropriate for bacteria growth for 7 days. After that transport the explants on media containing antibiotic (for bacteria removal) and incubate it in shaker incubator (antibiotic dispense to all parts of the explants and remove the bacteria) for 48h.

2- Take the genetically transformed explants and culture it on hormone free MS media after 4-6 weeks tumer will appear on explants.

Note:

* Liquid MS media is better to use in this method to ensure better growth of genetically transformed explants because it differs genetically from the original explants due to the bacteria infection.

Nanoparticles and plant tissue culture

This Nanoparticles are materials ranging in size from 1 nm to 100 nm (1nm=10^{-9} m) in at least one dimension. Due to their singular antimicrobial, electronic, optical, and structural strength enhancement properties, today NPs are used in practically all fields for example in the fields of chemistry, biology, energy saving, molecule engineering etc. Employing nanotechnology in plant tissue culture have resulted in positive outcome. NPs have been employed to :

1. boost crops production and plant growth
2. improvement seeds germination
3. enable plant genetic modification
4. attain plant protection and increase the production of bioactive compounds
5. enhance Rooting, Shoot Growth, Organogenesis and
6. Callus Induction
7. enhancement of secondary metabolites (were NPs are considered as a stress agent)
8. As antimicrobial agent (either by direct addition to the culture medium or by explant sterilization with it)

Most metals can be modified to NPs like Nano-oxides (Ag, Au, Zn, Si, Fe etc) used in P.T.C. were they are prepared as PGRs (heat tolerance).

Pomegranate is conventionally propagated by hard wood and soft wood cuttings. Yet, this traditional method of reproduction does not guarantee disease- free and healthy plants and it relies upon the season. In addition, this way is a very time-consuming and also needs a large workforce and a large numbers of cuttings do not persist during plantation, Plant regeneration of pomegranate is also an important step in the success of the generation of transgenic lines and it determines the efficiency of a transformation protocol.

Hence, developing an efficient *in vitro* technique for the propagation of pomegranate has a great importance. During the past decade, tissue culture techniques have been widely applied.

Culture procedure:

1. Wash the pomegranate leaves with tap water (why?)

2. Sterilize them with 3%NaOCL containing tween-20 for 5min. then wash them 3 times (no more or less?) with S.D.W.

3. Culture them up-side down (why?) on MS media containing 0.5mg/L of 2,4-D for 4 weeks.

4. subculture 50-100mg of the produced callus on MS media containing

a. 2, 4-D only as control media (why?)

b. 2,4-D and 10mg/L of MgO

c. 2,4-D and 10mg/L of CuO

5. harvest the callus cells after 21-30 days after culture (why this period of time not after or before it?) to measure the secondary plant products (secondary metabolites) using HPLC

Results

The cultured leaves produced callus which were submitted to the stress but non-toxic levels of the NPs resulting mainly in the increase of S.M. knowing that Mg molecules from MgO are microelements needed by the plant tissue and Cu from CuO is a macroelement found in the MS media composition (why then they were considered as stress?) lead to elevation in S.M levels manly tannins which are of medical importance recorded after HPLC test as follows:

S.M.(tannins)	Control (mg/L)	Conc. Of MgO NPs (10mg/L)
Gallic acid	27.38	115.25
Tannic acid	35.15	333.53
Ellagic acid	11.72	153.60

S.M.(tannins)	Control (mg/L)	Conc. Of CuO NPs (10mg/L)
Gallic acid	27.38	29.38
Tannic acid	35.15	92.05
Ellagic acid	11.72	41.45

As seen above there was an increase in S.M levels for the different Tannic acids at the mentioned NPs concentrations that serves the importance of these medical compounds produced in this period of time. We can notice that the effect of MgO is better than CuO on the levels of S.M at the same concentration because of the high consumption of Mg when compared to CuO.