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Plant Tissue Culture Regeneration and Aseptic Techniques

E. E. Ikenganyia[1*], M. A. N. Anikwe[1], T. E. Omeje[2] and J. O. Adinde[3]

[1]Department of Agronomy and Ecological Management, Enugu State University of Science and Technology, Agbani, Enugu State, Nigeria.
[2]Department of Agricultural Technology, Enugu State Polytechnic, Iwollo, Enugu State, Nigeria.
[3]Department of Horticultural Technology, Enugu State Polytechnic, Iwollo, Enugu State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The science or art of growing plant cells, tissues or organs isolated from the mother plant on artificial media is known as plant tissue culture. The merits of plant tissue culture in crop production is that it helps in disease elimination, quick international distribution of genetic resources, germplasm conservation and reduction in quarantine requirements, time and space of regeneration. This paper looks at the materials for *in vitro* plant tissue culture, different methods of plant tissue culture media preparations, protocols of hygiene and sterilization in plant tissue culture and techniques in plant tissue culture for crop improvement.

Keywords: Tissue culture; in vitro; media; explants; cell.

**Corresponding author: E-mail: eejike43@yahoo.com, emmanuel.ejike@esut.edu.ng;*

1. INTRODUCTION

Plant tissue culture is the science or art of growing plant cells, tissues or organs isolated from the mother plant on artificial media [1]. It is the growth of isolated plant cell or pieces of tissue under controlled environmental conditions in a sterile growth medium [2,3,4]. Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency) [5]. Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones [6,7,8]. The exact conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. Each variety of a species will often have a particular set of cultural requirements. Despite all the knowledge that has been obtained about plant tissue culture during the twentieth century, these conditions have to be identified for each variety through experimentation. Disease elimination, quick international distribution of genetic resources, germplasm conservation and reduction in quarantine requirements, time and space of regenerations are the merits of plant tissue culture practices [9,10,11,12].

This paper looks at the materials for *in vitro* plant tissue culture, different methods of plant tissue culture media preparations, protocols of hygiene and sterilization in plant tissue culture and techniques in plant tissue culture for crop improvement.

2. MATERIALS FOR *In vitro* PLANT TISSUE CULTURE

1. **Plants:** Any part obtained from any plant species can be employed to induce callus tissue. Younger and fresh explants are preferable as explants materials [13].
2. **Media:**
   - **Inorganic salts:** These are carriers of nutrients such as nitrate, potash and ammonium. A common inorganic salts medium is the Murashige and Skoog media [14] and B5 medium.
   - **Carbon sources:** Sucrose, glucose, fructose, maltose and other sugars are suitable carbon sources which are added to the basal medium to support the growth of various plant cells and serve as energy supplier for physiological activities in cell [15].
   - **Vitamins:** Vitamins are essentials for cell growth and help to stimulate the growth of cells. Examples are myo-inositol, nicotinic acid, pyridoxine and thiamine (vitamin B-complex).
   - **Phytohormones:** Phytohormone or growth regulators are required to induce callus tissues and to promote the growth of many cell lines Examples are auxin such as 2,4-Dichlorophenoxyacetic acid (usually called 2,4-D) and Naphthaleneacetic acid (NAA), kinetin or benzyladenine, cytokinin and gibberellic acid (also called gibberellin A3, GA, and GA_3) [13,16].
   - **Organic supplements:** Examples are amino acid, peptone, yeast extracts, malt extracts and coconut milk. The coconut milk is also known as a supplier of growth regulators [13].

3. METHODS OF PLANT TISSUE CULTURE MEDIA PREPARATIONS

3.1 Inorganic Salt Solution

Table 1 shows the various inorganic salt compounds and their respective concentrations (mg/100 ml) in the Murashige and Skoog micronutrient stock solution.

Table 1. The concentrations of some of inorganic salt compounds (store in refrigerator)

Ingredient	Concentration (mg/100 ml)
H_3BO_3	620
$MnSo_4 4H_2O$	2230
$ZnSo_4 7H_2O$	860
$Na_2MnO_4 2H_2O$	25
$CuSo_4 5H_2O$	2.5
$CoCl_2 6H_2O$	2.5

Source: [13]

3.2 Vitamins

Vitamins use in tissue culture is shown in Table 2 below.

Table 2. The concentrations of some vitamins (store in refrigerator)

Vitamins	Concentration (mg/100 ml)
Nicotinic acid	100
Thiamine	1000
Pyridoxine	100
Myo-inositol	10000

Source: [13]

3.3 Preparation of Stock Solution

Calcium chloride ($CaCl_2$ $2H_20$) - 15 g/100 ml (store in refrigerator).

Potassium iodide (KI) - 75 mg/100 ml (store in refrigerator).

Dissolve 50 mg of 2, 4-D in 2 to 5 ml ethanol, heat slightly and gradually dilute with 100 ml of distilled water (store in refrigerator).

Prepare NAA (2.8 mMl) as the same as 2, 4-D above.

Dissolve 21.5 mg of kinetin (1 mM) in a small volume of 0.5 N HCl, heat slightly and gradually dilute with 100 ml of distilled water (store in refrigerator).

3.4 Similar Procedure can be done for Cytokinins

A certain volume of each stock solution is mixed and an appropriate carbon source is added to the mixture. The pH is adjusted to around 5.5 with 0.2 N KOH or 0.2 NHCl. Distilled water is added to the mixture up to a certain volume required. Agar (0.6 to 1.0% wt/vol) is added for a solid medium [13].

The medium prepared is distributed into vessels such as Erlenmeyer flask, also known as a conical flask or titration flask (for example, 50 ml of the medium in a 300 ml volume Erlenmeyer flask and sterilized by using an autoclave at 120^{o}C for 15 minutes. The sterilization conditions should be varied based on the volume of the medium and the size of the vessel [13]. Explants are sterilized with 2% sodium hypochlorite solution and /or 70% ethanol solution. The period of time for submerging the plant materials in these solutions depends upon plant species, their parts and age. The explants should be rinsed with sterile distilled water. The stem or any other part of plants sterilized is cut to approximately 1cm in length using a sterilized scalpel and each piece is transferred with tweezers to a solid medium in a flask or a petri-dish. The plant material is incubated aseptically at around 25°C on the solid medium for several weeks or more and a callus is produced. The callus is sub cultured by transferring a small piece to fresh solid medium. After several subsequent transfers, the callus becomes soft and fragile [13].

3.5 Protocols of Hygiene and Sterilization in Plant Tissue Culture

Hygiene is a pertinent precaution necessary in the preparation of tissue culture media. The protocols of hygiene and sterilization in tissue culture encompasses all the wholesome practices that is used in the identification of possible sources of contamination and determining measures of prevention during preparation of a tissue culture media and culturing plants for regeneration.

3.6 Sources of Contamination

- **Environment:** The environment is an amalgam of many climatic factors such as air temperature, radiation, moisture etc. In tissue culture practice, the kind of environment that is needed is an aseptic one, (sterile that is free from micro-organisms). The flow of air current carries along with it spores of micro-organisms which is a potential source of contamination. The use of laminar air flow cabinets is a technique in preventing contamination due to air constituents [1].
- **Culture media:** Media used to grow plant tissue can support the growth of micro-organisms such as fungi and bacteria, which can cause the death of the plant material in culture solution. All contaminated culture should be autoclaved for 40 mins before disposing to prevent subsequent contamination of other instrument and plants [1].
- **Plant materials:** Plant surfaces carry a significant amount of micro-organisms which can be removed by surface sterilization after the initial explants are being removed from the mother plant. Plant materials from the field should be confined to a preparation room, whereby unwanted plant parts are disposed of immediately, then followed by a gentle wash of the plant parts required for inoculation.
- **Culture media containers and instruments:** Equipments such as sensitive weighing balance, autoclave, pH meter, camera, and microscope are used in the practice of plant tissue culture. Instruments in contact with contaminated substances should also be autoclaved for 40 minutes. It is essential to wash scapel and glass beakers with hot water after

being used with boiled agar. This is because agar solidifies at 41°C. Glass wares should be washed thoroughly with powerful and effective scouring pads.

- **Management practices:** Management practices such as irrigation, handling of explants and formulation of culture media introduces contaminants into the culture medium. For instance, plants grown in containers within a glasshouse are more heavily contaminated on the surface where they have been watered from overhead. Overhead watering results in the splash of microbes from compost or soil onto the plants.

3.7 Sterilization Procedure

After the identification of all possible source of contamination, the next step is to use appropriate sterilization procedure to clean the instruments such as glassware, flask, bottles or jars used or to be used in tissue culture preparation. The procedures are classified as follows:

1. **Chemical method:** This is the use of chemical reagents to sterilize and check the growth of bacteria and other micro-organisms. Table 3 shows some sterilization chemical which can be use in tissue culture preparation.
2. **Dry heat method:** For dry heat sterilization, laboratory drying ovens may be used. Approximately one hour heating period is allowed for the entire load in the oven to reach sterilization temperature. Recommended holding periods at different sterilization temperatures are 45 mins/ 160°C, 18 mins/ 170°C, 7.5 mins/180°C and 1.5 mins/ 190°C. Dry heat method is used for glassware, metal instruments, or the other materials that are not burnt by high temperatures. Objects containing cotton, paper or plastics should not be sterilized by dry heat [1].
3. **Wet heat method:** Wet heat method employs an autoclave operated with steam under pressure. This method is used mainly for media, liquids and sometimes glassware as well. A time setting of 15 mins at 121°C under 1.6kgcm^2 pressure is commonly used; this condition is perfectly adequate to kill bacteria and fungal spores. It is important to note that leaving items too long in the autoclave can result in unacceptable chemical changes in the media and therefore poor plant growth [1].
4. **Ultra-filtration method:** This method is used mainly for some media components that are unstable at high temperatures and must be sterilized by ultra-filtration at room temperature (e.g. hormones, enzymes, etc.) usually a small volume is sterilized by passage through a membrane filtration unit attached to a graduated syringe [1].

3.8 Techniques in Plant Tissue Culture for Crop Improvement

- **Meristem culture:** Meristem is the region of active cell division in plant. This region occurs at the tip of shoots, root and leaves of plants. In this practice, very small shoot apices, each consisting of the apical meristematic dome with or without leaf primordium is inoculated. For most dicotyledonous plants, the size depends on plant species and is usually less than 1 cm [17]. This technique is use in the disease elimination and quick distribution of genetic materials [9].
- **Shoot tip or shoot culture:** Shoot tip culture consist of shoot tips or buds having several leaf primodia and are larger than shoot meristems which are inoculated on culture medium. This is appliedin horticultural plants like *Musa* germplasm. The constituents of the medium are designed to induce lateral buds and the shoot growth.

Table 3. Sterilization chemicals use in tissue culture preparation

Sterilizing agent	Concentration	Sterilization time (min)	Effectiveness
Sodium hypochlorite	10%	5-30	Very good
Calcium hypochlorite	9-10%	5-30	Very good
Antibiotics	4-50 mg/l	30-60	Fairly good
Bromine water	1-2%	2-10	Very good
Silver nitrate	1%	5-30	Good
Mercuric chloride	0.1-1%	2-10	Satisfactory
Hydrogen peroxide	10-12%	5-15	Good

Source: [1]

- **Nodal culture:** In nodal culture, lateral buds each carried on a small piece of stem tissue or stem pieces carrying either single or multiple nodes may be cultured. Each bud is cultivated to provide a single shoot. This technique is normally employed in yam crop improvement [18].
- **Protoplast culture:** Protoplasts are plant cells without a cell wall. The plant cell wall can be removed mechanically or by the action of an enzyme known as pectinase which degrade the cell wall. When the plant cell is degraded the plants becomes exposed or cellulase to the external environments. Protoplast cell wall is becoming a useful step in several crop breeding programmes as it facilitates mutant selection, somatic hybridization and gene transfer [19,20]. Example of some crops in which protoplast regeneration have be achieved are *Actinidia arguta, Glycine max, Zea mays, Oryza sativa and Triticum aestivum* [21].
- **Somatic embryogenesis:** This is the development of embryo from somatic cells and tissues that are not the product of gamete fusions. In fact they are developed directly from somatic cells without gametegenesis [22,23]. Somatic embryos closely resemble their zygotic counter parts from structural and biochemical point of view [24]. Examples of plants that have being improved through this method are *Asparagus officinalis, Carica papaya, Elaeis guinnensis, Glycine max, Pisum sativum, Solanum melongena* [21].

4. CONCLUSION

Disease elimination, quick international distribution of genetic resources, germplasm conservation and reduction in quarantine requirements, time and space of regenerations are the positive gains of plant tissue culture practices. Hygiene is a pertinent precaution necessary in the preparation of a tissue culture media. Crop improvement can be done through any of the plant tissue culture technique such as meristem culture, shoot tip or shoot culture, nodal culture, protoplast culture and somatic embryogenesis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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