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CHAPTER 3

Sterilization Techniques in Plant Tissue Culture

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INTRODUCTION

Plant tissue culture techniques have been used routinely in the study of totipotency and the roles of hormones in cytodifferentiation and organogenesis. Tissue-cultured plants that have been genetically engineered provide insight into plant molecular biology and gene regulation which are key to plant and agricultural biotechnology. Clonal selection of elite plants and production of secondary metabolites using cultured suspended cells. Tissue culture methods are often used in the formation of somatic haploid embryos from which homozygous plants can be generated.

Tissue culture involves the use of small pieces of plant tissue called explant which are cultured in a nutrient medium under sterile conditions. Using the appropriate growing conditions for each explant type, plants can be induced to rapidly produce callus (a group of undifferentiated cells), somatic embryos (embryos developing from vegetative cells), suspension cultures, new shoots, and, with the addition of suitable hormones new roots. The new plants are then hardened in the control conditions, placed in soil and grown in the field condition as a normal plant grows in nature.

Plant tissue or cell culture requires various combinations of nutrients, minerals, plant growth substances, vitamins and sugars (as carbon source). Unfortunately, these culture media are also suitable for the rapid growth of bacteria and fungi. Once these microbes invade the plant tissue or cell culture they usually grow quickly, depleting the medium of nutrients and producing toxins that can affect the growth of, and ultimately kill, the cultured plant tissue.

Thus, it is imperative to use sterile techniques for all *in vitro* plant culture manipulations. The preparation and maintenance of tissue culture system requires the sterilization of the culture medium, the culture container, surface-sterilization of the seed or plant tissue to be cultured, and sterilization of any instruments used to handle or manipulate the plant tissue. In addition, any subsequent manipulation of the plant tissue must typically be carried out in a sterile room or cabinet and filtered-air environment like laminar air-flow cabinet. Contamination by bacteria and fungi is a frequent problem that continually threatens plant tissue cultures. Even a fungal spore or bacterial cell that comes into contact with the growth

media will rapidly contaminate the plant tissue culture materials and instruments as well. Killing or removing all forms of microbial life (including endospores) in a material or an object is defined as the process of sterilization. This review deals with the techniques and methods of sterilization to maintain the laboratory, equipments and the plant materials used in tissue culture.

ASEPTIC STERILIZATION TECHNIQUE

The essence of sterilization or aseptic technique is the exclusion of invading microorganisms during experimental procedures. If sterile tissues are available, then the exclusion of microorganisms is accomplished by using sterile instruments and culture media concurrently with standard bacteriological transfer procedures to avoid extraneous contamination. Media and apparatus are rendered sterile by autoclaving at 15 lbs/inch^2 (121°C) for 15 minutes. The uses of disposable sterile plastic ware are convenient. Filter sterilization is employed for heat-labile substances like cytokinins. Aseptic transfers can be made on the laboratory bench top by using standard bacteriological techniques using a laminar flow hood. Plant tissue collected from field or which have a probability of microbial contamination needs surface sterilization with ethyl alcohol and/or chlorox and added surfactant. Concentration of thesurface-sterilant and exposure time are determined empirically depending on the type of explant used.

STERILIZING CULTURE ROOMS AND TRANSFER HOODS

Tissue culture lab is compartmentalized into work elements in separately specified areas such as, media preparation, glassware washing, sterilization, microscopy, and aseptic transfers, in order to facilitate all operations and enhances cleanliness. Tissue culture media and nutrient agar, and Laminar flow hoods are available from several suppliers.

Large transfer rooms are best sterilized by exposure to ultraviolet (UV) light. Sterilization time varies according to the size of the room and should be done when no experiment is in progress. Ultraviolet radiation is harmful to the eyes. Transfer rooms can also be sterilized by washing them 1-2 times a month with a commercial brand of antifungal liquids. Smaller transfer rooms and hoods also can be sterilized with UV lights or by treatment with bactericides and/or fungicides. Laminar flow hoods are easily sterilized by turning on the hood and wiping down all surfaces with 95% ethyl alcohol 15 min before initiating any operation under the hood.

Culture rooms should be initially cleaned with detergent-brand soap and then carefully wiped down with a 2% sodium hypochlorite solution or 95% ethyl alcohol. All floors and walls should be washed gently on a weekly basis with a similar solution; extreme care must be used to avoid stirring up any contamination that has settled. Commercial disinfectants diluted at manufacturer's recommended rates can be used to disinfect work surfaces and culture rooms. Formaldehyde gas is an excellent disinfectant for larger culture area and rooms. Commonly used as formalin, a 37% aqueous solution inactivates viruses and bacteria by inactivating proteins by forming covalent cross-links with several functional groups.

STERILIZATION OF INSTRUMENTS AND EQUIPMENTS

The decontamination, cleaning, maintenance, handling, storage, and/or sterilization of tissue culture instruments and equipments are prerequisite for any tissue culture lab. There are clear instructions for cleaning and sterilization of these equipments by the manufacture. It is always advisable to follow the manufacturer's instruction. Otherwise, the following protocols may be followed as proper care and cleaning ensures the effectiveness, reduces the likelihood of delays, and also helps reduce the risk of infection and injury through the instruments.

Instruments should be kept free of dust or soil particles by wiping with sponges moistened with sterile water. Inadequate cleaning of instruments results in retaining microorganisms. Corrosion and rusting occurs when biological materials and debris adhere to tissue culture instruments. Washing the instruments with sterile water removes residues and prevents tissue damage. Saline causes deterioration of instrument surfaces. Automated cleaning methods and/or manual cleaning methods are equally effective.

Instruments should be taken apart at the point of use and arranged in an orderly fashion in mesh bottom trays so that all surfaces are exposed to the action of the automatic cleaner. The following activities should be completed:

- open instrument box locks;
- disassemble instruments with removable parts;
- place scissors, lighter-weight instruments, and microsurgical instruments on top;
- place heavy instruments in a separate tray; and
- separate all reusable sharp instruments from the general instrumentation.

Instruments may be covered with a damp towel to prevent drying during transportation to the decontamination area.

All the instruments can be sterilized by dry or stem sterilization methods as described below:

i. Steam Sterilization

Autoclaving is a method of sterilizing with water vapor under pressure. Cotton plugs, gauze, lab ware, plastic caps, glassware, filters, pipettes, water, and nutrient media can all be sterilized by autoclaving. Nearly all microbes are killed by exposure to the super-heated steam of an autoclave for 10-15 minutes. All objects should be sterilized at 121°C and 15 psi for 15-20 min. In place of glassware, some type of good quality plastic lab ware are used (polypyrene, polymethylpentene, polyallomer, Tfzel, ETPE, Teflon FEP), which can be repeatedly autoclaved. A large variety of pre-sterilized culture vessels are also available which could be directly used to pour autoclaved media.

Metallic instruments may rust and become blunt by steam sterilization. So, these instruments *e.g.* forceps, scalpel, needle, spatula and scissors etc. are preferably sterilized by dry heat method.

ii. Dry Sterilization

(a) Ultrasonic cleaner

The ultrasonic cleaner or a washer/decontaminator may be used followed by several cycles of cleaning with cold water, pre-rinse, high-temperature wash with alkaline detergent, neutralizing cycle, final rinse with deionized solution, lubrication, and drying. Enzymatic detergent solution effectively removes all visible debris, thus provide an alternative to manual cleaning. Ultrasonic cleaner transmits ultrasonic waves through the cleaning solution in a mechanical process known as cavitation. The ultrasonic waves produce tiny air bubbles on the surfaces of instruments, which in turn make a scouring action that cleans the instrument surfaces. Ultrasonic cleaning is particularly effective in removing soil or dust deposits from hard-to-reach areas.

(b) Heat Sterilization Treatments

i. Flame sterilization

Metalic instruments viz. forceps, scalpel, needle, spatula and scissors are dipped in 70% ethanol followed by flaming with a burner (Bunsen burner or spirit lamp) and cooling. However, ethanol is volatile and inflammable. So this procedure involves the risk of fire during sterilizing the equipments.

ii. Hot dry air circulating oven

Metal instruments, glassware, aluminum foil, etc., can also be sterilized by exposure to hot dry air (130°-170°C) for 2-4 hr in a hot-air oven. All items should be sealed before sterilization but not in paper, as it decomposes at 170°C. Autoclaving is not advisable for metal instruments because they may rust and become blunt under these conditions.

iii. Glass bead sterilizer

Metal Instruments are best sterilized using a glass bead sterilizer or steri-pots. These sterilizers heat to approximately 275-350°C and will destroy bacterial and fungal spores that may be found on your instruments. The instruments simply need to be inserted into the heated glass beads for a period of 10 to 60 sec. The instruments should then be placed on a rack under the hood to cool until needed.

iv. Infra-red sterilizer

The infra-red sterilizers are quick and efficient in large scale tissue culture production units or industrial applications. The sterilizer temperature rises to almost 700°C by the infrared wave produced by a device. Due to such a high heat capacity, sterilization is obtained by a 2-5 seconds exposure. These instruments are also safer than burner which could cause fire hazard.

Instruments that have been sterilized in hot dry air should be removed from their wrapping, dipped in 95% ethyl alcohol, and exposed to the heat of a flame. After an instrument has been used, it can again be dipped in ethyl alcohol, re-flamed, and then reused. This technique is called flame sterilization.

v. Dry sterilization using gaseous substances

Sterilization depends on contact of the sterilant with all surfaces of the equipment. Ethylene oxide gas sterilization should be used only when equipments cannot withstand pre-vacuum steam sterilization. Ethylene oxide is an alkylating agent that, under the right conditions of time, temperature, concentration, and humidity, can result in microbial death. Ethylene oxide sterilization may not sterilize the internal mechanisms of powered equipment, as gas does not diffuse readily through lubricants.

POST-STERILISATION CARE OF THE INSTRUMENTS

Abrasives will damage the protective surfaces of the instruments, contribute to corrosion, and impede sterilization. After initial cleaning, instruments may be processed in ultrasonic cleaners to remove small particles and debris from instruments crevices. Instruments may be damaged by mechanical vibrations and so this may be avoided. Moving parts of the instruments must be lubricated with water-soluble lubricants after every cleaning. Instruments must be dried thoroughly before storage to prevent rust formation. Otherwise, washer/sterilizer that cleans by a spray-force action known as impingement combining a vigorous agitation bath with jet-stream air to create underwater turbulence can be used. A sterilization cycle follows the washing cycle. Pre-vacuum steam sterilization is another useful method for equipment sterilization.

CLEANING AND STERILISING GLASSWARE

Use clean and decontaminated glassware only for tissue culture purposes. Toxic metals usually bind to the silicate surface of glassware, which can be troublesome. Washing glassware with liquid detergent followed by several rinses with tap water and finally with deionized, purified and sterile water can minimize these problems.

The conventional method of washing glassware is by soaking it in acid bath (chromic acid-sulfuric acid) followed by several cycles of rinsing with tap water, distilled water, and finally double-distilled water. However, acids are corrosive and so this procedure is as much possible avoided until and unless the glassware is soiled or highly contaminated. Most of the glassware for tissue culture can be properly washed in hot water (70°C+) with commercial detergents, rinsing with hot tap water (70°C+) and finally rinsing with distilled and double-distilled water. However, highly contaminated glassware should be cleaned in a chromic acid-sulfuric acid bath or by some other proven method such as ultrasonic cleaning, washing with sodium pyrophosphate, or boiling in metaphosphate, rinsing then boiling in a dilute hydrochloric acid solution, and then finally re-rinsing. Cleaned glassware should be inspected, dried at 150°C in a drying oven, capped with aluminum foil, and stored in a closed cabinet.

WEIGHING INSTRUMENTS

The preparation of media requires careful and accurate weighing of all components for consistency and repeatability in tissue culture practices. Various types of balances used in the tissue cultured laboratory include top-loading single-pan balance, triple-beam balance,

double-pan torsion balance, analytical single-pan balance, and top-loading electronic balance. The last type has become quite popular in recent years due to its accuracy, ease of use, and durability. The balance or weigh area should always be kept clean.

LIQUID AND LIQUID HANDLING SYSTEMS

High-purity water or double glass distilled water or deionized water from an ion-exchanger are used in tissue culture methods. Calibrated glassware (*e.g.,* beakers, flasks, and pipettes) are required for the preparation of culture media. Graduated cylinders of 10, 25, 100, and 1000 ml capacities are used for many measuring operations, but volumetric flasks and pipettes are required for more precise measurements. Pipettes should be filled with a hand-operated device, called a pipettor, which eliminated the hazards of pipetting by mouth. Never pipette by mouth!! (bulb-type pipettor, pipettor operated by rotating a small wheel on the side of the handle or pipettor which utilizes an electric air pump).

STERILIZATION OF NUTRIENT MEDIA

The most tedious parts of *in vitro* techniques are sterilizing plant materials and media and maintaining aseptic conditions. Bacteria and fungi are the common contaminants observed in tissue and cell cultures. Fungal spores are lightweight and present throughout our environment. When a fungal spore comes into contact with the culture media used in tissue culture, conditions are optimal for germination of the spore and subsequent contamination of the culture.

Two methods (autoclaving and membrane filtration under positive pressure) are commonly used to sterilize culture media.

i. Steam Sterilization

Culture media, distilled water, and other stable mixtures can be autoclaved in glass containers that are sealed with cotton plugs, aluminum foil, or plastic closures. However, solutions that contain heat-labile components must be filter-sterilized.

Generally, nutrient media are autoclaved at 15 psi and 121°C. For small volumes of liquids (100 ml or less), the time required for autoclaving is 15-20 min, but for larger quantities (2-4 liter), 30-40 min is required. The time period is not linearly related to the medium volume, but it can be theoretically presented as shown in Fig. 1. The pressure should not exceed 20 psi, as higher pressures may lead to the decomposition of carbohydrates and other thermo-labile components of a medium. In order to ensure proper sterilization of the media, with an increase or decrease in volume and keeping the pressure constant, only the time period is varied.

Autoclaving can cause the following changes in the medium:

- Lowering of the pH of the medium by 0.3-0.5 units.
- Autoclaving at high temperature can caramelize sugars, which may have toxic effect.
- Volatile substances can be destroyed by the use of autoclave.
- Long periods of autoclaving can precipitate salts and depolymerize agar.
- So correct temperature and duration of time is a pre-requisite for proper medium preparation and its stability.

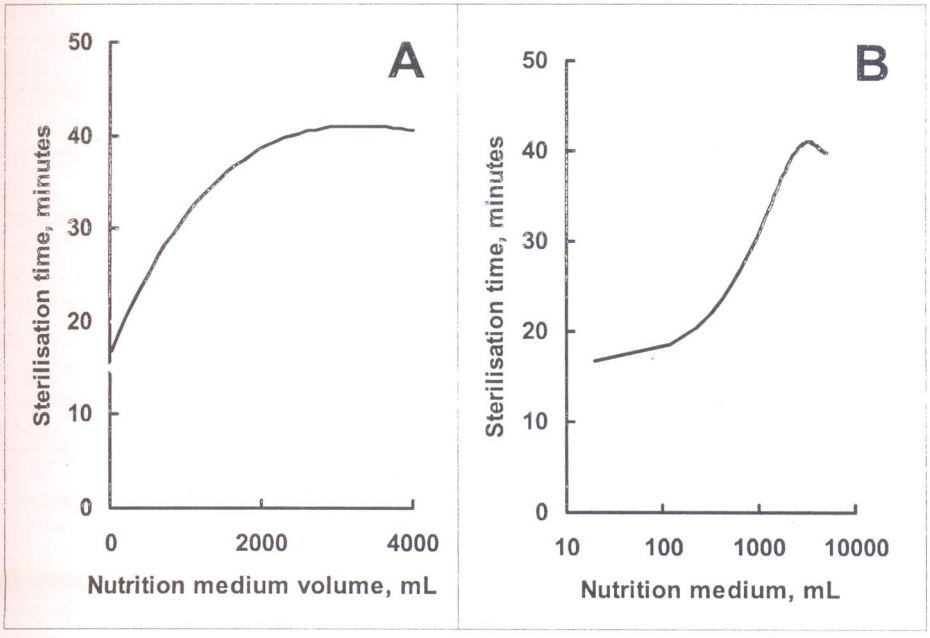

FIG. 1: Relationship between nutrition volumes with sterilization time. **(A)** In linear scale taking into consideration maximum 5 Litre volume. **(B)** The same values in log values for medium volume. The sterilization time is linear upto 500 mL and is log linear upto 2 Litres, after this the sterilization time reaches the plateau of 40 minutes.

ii. Filter Sterilization

Since many proteins, vitamins, amino acids, plant extracts, hormones, and carbohydrates are thermo-labile and may decompose during autoclaving, filter sterilization may be required. A Millipore or Nalgene or Sartorius or Seitz filter can be used; the porosity of the filter membrane should be no larger than 0.2 microns (μm). Empty glassware that is to hold media must be sterilized in an autoclave before filter sterilization. Most filters are of cellulose acetate or cellulose nitrate and are available in pre-sterilized, plastic disposable units. During filter sterilization, all the particles, microorganisms and virus that are bigger than the pore diameter of the filter are removed.

Nutrient media that contain thermo-labile components can be prepared in several steps. That is, a solution of the heat-stable components is sterilized in the usual way by autoclaving, then cooled to 50°-60°C under sterile conditions; in a separate operation, solutions of the thermo-labile components are filter-sterilized. For large volumes, filter sterilization can be carried out using a vacuum filtering set up. The sterilized solutions are then combined under aseptic conditions to give the complete media.

A comprehensive summary of the different sterilization techniques described above is given in Table 1.

TABLE 1: Sterilization techniques used in Plant Tissue Culture

Technique	Materials sterilized
- Steam sterilization/Autoclaving - (121°C at 15 psi for 20-40 min)	- Nutrient media, culture vessels, glassware and plastic wares
- Dry heat (160-180°C for 3h)	- Instruments (scalpel, forceps, needles etc.), glassware, pipettes, tips and other plasticwares
- Flame sterilization	- Instruments (scalpel, forceps, needles etc.), mouth of culture vessel
- Filter sterilization (membrane filter - made of cellulose nitrate or cellulose acetate of 0.45-0.22 µm pore size)	- Thermolabile substances like growth factors, amino acids, vitamins and enzymes.
- Alcohol sterilization	- Worker's hands, laminar flow cabinet
- Surface sterilization (Sodium hypochlorite, hydrogen peroxide, mercuric chloride etc)	- Explants

STERILIZATION OF PLANT MATERIAL

Actively growing and healthy parts of the plant are used in tissue culture. Stressed plants/parts usually do not grow in tissue cultures. Insect and disease-free greenhouse plants can be sterilized or made aseptic more readily, so these plants are preferred in tissue culture. Seeds are surface sterilized to produce contamination-free plants under clean greenhouse conditions for later experimental use in tissue culture methods.

Aseptic technique is absolutely necessary for the successful establishment and maintenance of plant cell, tissue and organ cultures. The *in vitro* environment in which the plant material is grown is also ideal for the proliferation of microorganisms. In most cases the microorganisms outgrow the plant tissues, resulting in their death. Contamination can also spread from culture to culture. The purpose of aseptic technique is minimize the possibility that microorganisms remain in or enter the cultures.

Contaminants

Some of the common contaminants are dust particles, bacteria, fungi and insects

i. Bacteria

Bacteria are the most frequent contaminants. They are usually introduced with the explant and may survive surface sterilization of the explant because they are in interior tissues. So bacterial contamination can first become apparent long after a culture has been initiated (see below). Some bacterial spores can also survive the sterilization procedure even if they are on the tissue surface. Many kinds of bacteria have been found in plant tissue cultures including *Agrobacterium, Bacillus, Corynebacterium, Enterobacter, Lactobacillus, Pseudomanas, Staphylococcus,* and *Xanthomonas*. Bacteria can be recognized by a characteristic "ooze", the ooze can be many colors including white, cream, pink, and yellow. There is also often a distinctive odor.

ii. Fungi

Fungi may enter culture on explants or spores may be airborne. Fungi are frequently present as plant pathogens and in soil. They may be recognized by their "fuzzy" appearance and occur in a multitude of colors.

iii. Yeast

Yeast is a common contaminant of plant cultures. Yeasts live on the external surfaces of plants and are often present in the air.

Obtaining sterile plant material is difficult, and despite any precautions taken, 95% of cultures will end up contaminated if the explant is not disinfected in some manner. Because living materials cannot be exposed to extreme heat and retain their biological capabilities, plant organs and tissues are sterilized by treatment with a disinfecting solution. Solutions used to sterilize explants must preserve the plant tissue but at the same time destroy any fungal or bacterial contaminants. A summary of the sterilizing agents commonly used in plant tissue culture is shown in Table 2.

TABLE 2: A comprehensive list of chemicals, concentration and treatment time period range used as sterilizing agents for plant explants. Besides these surface sterilsing agents, use of antibiotics separately or with these agents are more efficient. Antibiotics in the concentration of 4-50 mg/L can be used for 30-60 minutes

Chemicals used as sterilizing agent	Concentration (% w/v)	Treatment time, (min)
Sodium hypochloride (commonly used)	1-1.4	5-30
Hydrogen peroxide	10-12	5-15
Calcium hypochloride	9-10	5-30
Silver nitrate	1	5-30
Mercuric chloride	0.01-1	2-10
Bromine water	1-2	2-10

Once explants have been obtained, they should be washed in a mild soapy detergent before treatment with a sterilizing solution. Some herbaceous plant materials (*e.g.,* African violet leaves) may not require this step, but woody material, tubers, etc., must be washed thoroughly. After the tissue is washed, it should be rinsed under running tap water for 10-30 min and then be submerged into the disinfectant under sterile conditions. All surfaces of the explant must be in contact with the sterilant. After the allotted time for sterilization, the sterilant should be decanted and the explants washed at least three times in sterile distilled water. For materials that are difficult to disinfect, it may be necessary to repeat the treatment 24-48 hr before making the final explants. This allows previously unkilled microbes time to develop to a stage at which they are vulnerable to the sterilant. The protocol for sterilizing an explant is depicted in Fig. 2.

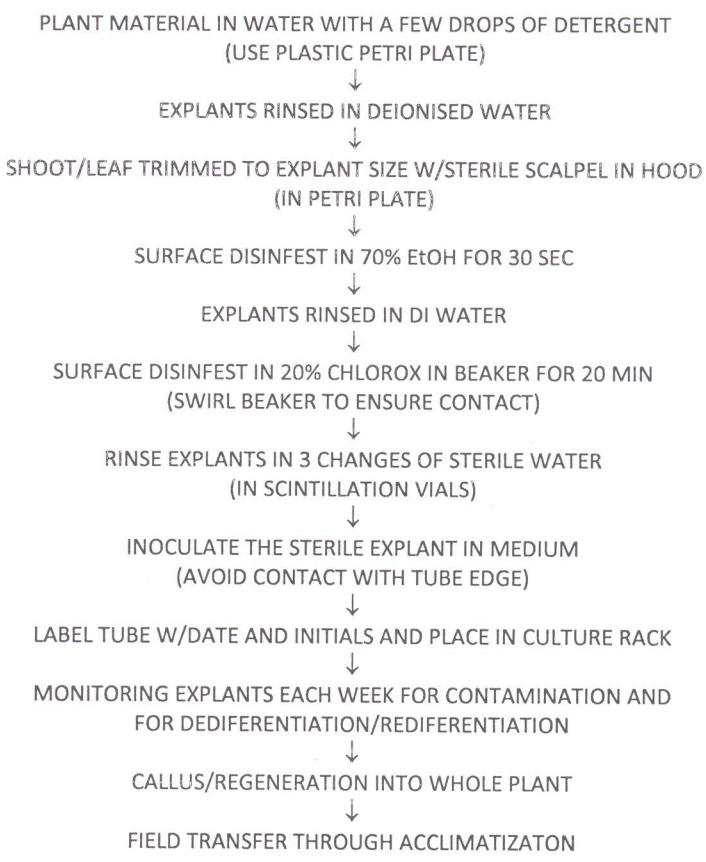

Fig. 2: Flow chart for the sterilization of plant explants for tissue culture.

The success of tissue culture lies in; i) to develop contamination-free culture ii) induction/regeneration of plantlets and iii) finally to transfer the *in vitro* regenerated plantlets to field conditions. In the first step the operator has a great role to play as he has to perform the inoculation process as quickly as possible to overcome the contamination, if any, from the ambience. In addition to surrounding, the worker himself is a potential source of contaminations. To minimize the risk of contamination it is advisable to wear a sterile cloth (Lab-coat), cover the face (mouth and nose) with mask and to put a sterile headgear.

It is most important to wash the hand with 95% alcohol before any experiment is to be started. Precaution is also taken that the explants should not pushed deep into the medium and should not touch the edge of the of the culture vessels.

An alternative measure for obtaining sterile plant material is to develop *in vitro* seedlings from the thoroughly sterilized seeds. The differentiated and regenerated plantlets in the culture vessels can be grown ascetically in the field through gradual acclimation, hardening and field transfer methods.

CONCLUSION

Despite the most stringent use of sterile techniques by the skilled artisan, however, the contamination of plant cultures remains a persistent problem that can result in losses ranging from small numbers of cultures to the catastrophic loss of whole batches of culture medium and tissue cultures. Contamination by bacteria and fungi is an insidious process that continually threatens plant tissue cultures throughout the duration of the culture period. Despite the fact that plant tissue cultures may be sterile when initiated, microorganisms can often contaminate cultures at any point during subsequent tissue culture manipulations. The review covers onfundamental aspects of sterilization procedures. There are several other methods used in sterilizing tissue culture laboratory, equipments and the plant materials under special and specific need.

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