TOPIC :	General techniques of aseptic manipulation
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General techniques of aseptic manipulation

Most important and challenging aspect of *in vitro* culture is to perform the aseptic manipulation techniques with utmost care. The microbes like bacteria, fungi and virus (to some extent) are present everywhere. Bacteria and fungi are the most common contaminant which if present in the media grow faster than the cultured tissues. They compete with the cultured tissues and release toxic compounds in the culture vessels leading to the death of the cultured tissues. So, it is very important to take appropriate measures to eliminate all contaminants and make the plant tissue culture free from contaminants.

The followingsteps can be performed for aseptic manipulation:

1. Sterilization of the Culture Vessels and Instruments
2. Sterilization of Nutrient Media
3. Sterilization of Culture Rooms and Transfer-Area
4. Sterilization of Explants
5. Aseptic Transfer of Explant/Sub-Culturing.

1. Sterilization of the Culture Vessels and Instruments

For sterilization of culture vessels, glassware, cotton plugs, gauze, plastic caps, lab wares, filters and pipettes it is better to use steam sterilization techniques i.e., by autoclaving at high pressure (15 psi) and high temperature (121°C) for 15-20 mins. All the items should be properly covered with aluminium foil before sterilization. Glassware metal instruments can also be sterilised by exposure to dry hot air oven (160°-180°C) for 2-4 hrs.

The metal instruments like forceps, scalpels, needles, and spatula are further sterilised by flame sterilization technique before use by dipping in 95% alcohol, and followed by flaming and cooling. Metal equipments are generally not sterilized by using autoclave to avoid rusting. Now-a-days flame sterilization is replaced by glass bead sterilisers for safety purposes.

Hot-air oven

Autoclave

Glassbead sterlizer

Portable autoclave

2. Sterilization of Nutrient Media:

The nutrient media used in tissue culture are commonly sterilised by autoclaving and filter sterilization. Macro-, micronutrients, double distilled water and other stable compound mixtures are autoclaved, whereas the thermolabile compounds are filter-sterilised separately and mixed with the media whenever necessary. Some vitamins, amino acids, plant extracts and hormones are thermolabile, they require filter sterilization. The solutions are passed through a bacterial membrane filter under positive pressure. A Millipore or Seitz filter (pore size 0.2 µm) is used for filter sterilization. The sterilised compound is then mixed with the autoclaved media.

Table 1. Minimum time required for autoclaving nutrient media

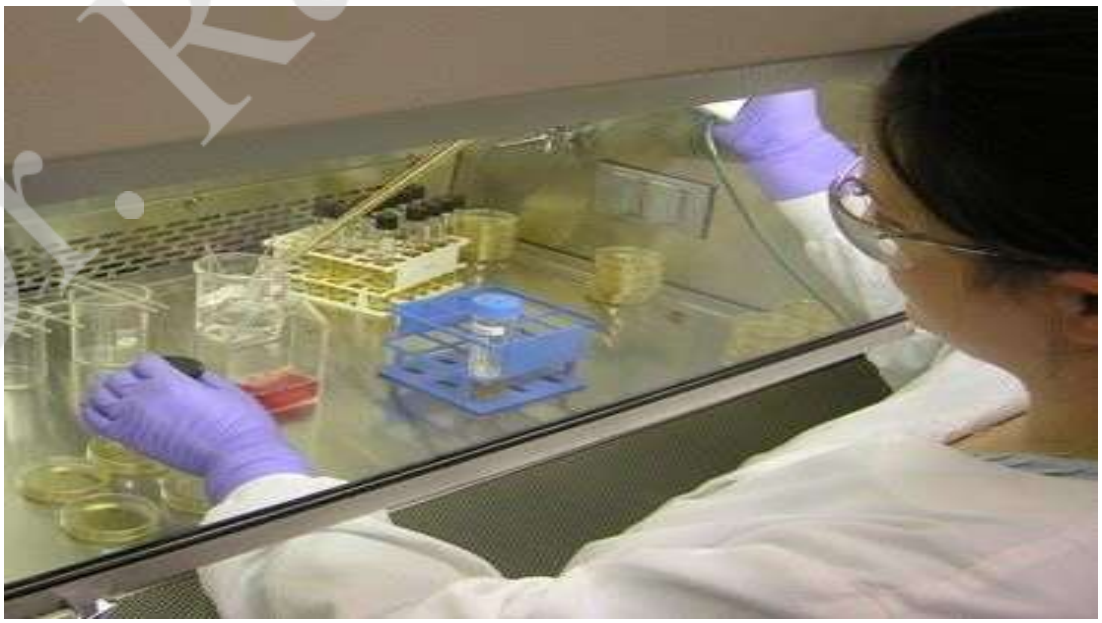
Volume (ml)	Sterilization time[1] (min)
1-200	15
200-1000	30
1000-2000	40

1-- at 121ºC and 15 psi

3. Sterilization of Culture Rooms and Transfer-Area:

The floor and walls of culture rooms are cleaned by gently washing with detergent then by wiping them with 2% sodium hypochlorite solution and then using 95% ethanol. The process of surface sterilization of culture rooms should be done at regular intervals.

The transfer-area is also sterilised once or twice a month by washing with commercial brand of antifungal liquid/powder. Transfer rooms which are large are further sterilised by UV light. UV radiation is harmful for eyes, so UV radiation should be done before performing any laboratory work. Time of sterilization varies according to the size of the room. Where laminar air flow cabinet is used for transfer, the surface is cleaned by wiping with 95% ethanol 20 min before initiating the operation and chamber is sterilised by UV light before work in progress. Then the sterile air is flowed through HEPA filter during works.



4. Sterilization of Explant:

All different kinds of plant materials or explants should be surface sterilised by a variety of chemicals before using in tissue culture. It is the eradication of surface microorganisms with the help of different chemicals. The plants and organ tissues are surface sterilized to eliminate bacteria and fungi only and they should not lose their biological activity. The type and concentration of different chemical sterilant to be used for sterilization of different types of explants and exposure time must be decided experimentally. Some common disinfectants used for sterilizing plant materials are listed below Table 2. Sometimes the sterilization procedure may lead to lethality to plant tissue, so the use of disinfectants should be tested. Here are few disinfectants which are used commonly for surface sterilization of different explants.

Table 2. Disinfectants, concentration and duration of treatment for surface sterilization of explants

Disinfectant	Concentration	Duration of treatment(min)
Sodium hypochlorite	0.5-5%	5-30
Calcium hypochlorite	9-10%	5-30
Ethyl alcohol	75-95%	_[2]
Hydrogen peroxide	3-12%	5-15
Mercuric chloride	0.1-1.0%	2-10

2 --several seconds to several minutes

(a) 1% solution of Sodium hypochlorite (NaClO), commercial bleach having 5% active chlorine can be used.

(b) 4%-l0% solution of Calcium hypochlorite [$Ca(ClO)_2$] can be used, it enters within the plant tissue slower than sodium hypochlorite.

(c) 1% solution of Bromine-water.

(d) 0.01-0.1% solution of ($HgCl_2$) which is an extremely toxic substance for plant tissue, repeated rinsing with water is very much essential.

(e) 70% Ethyl alcohol is used for sterilization of plant material dipping them for 30 sec-2 mins.

(f) 10% Hydrogen peroxide (H_2O_2) solution is effective for end surface sterilization.

All these sterilants should be washed out properly before using the explant as the retention of these chemical substances may affect the establishment of successful tissue culture. But in most of the cases it becomes difficult to determine the optimal conditions for each kind of tissue.

So to avoid this problem the explants can be taken from aseptically grown plants developed from the surface sterilised seeds as these seeds are more resistant to chemicals due to presence of seed coat.

For this purpose, the seeds are surface sterilised and then cultured aseptically in basal nutrient media. These give rise to aseptic seedlings from which the different explants can be used. Explants from such seedlings need no further sterilization.

But for anther culture and shoot tip culture the explants are collected from outside grown plant. For these kinds of explants addition of few drops of surfactant (Trito -X or Tween-20) to the solution or treating the plant material in a solution of Cetavlon for 2 min. before exposing to sterilant may enhance sterilization efficiency.

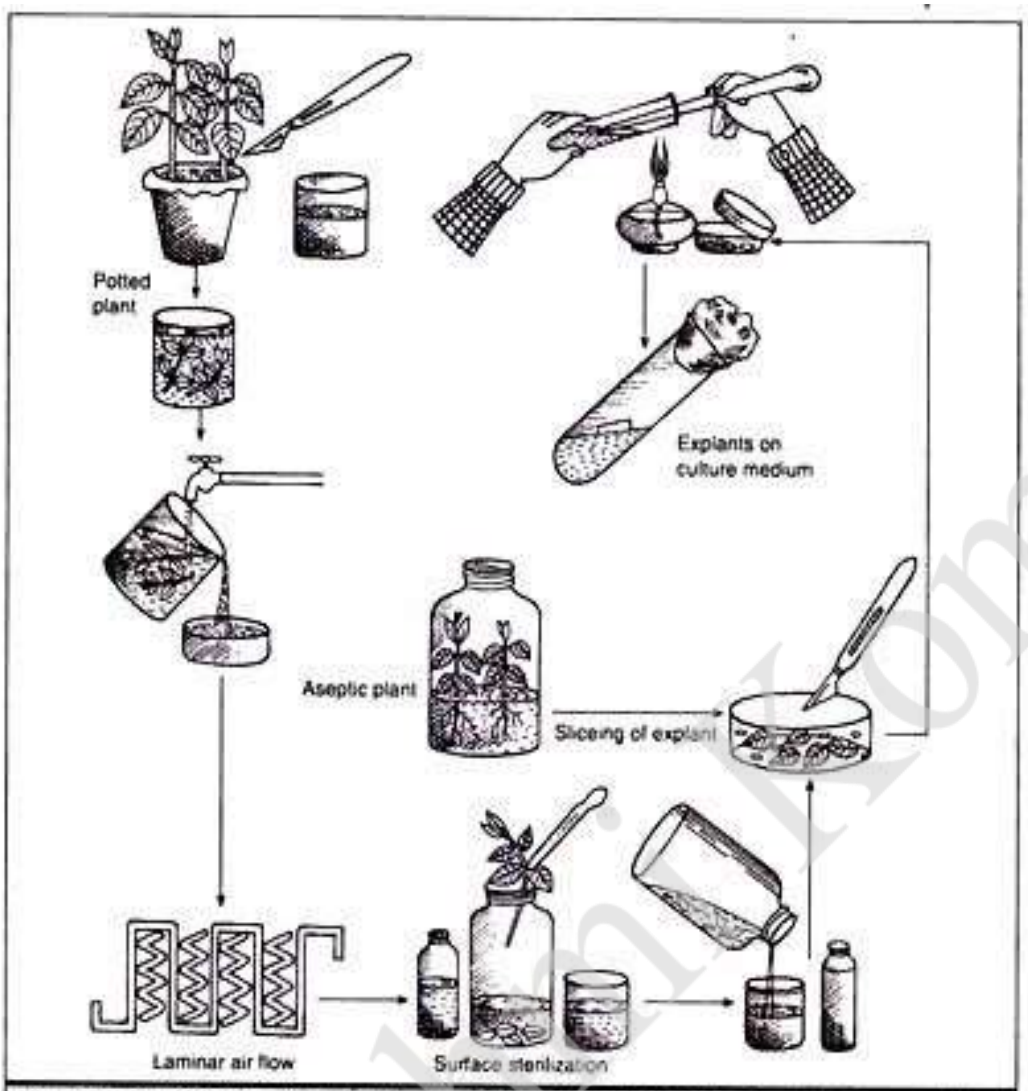


Fig. 16.3: Flow diagram illustrating the procedure for surface sterilization of plant material and inoculation of explant for culture

5. Aseptic Transfer of Explant/Sub-Culturing:

Control of contamination largely depends upon the operator's technique while transferring the sterilised explant/sub-culturing into the sterile culture vial containing nutrient media under aseptic condition. Dust from the surface, hair, hands and clothes are the potential sources of contamination.

Before starting the transfer procedure the surface of transfer area and hands should wiped with 95% ethanol; sterile clothes (aprons) should be used. All the metallic equipment's used for transfer (inoculating needle, forceps, scalpel) should be dipped into 95% ethanol and then flamed and cooled. The tissue material should not touch the edge of culture vessel during transfer.

Precautions taken during aseptic manipulation in tissue culture laboratory :

1. Laboratory should be entered wearing lab shoes and lab coats and keeping long hair tied back.
2. Avoid handling alcohols around open flames.
3. Never pipette by mouth.
4. Mask should be worn during inoculation.
5. Inspect all equipment and media for visible contamination before use.
6. Stay as organised as possible—label everything and set up all of yourmaterials before getting started.
7. Do not pass your hands/arms over open bottles, plate or tube.
8. Wipe done working surface with ethanol and turn on UV lamp for 10 min after finishing the experiment.

THANK YOU