Sterile (Aseptic) Technique

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.

The environmental control of air is also of concern because room air may be highly contaminated. Example: Sneezing produces 100,000 - 200,000 aerosol droplets which can then attach to dust particles. These contaminated particles may be present in the air for weeks. (Have you ever viewed the air around you when you open the curtains on a sunny day?)...Air may also contain bacterial and fungal spores, as do we.

I. Contaminants

A. Bacteria, fungi, and insects

1. 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.

2. Fungi

Fungi may enter cultures 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.

3. Yeast

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

4. Viruses, etc.

Viruses, mycoplasma-like organisms, spiroplasmas, and rickettsias are extremely small organisms that are not easily detected. Thus, plant culture is not necessarily pathogen-free even if microorganisms are not detected, and this can influence culture success. Special measures such as meristem culture are often necessary to eradicate such contaminants.

5. Insects

The insects that are most troublesome in plant cultures include ants, thrips, and mites. Thrips often enter cultures as eggs present on the explants. Ants and mites, however, usually infest already established cultures. Mites feed on fungus and mite infestations are often first detected by observing lines of fungal infection that lead from the edge of the culture vessel to the plant tissue, having been introduced by the insect. It is very difficult to eradicate insect infestations. Careful lab practices and cleanliness should prevent most infestations.

B. Initial Contaminants

Most contamination is introduced with the explant because of inadequate sterilization or just very dirty material. It can be fungal or bacterial. This kind of contamination can be a very difficult problem when the plant explant material is harvested from the field or greenhouse. Initial contamination is obvious within a few days after cultures are initiated. Bacteria produce “ooze” on solid medium and turbidity in liquid cultures. Fungi look “furry” on solid medium and often accumulate in little balls in liquid medium.

C. Latent Contamination

This kind of contamination is usually bacterial and is often observed long after cultures are initiated. Apparently the bacteria are present endogenously in the initial plant material and are not obviously pathogenic in situ. Once in vitro, however, they increase in titer and overrun the cultures. Latent contamination is particularly dangerous because it can easily be transferred among cultures.

D. Introduced Contamination

Contamination can also occur as a result of poor sterile technique or dirty lab conditions. This kind of contamination is largely preventable with proper care.

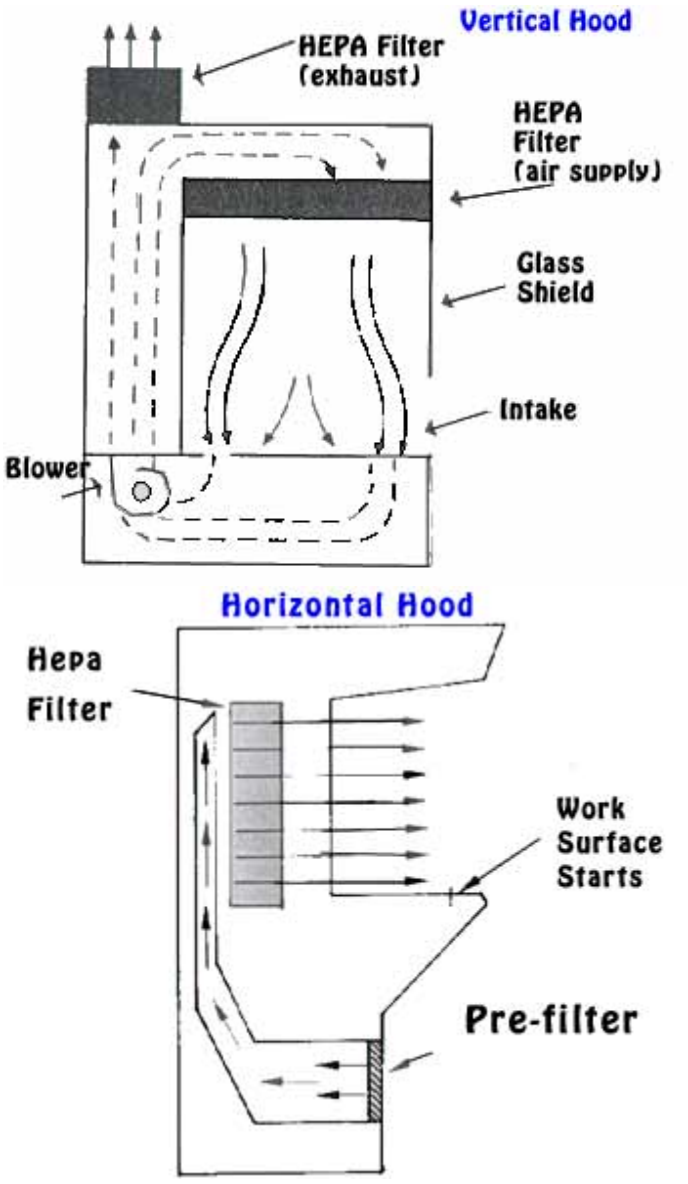
E. Detection of Contaminants

Contamination is usually detected by the "eyeball" method in research labs. However, indexing is possible, and is frequently done in commercial settings. This involves taking a

part of the plant tissue and culturing it in media that are specific for bacteria and fungi. Media that have been used for this purpose include PDA (potato dextrose agar) and NB broth (with salts, yeast extract and glucose). This is the most reliable method for detecting bacteria and fungi, but, as indicated above, there may be infecting organisms that won't be detected.

II. The Transfer Hood

Laminar airflow hoods are used in commercial and research tissue culture settings. A horizontal laminar flow unit is designed to remove particles from the air. Room air is pulled into the unit and pushed through a HEPA (High Energy Particle Air) filter with a uniform velocity of 90 ft/min across the work surface. The air is filtered by a HEPA (high efficiency particulate air) filter so nothing larger than 0.3 micrometer, which includes bacterial and fungal spores, can pass through. This renders the air sterile. The positive pressure of the air flow from the unit also discourages any fungal spores or bacteria from entering. Depending on the design of the hood, the filters are located at the back or in the top of the box.



III. Sterilization and Use of Supplies and Equipment:

A. Sterilizing tools, media, vessels etc.

1. Autoclaving

Autoclaving is the method most often used for sterilizing heat-resistant items and our usual method for sterilizing items. In order to be sterilized, the item must be held at 121°C, 15 psi, for at least 15 minutes. It is important that items reach this temperature before timing begins. Therefore time in the autoclave will vary, depending on volume in individual vessels and number of vessels in the autoclave. Most autoclaves automatically adjust time when temperature and psi

are set, and include time in the cycle for a slow decrease in pressure. There are tape indicators that can be affixed to vessels, but they may not reflect the temperature of liquid within them. There are also “test kits” of microorganisms that can be run through the autoclave cycle and then cultured.

Empty vessels, beakers, graduated cylinders, etc., should be closed with a cap or aluminum foil. Tools should also be wrapped in foil or paper or put in a covered sterilization tray. It is critical that the steam penetrate the items in order for sterilization to be successful.

2. Autoclaving and Fiter-sterilizing Media and Other Liquids

Two methods (autoclaving and membrane filtration under positive pressure) are commonly used to sterilize culture media. Culture media, distilled water, and other heat 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. 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 to complete the cycle. The pressure should not exceed 20 psi, as higher pressures may lead to the decomposition of carbohydrates and other components of a medium. Too high temperatures or too long cycles can also result in changes in properties of the medium.

Organic compounds such as some growth regulators, amino acids, and vitamins may be degraded during autoclaving. These compounds require filter sterilization through a 0.22 µm membrane. Several manufacturers make nitrocellulose membranes that can be sterilized by autoclaving. They are placed between sections of a filter unit and sterilized as one piece. Other filters (the kind we use) come pre-sterilized. Larger ones can be set over a sterile flask and a vacuum is applied to pull the compound dissolved in liquid through the membrane and into the sterile flask. Smaller membranes fit on the end of a sterile syringe and liquid is pushed through by depressing the top of the syringe. The size of the filter selected depends on the volume of the solution to be sterilized and the components of the solution.

Nutrient media that contain thermo labile components are typically prepared in several steps. A solution of the heat-stable components is sterilized in the usual way by autoclaving and then cooled to 35°-50° C under sterile conditions. Solutions of the thermo labile components are filter-sterilized. The sterilized solutions are then combined under aseptic conditions to give the complete medium.

In spite of possible degradation, however, some compounds that are thought to be heat labile are generally autoclaved if results are found to be reliable and reproducible. These compounds include ABA, IAA, IBA, kinetin, pyridoxine, 2-ip and thiamine are usually autoclaved.

3. Ethylene Oxide Gas

Plastic containers that cannot be heated are sterilized commercially by ethylene oxide gas. These items are sold already sterile and cannot be resterilized. Examples of such items are plastic petri dishes, plastic centrifuge tubes etc.

4. UV Radiation

It is possible to use germicidal lamps to sterilize items in the transfer hood when no one is working there. We do not do this. UV lamps should not be used when people are present because the light is damaging to eyes and skin. Plants left under UV lamps will die.

5. Microwave

It is also possible to sterilize items in the microwave; we do not do this.

6. More Comments

- Know which of your implements, flasks, etc. are sterile and which are not. Sterile things will have been autoclaved and should be wrapped with some kind of protective covering, e.g. foil, for transport from the autoclave to the hood.
- Our usual autoclave time of 20 minutes is intended for relatively small volumes. Large flasks of media, water, etc. may require longer autoclaving periods. It is preferable to put no more than one liter of liquid in a container to be autoclaved. Also, be sure to leave enough room in the container so that the liquid does not boil over.
- Items that come packaged sterile, e.g. plastic petri plates, should be examined carefully for damage before use. If part of a package is used, seal up the remainder and date and label. Use up these items unless there is some question about their sterility; they are expensive.