

Surface-sterilizing plant material

1. Preparation of Stock Plants

Prior good care of stock plants may lessen the amount of contamination that is present on explants. Plants grown in the field are typically more “dirty” than those grown in a greenhouse or growth chamber, particularly in humid areas like Florida. Overhead watering increases contamination of initial explants. Likewise, splashing soil on the plant during watering will increase initial contamination. Treatment of stock plants with fungicides and/or bacteriocides is sometimes helpful. It is sometimes possible to harvest shoots and force buds from them in clean conditions. The forced shoots may then be free of contaminants when surface-sterilized in a normal manner. Seeds may be sterilized and germinated in vitro to provide clean material. Covering growing shoots for several days or weeks prior to harvesting tissue for culture may supply cleaner material. Explants or material from which material will be cut can be washed in soapy water and then placed under running water for 1 to 2 hours.

2. Sodium Hypochlorite

Sodium hypochlorite, usually purchased as laundry bleach, is the most frequent choice for surface sterilization. It is readily available and can be diluted to proper concentrations. Commercial laundry bleach is 5.25% sodium hypochlorite. It is usually diluted to 10% - 20% of the original concentration, resulting in a final concentration of 0.5 - 1.0% sodium hypchlorite. Plant material is usually immersed in this solution for 10 - 20 minutes. A balance between concentration and time must be determined empirically for each type of explant, because of phytotoxicity.

3. Ethanol (or Isopropyl Alcohol)

Ethanol is a powerful sterilizing agent but also extremely phytotoxic. Therefore, plant material is typically exposed to it for only seconds or minutes. The more tender the tissue, the more it will be damaged by alcohol. Tissues such as dormant buds, seeds, or unopened flower buds can be treated for longer periods of time since the tissue that will be explanted or that will develop is actually within the structure that is being surface-sterilized. Generally 70% ethanol is used prior to treatment with other compounds.

4. Calcium Hypochlorite

Calcium hypochlorite is used more in Europe than in the U.S. It is obtained as a powder and must be dissolved in water. The concentration that is generally used is 3.25 %. The solution must be filtered prior to use since not all of the compound goes into solution. Calcium hypochlorite may be less injurious to plant tissues than sodium hypochlorite.

5. Mercuric Chloride

Mercuric chloride is used only as a last resort in the U.S. It is extremely toxic to both plants and humans and must be disposed of with care. Since mercury is so phytotoxic, it is critical that many rinses be used to remove all traces of the mineral from the plant material.

6. Hydrogen Peroxide

The concentration of hydrogen peroxide used for surface sterilization of plant material is 30%, ten times stronger than that obtained in a pharmacy. Some researchers have found that hydrogen peroxide is useful for surface-sterilizing material while in the field.

7. Enhancing Effectiveness of Sterilization Procedure

Surfactant (e.g. Tween 20) is frequently added to the sodium hypochlorite.

A mild vacuum may be used during the procedure.

The solutions that the explants are often shaken or continuously stirred.

8. Rinsing

After plant material is sterilized with one of the above compounds, it must be rinsed thoroughly with sterile water. Typically three to four separate rinses are done.

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TABLE 1: Sterilization techniques used in Plant Tissue Culture

Technique	Materials sterilized
<ul style="list-style-type: none">- Steam sterilization/Autoclaving- (121°C at 15 psi for 20-40 min)- Dry heat (160-180°C for 3h)	<ul style="list-style-type: none">- Nutrient media, culture vessels, glassware and plastic wares- Instruments (scalpel, forceps, needles etc.), glassware, pipettes, tips and other plasticwares
<ul style="list-style-type: none">- Flame sterilization	<ul style="list-style-type: none">- Instruments (scalpel, forceps, needles etc.), mouth of culture vessel
<ul style="list-style-type: none">- Filter sterilization (membrane filter- made of cellulose nitrate or cellulose acetate of 0.45-0.22 µm pore size)	<ul style="list-style-type: none">- Thermolabile substances like growth factors, amino acids, vitamins and enzymes.
<ul style="list-style-type: none">- Alcohol sterilization	<ul style="list-style-type: none">- Worker's hands, laminar flow cabinet
<ul style="list-style-type: none">- Surface sterilization (Sodium hypochlorite, hydrogen peroxide, mercuric chloride etc)	<ul style="list-style-type: none">- 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.

TABLE 2: A comprehensive list of chemicals, concentration and treatment time period range used as sterilizing agents for plant explants. Besides these surface sterilising 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 unkilld 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.

Otázky:

1. Proč se musí rostliny nebo jejich části desinfikovat před jejich zavedením do *in vitro* kultur?
2. Jaké chemikálie se nejčastěji používají na desinfekci rostlinného materiálu? (vyjmenuj alespoň 3)
3. Proč se používají různé časy pro desinfekci různými roztoky a pro různý rostlinný materiál?
4. Jak se desinfikují živná média?
5. Jak se desinfikují termolabilní látky?
6. Co se desinfikuje pomocí plamene?
7. Jak se desinfikují skleněné Petriho misky, kovové skalpely a pinzety?
8. Jaký proces následuje po desinfekci rostlinného materiálu některou z desinfekčních sloučenin?
9. Čím lze zvýšit účinnost desinfekce rostlinného materiálu?