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PREPARATION OF PLANTS FOR MICROPROPAGATION

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Several researchers have experienced the frustration in trying to obtain viable, sterile explants. This step, frequently referred to as stage 1 (17), is initially the most important area with which researchers should be concerned. Simply stated, a plant cannot be multiplied effectively if a plant part cannot be properly sterilized. It is the purpose of this paper to reexamine this area and offer possible solutions to these problems.

Whenever possible, the stock plant used for multiplication should be healthy, vigorous, and preferably virus-indexed. If the plant is indexed for certain viruses, care should be taken to prevent reinoculation of that plant. Screening methods have been developed to detect systemic contaminants (2,14).

As in conventional propagation, timing is very important. The physiological state of the plant part will partially determine whether or not the plant will grow, stay dormant, or die.

The environment in which the stock plant is grown is an important consideration. If possible, plants should be grown in a greenhouse. Water should be applied only to the soil so as not to wash contaminants into the axils of the leaves. Plant diseases should be controlled with appropriate fungicides, insecticides, and antibiotics. Systemic pesticides have proven effective. The plants should be grown in a loose, sterile soil mix. This is especially true if attempting to use underground structures as a tissue source. To reduce soil-borne contaminants, plants should be potted to expose those tissues needed for sterilization. When shipping or receiving plant material, it should be sent as quickly as possible and packed in a moist but not wet material. Care should be taken to prevent sweating in the container as contaminants spread quickly in this moist environment.

Frequently stock plants are available only in the field. If above-ground parts are used, they may be brought in before bud break to force growth, or dormant material may be used. It is often difficult to obtain clean explants after the spring or summer rains have started. This can be partially overcome by loosely covering the developing bud or shoot with a sterile bag (8).

The choice of the explant will determine the type of sterilization needed. Although meristems are relatively easy to sterilize, their survival rate is generally low. Meristems have been useful for virus eradication (16) and obtaining sterile explants from material difficult to sterilize. The removal of bud scales appears to satisfy the cold requirement of the plant.

Shoot tips are probably the most common explant. Stem tips with swollen buds, 2 to 6 cm. in length, appear more tolerant to culture environment and sterilization than shoot apices. Since there is more surface area, sterilization can be more rigorous and, therefore, contamination is more easily controlled.

Underground structures are difficult to sterilize. Tubers may have to be vigorously scrubbed with a brush to remove soil particles before sterilization can begin (7). Obtaining etiolated shoots from excised root pieces may be a way of obtaining clean material from underground structures (2).

Seeds and seedlings are a useful source of explants. Plants arising are juvenile and generally respond much faster to culture conditions. They are a useful tool for determining media and culture requirements of mature plants (1,4). Generally seeds are easy to sterilize. However, seeds with very permeable seed coats should be sterilized carefully in order to prevent damage to the embryo.

The terminology used to describe the actions of antimicrobial agents is confusing due to differing definitions from various sources (10,18). **Sterilization** is generally referred to as the destruction of all viable organisms. A **disinfectant** is a substance applied to inanimate objects that kills or removes infectous microorganisms. An **antiseptic** is an agent closely related to disinfectants, except being applied to living tissue. **Germicides** are agents that destroy microorganisms, which includes both antiseptics and disinfectants.

Freeing plant material of microorganisms is brought about by controlling three factors: concentration of the germicide, amount of tissue, and time the tissue is exposed to the germicide. It would be helpful if researchers consider including average fresh weight of material used, besides germicide concentration and exposure time. We routinely use 400 ml of germicide with 250 to 500 g (fresh weight) of material for various times. The length of time the plant material is in the germicide is dependent upon several factors and should be experimentally determined by removing plant material from the germicide at different time intervals.

Vigorous agitation by shaking is a useful way of obtaining cleaner explants. Stirring may also be used but is not as effective. Use of an ultrasonic cleaner caused intracellular destruction and necrosis of the explants in our experiments.

At Briggs Nursery, we have conducted experiments to determine the efficiency of several germicides on shoots of *Rhododendron* 'Molly Ann'. These germicides and their descriptions are presented below:

ANTIBIOTICS

Antibiotics are not used by many researchers as a germicide. Antibiotics may only prove useful in freeing cultures contaminated with systemic or persistent bacteria. Generally these organic compounds break down under the high pressure and temperature of autoclaving and need to be filter sterilized to a previously autoclaved medium. Descriptions of antibiotics follow:

Ampicillin — is a penicillin-like compound commonly used in protoplast and suspension cultures to prevent bacterial contamination (19).

Chloramphenicol — has a fairly wide spectrum, effective against soil bacteria.

Gentimicin — is shown to inhibit cell division and decrease callus formation at high concentrations (50 μg/ml)(9); recommended concentration is 10 μg/ml, and it is autoclavable.

Incyte™ (Alcide Co., Westport CT.) — is a relatively new compound; preliminary results indicate some use in cleaning up contaminated cultures. One researcher notes it more bactericidal, but less fungicidal than sodium hypochlorite (12), and is autoclavable.

Novobiocin — is used to prevent growth of soil bacteria, has some fungicidal activity, and may be autoclaved. (20).

Tetracycline — inhibits soil bacteria, has a broad spectrum, is readily available, but with limited success in certain instances.

COMPLEX ORGANICS

Alcohols are weak germicides commonly used at concentrations from 70% to 95%. Alcohols are useful in removing surface waxes, allowing proper wetting of the surface for other germicides. Alcohols are bactericidal, but there are bacteria species that survive and can even grow in these chemicals. Ethanol is not sporicidal and therefore should not be used alone as a germicide (11). Isopropanol is slightly more germicidal and phytotoxic than ethanol. Generally germicidal activity of alcohols increases with increasing chain length. In other words, methanol is least active, followed by ethanol and propanol. Branching and additional hydroxyl groups lower potency (11), therefore isopropanol is less toxic than propanol.

Phenol and phenol-like compounds such as Lysol® (Sterling Drug, Inc. Montvale, N.J.) are effective germicides. Unfortunately, plant tissues appear sensitive to these compounds. After application, plant shoots become necrotic and stain the medium a dark brown.

Benzalkonium chloride (Zephiran) is used to control bacteria. Although this compound is somewhat effective, it is not sporicidal, relatively slow acting, and only moderately effective against fungi (11). This chemical is absorbed by porous material such as rubber gloves, cotton, and human skin (11). This absorption can reduce the effective concentration of the chemical and its germicidal activity. At Briggs Nursery benzalkonium chloride has never proved more effective than sodium hypochlorite.

DYES

Methylene blue is a weak germicide. Concentrations up to 0.1 ml of methylene blue per liter of water were used on explants for up to 32 minutes, but failed to obtain clean cultures.

Chlorophenol red has been added to media by one researcher to detect changes in pH caused by contaminants (21). Frequently, bacteria cause a pH change by fermentation. This indicator would turn yellow in a low pH solution.

HALOGENS

Chlorine is an effective germicide. Both chloride and hypochlorite ions possess antibacterial action. Harvey (11) states that in neutral or slightly acidic solutions, hypochlorous acid is highly bactericidal. In acidic solutions, the rate of deterioration and production of gaseous chlorine increases. In alkaline solutions, the bactericidal activity is greatly reduced.

After conducting a number of experiments, we have concluded that in low pH sodium hypochlorite solutions, the bactericidal activity is increased but the fungicidal activity decreased compared to an alkaline solution. The germicidal activity of an acidic solution of hypochlorite was always lower than that of a nonacidified solution.

Organic matter appears to affect the activity of chlorine. Hypochlorite and chlorine are bound by organic matter (10). Solutions of hypochlorite tend to be relatively unstable. Tests at Briggs Nursery indicate that organic matter can bind chlorine from irrigation water, but not to a significant amount.

Hypochlorite is commonly available as two salts: sodium hypochlorite (laundry bleach) and calcium hypochlorite (chlorinated lime). Some researchers report that calcium hypochlorite produced less browning of tissue compared to that of the sodium salt (22). We have found little difference between the two salts and prefer to use the sodium salt due to the ease of preparation.

Some researchers have noticed, particularly with hypochlorite germicides, burning or browning of explants after application. Antioxidants, such as ascorbic acid or citric acid, have been used to counteract this effect (15). We found little benefit from using these chemicals.

At Briggs Nursery, we found combining molasses at 10 ml/l, when using high nitrogen fertilizer, could reduce burning of field plants. We examined the benefits of sucrose and molasses (a complex sugar) added to 0.5% sodium hypochlorite (NaOCl). After 30 minutes in these solutions, *Rhododendron* 'Molly Ann' shoots were not burned or showed only slight bud burning compared to NaOCl with no sugar or molasses. Sucrose at concentrations of 20 to 50 g/l or molasses at 10 to 50 ml/l added to NaOCl may be useful in preventing burning.

Iodine is a common but useful germicide. Elemental iodine is the most active germicide of the halogens. Most bacteria are killed within a few minutes at concentrations of 0.005%.

In tests using iodine tincture at a concentration of 0.005%, shoots were effectively sterilized after 15 to 20 minutes in this solution. Decolorized iodine tincture was used since it resulted in less staining.

Iodine is also available in an organic form as povidone-iodine. This is a complex of elemental iodine and polyvinylpyrrolidone (PVP). In experiments we have found it to be less effective than iodine tincture. Although povidone-iodine does cause less burning on human flesh, it did not prove any better than iodine tincture when browning of tissue was compared.

Bromine is a little used germicide. Methyl bromide is commonly used as a soil sterilant. Bromine in the form of bromine water may be used to sterilize plant tissue (5).

HEAVY METALS

Inorganic mercury compounds were used for many years as a disinfectant and antiseptic. It is still believed to be an effective germicide by some people. This is questionable since these compounds tend only to be highly bacteriostatic (11).

Mercuric chloride at 0.01% was once widely used as a surface disinfectant. Organic mercurials, such as mercurochrome, have been used to disinfect instruments and as antiseptics. These agents are primarily bacteriostatic and not sporicidal. Organic mercurials are not effective in sterilizing instruments (11).

OXIDANTS

Hydrogen peroxide is a feeble germicide. Explants placed in a 3% solution quickly became contaminated even after 32 minutes in this oxidant. Hydrogen peroxide may have some use in preparing explants covered with tomentose hairs or indumentum by cleansing the surface. Then a more powerful germicide may be used to penetrate these cracks and clean the explant.

Potassium permanganate is a more powerful oxidant than hydrogen peroxide. It has proven to be an effective antiseptic at a concentration of 0.01% from 4 to 32 minutes.

PRESERVATIVES

Preservatives, such as benzoic acid and propionic acid, have been used in food proparations to inhibit bacteria and fungi growth. Sodium benzoate used at 0.1% added to the stage 1 medium, did inhibit growth of bacteria and fungi. However, growth of the plant shoot was also inhibited and toxicity appeared at 0.1%. Propionic acid (calcium salt) was phytotoxic at 1% to 10% concentrations.

Woody plants are best started on a low salt medium. Cheng (6) reports that preconditioning plants on a low salt medium with no growth regulators gave more uniform growth when shoots were subcultured from these explants. A liquid medium causes faster bud break of cultured shoots, lower water stress, and generally lower contamination than a solidified medium. If shoots become contaminated in a liquid media the upper portion can be resterilized easier than when the shoots are lying on a solid medium. If a solidified medium is used, a clear agar will allow easy visual detection of contaminants.

Plant material is never freed of contaminants and sometimes material is not available to be sterilized again. This is when and why plant material should be resterilized. We have found that using a different germicide on contaminated plant material is useful in removing bacteria contaminants. Sodium hypchlorite (0.5%) is useful as a fungicidal agent. Jones (13) resterilized *Malus* shoots for up to 40 minutes in 0.42% sodium hypochlorite. We resterilize shoots in 0.5% NaOCl for 5 to 20 minutes.

Several germicides are useful for preparing plant material for micropropagation. In our experiments we found iodine tincture, sodium hypochlorite, and potassium permanganate to be the most effective. Although there are many more germicides as yet untested, in the near future several more active chemicals will become available. Anderson (3) reports that one such chemical has shown promising results for sterilization of hard to clean material. We are confident that many more germicides and useful ways of handling them will be found.

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ASEXUAL PROPAGATION OF TROPICAL PLANTS USED IN THE LANDSCAPE

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You can probably understand why Kauai's nickname of the "Garden Island" is so appropriate, for we have some of the most beautiful gardens in the United States right here. But these gardens did not happen naturally. It took a great deal of planning, preparing, and designing, or in other words, intentional landscaping to create the lush tropical feeling that is so prevalent here.

In most places, creating a garden involves getting plants from a nursery. Somewhere along the line those plants were carefully propagated by someone, and chances are, they made use of such supplies and equipment as rooting hormones, disinfectants, pots and rooting media, special timers, bottom heaters, mist beds, and temperature controlled greenhouses. Then