The Most Tricky Part of Micropropagation: Establishing Plants in Greenhouses and Fields©

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When they are first removed from the culture vessel, micropropagated plants and shoots are unique in that they are very poorly adapted for the growing conditions of a greenhouse or field. While in culture, these shoots develop under low light, in a sealed glass or plastic container that provides approximately 100% relative humidity, limited gas exchange, and an environment free from microorganisms.

Propagators are familiar with growing bedding plants and rooting softwood and hardwood stem cuttings under intermittent mist, fog, or high humidity. These plants require hardening-off prior to transplanting to ensure good survival. The same logic holds for micropropagated plants; however, they are even more sensitive than plants that are propagated by more traditional methods.

Cuttings are weaned from the propagation bench by gradually reducing the frequency and duration of misting, or by slowing exposing them to lower relative humidity levels. Bedding plants are hardened off by reducing watering, withholding most fertilizers, and lowering the temperature. The result is tougher plants that will survive transplanting and grow and thrive in the greenhouse or field. The same is true for plants that are micropropagated; however, they are smaller and more tender than cutting or seed-propagated plants and, therefore, the risk of loss is greater.

STAGES OF MICROPROPAGATION

To understand better the plant or shoot that is removed from culture, it is useful to know their general history. Although micropropagation is a continuous cycle, there are different objectives for each part of the process. Initially, small pieces of the plant, such as shoot tips or nodes are removed from a stock plant, microorganisms are killed on the surface of the explants using a dilute bleach solution, and they are placed in an aseptic environment inside a culture tube or other vessel. The objective during this stage is to achieve new growth that is adapted to the in vitro environment. This is known as Stage I, explant establishment.

Stage I gradually moves into the next phase. Branching is induced because of the medium formulation and the inclusion of cytokinins. It is important that the new shoots arise as “normal” branches from the axils of leaves, just like the branching on a stock plant from which stem cuttings are collected. Lab managers try to keep the cytokinin level sufficiently low so that all shoots are branches, rather than adventitious shoots growing from callus. Adventitious shoots often develop into plants that are different from the original stock plant and that is not desirable in a clone. This phase of multiplication of axillary shoots is known as Stage II, shoot proliferation. This stage can be cycled almost indefinitely to bulk up the number of shoots to meet demand.

When sufficient shoots are produced, they are cut and rooted in a manner somewhat similar to rooting “normal” cuttings. Rooting can be done in vitro in a culture medium that is frequently supplemented with an auxin, such as indolebu-

tyric acid (IBA). Alternately, rooting can be in a greenhouse rooting medium under high relative humidity in a propagation area. If rooting is done in this "ex vitro" manner, both rooting and acclimatization can be combined. This pretransplant phase where rooting is the primary objective is known as Stage III.

Although Stages I to III can be tricky and labor intensive, getting the micropropagated plants to survive and grow outside of the culture vessel can be the most challenging and may result in the greatest losses of plant material. The process of gradually conditioning the tender microplants to the ambient environment is know as acclimatization (the term acclimation is what happens in nature as plants acclimate; the word acclimatization means that humans have interceded). Stage IV is the acclimatization stage.

Growers can purchase microshoots that have not yet been rooted (Stage II microshoots), Stage III nonacclimatized microplants, or Stage IV plants. The earlier the stage, the less expensive the units. Prices are often reduced because the micropropagation labs do not have to put as much time and resources into production. That burden gets shifted to the grower. Stage IV microplants are acclimatized and can be handled and transplanted the same as any liner. Acclimatization is the focus with Stage III microplants and rooting combined with acclimatization must be accomplished with Stage II microshoots.

MICROORGANISMS

While growing in vitro, the plants are in an environment that is free from most or all microorganisms. Microorganisms flourish in the greenhouse and field. The majority of microbes are beneficial and only a few cause plant diseases. If normal sanitation procedures for a propagation facility are followed, losses from disease should be minimal during acclimatization.

Inoculation with beneficial microorganisms can increase acclimatization success and improve microplant growth and health (Cordier et al., 2000). Typically, microplants are planted in a soilless medium that has a relatively small microbial population. In fact fungicidal drenches are often used to keep these populations low.

A promising approach is inoculation with mycorrhizal fungi during the acclimatization phase (Cordier et al., 2000). Starrett et al. (2001) placed rooted *Pieris floribunda* microshoots in vials that had been previously inoculated with the mycorrhizal fungus *Hymenoscyphus ericae*. The plantlets remained on this medium for 1 month and were then gradually acclimatized in a greenhouse. Inoculation resulted in greater shoot growth and plant survival after 3 months in the greenhouse. Survival increased from 16% in the controls to 42% in the inoculated plants. While this is a considerable improvement in survival, it underscores the challenges of acclimatization since fewer than half of the inoculated plants survived.

WATER STRESS

The leaves that are formed in vitro have two distinctive characteristics. They generally have less epicuticular wax on their surfaces and the stomata function poorly (Preece and Sutter, 1991). Consequently they have a poor water retention capacity (De Klerk, 2000) and when initially removed from the culture vessel, shoots and plants tend to wilt. This appears to account for most of the losses of micropropagated plants during acclimatization. Therefore, to be successful with acclimatization, high relative humidity conditions are necessary during the early

phases of establishment outside of the culture vessel. Relative humidity can be gradually reduced when new roots and leaves form on micropropagated plants.

Gilly et al. (1997) found that when micropropagated plants were acclimatized, the cuticle that developed on the new leaves that formed under ex vitro conditions was thicker than what they measured on young leaves that developed in vitro. The new ex vitro environmental conditions, especially the lower relative humidity, progressively activated cuticle biosynthesis on these new leaves. However, they also reported that there was a decrease in cuticle biosynthesis (compared to leaves that remained in culture) when older leaves that formed in vitro are removed from culture conditions. That means that the new leaves that form during acclimatization are important and that the leaves that formed in vitro do not adapt well to the ex vitro conditions.

It is often reported that the stomatal apparatus that is so important for controlling transpirational water loss does not function well on the leaves of plantlets taken out of culture (Pospisilova et al. 1999). There are exceptions to this depending on species (Shackel et al., 1990).

Ticha et al. (1999) found that on tobacco, stomatal density decreased on both leaf sides on leaves that formed during acclimatization and that stomatal size increased compared to in vitro leaves. However, others have reported the reverse during acclimatization (Pospisilova et al. 1999). More dense stomata is an indication of increased transpirational water loss from plants.

The poor water retention capacity of micropropagated shoots can be worse if they do not take up water readily. De Klerk (2000) reported that there was a correlation between root number and length at the time of removal from culture and ex vitro growth and plantlet performance. This appears to be because these roots can replace water that is lost from the in-vitro-formed leaves. However, it is possible to root and acclimatize plantlets simulataneously.

PHOTOSYNTHESIS.

Most plants that are micropropagated rely on sugar that is added to the medium, rather than photosynthesis. There tends to be little net photosynthesis when plantlets are first removed from culture, rather, as new leaves develop, the net photosynthetic rate increases (Pospisilova et al., 1999). It is, therefore, probably best to acclimatize plants under shade or low light initially to avoid problems with high light destroying the chlorophyll in the in-vitro-formed leaves. When new leaves form ex vitro, shade can be gradually removed.

PLANTLET QUALITY

The quality of the shoot that is produced in vitro can have a profound influence on success of acclimatization (Debergh et al., 2000). If the plant growth regular balance is not optimal in culture, shoots can have multiple apices and result in excessively branched plants that acclimatize poorly. An even worse problem is hyperhydricity (formally known as vitrification). These shoots are abnormal and have a glassy, water-soaked appearance. They do not establish well under greenhouse or field conditions. There is evidence that when the hormonal inhibitor, abscisic acid (ABA) is added to the medium, there is less transplant shock (Pospisilova et al., 1999). Therefore, the laboratory that produces the plants can have a major influence on success of acclimatization.

ACCLIMATIZATION PROCEDURES

Acclimatizing Stage III plantlets (rooted microshoots) is often more successful than Stage II nonrooted microshoots, which must be rooted and acclimatized at the same time. The presence of roots on the State III plantlets can help the water relations of the plantlet, thereby increasing acclimatization success.

It is important for new leaves to form on microshoots during acclimatization. These new leaves have a lower transpiration rate and higher net photosynthesis than in-vitro-produced leaves. Once these new leaves form, relative humidity can be gradually reduced and light gradually increased.

There are various facilities that are used for acclimatization. These range from high humidity tents or domes to intermittent mist to fog systems. Frequently the plantlets are shaded for a time. Supplementation of the atmosphere in the acclimatization area with CO_2 can both increase the rate of photosynthesis and the efficiency of water use (Pospisilova et al., 1999). Research looks promising for the inoculation of micropropagated plantlets with mycorrhizal fungi during the early stages of acclimatization.

Losses of micropropagated plants during acclimatization can be reduced by very gradually changing the environment as new leaves and roots emerge on the plants. The length of time for this process varies with the species, the in vitro history and quality of the plantlets, and the environmental conditions in the acclimatization facility.

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