Novel Methods in Micropropagation

John E. Preece
Dept. of Plant, Soil & General Agriculture
Southern Illinois University
Carbondale, IL 62901-4415, USA
jpreece@siu.edu

Paul E. Read
Dept. of Agronomy and Horticulture
University of Nebraska
Lincoln, NE 68583-0724, USA
pread@unl.edu

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Abstract

The focus of this paper is on two novel methods for micropropagation, forcing softwood shoots from large stem sections and encapsulation of buds and shoot tips for storage and facilitation of Stage IV, acclimatization. Neither method is being used widely by commercial micropropagation laboratories. Branches are excised from within the cone of juvenility of woody plants that were originally propagated from seed. The branches are cut into sections 30-50 cm long and placed horizontally in flats or benches filled with perlite. Irrigation must be done with care to avoid water contacting the stem segments or new softwood shoots. The softwood shoots are excised, surface disinfested and used as explants. If the softwood shoots are to be used as macrocuttings, forcing is best under intermittent mist. However, forcing in mist will result in microbial contamination if micropropagation is the goal. Alginate gel is used to encapsulate somatic embryos to produce synthetic seeds. The same gel matrix can be used to encapsulate buds and shoot tips from Stage II proliferating cultures. The encapsulated buds can be stored under refrigerated conditions or dehydrated and placed in liquid nitrogen. Additionally, shoots and roots can grow from the encapsulated shoot tips and buds and these can be transplanted to a greenhouse medium for acclimatization. Because of the nutrient salts and sucrose in the gel matrix, the incorporation of fungicides into the gel has reduced fungal growth during Stage IV and has increased the percent survival.

INTRODUCTION

Plants have been micropropagated commercially for decades. Many of the laboratories that are currently producing plants for production are successful and have developed their protocols based on past research. However, there are some techniques that are currently being developed that have the potential to impact commercial micropropagation in a positive manner. Breakthroughs are largely in the area of woody plant micropropagation, but may also be utilized in the micropropagation of herbaceous species.

The focus of this paper is on two of these newer techniques, forcing softwood shoots from large stem segments and encapsulation. Neither technique is in widespread use in commercial micropropagation laboratories, but both appear to have great potential and may become adopted by labs in the near future.

JUVENILITY

Plants in the juvenile phase of growth generally are easier to propagate vegetatively than plants that have reached the adult phase (Preece, 2002). Cuttings taken from adult shoots of plants can be rooted, but the frequency of success is low, especially with woody plants. Likewise, researchers have had great challenges when attempting to micropropagate adult forms of many woody species. In fact, the change from the juvenile to adult phase is considered to be the most serious constraint to rooting cuttings (Howard, 1990) and micropropagation of shrubs and trees. Most of the difficulty experienced in rooting mature shoots seems to be caused by their altered physiology, but can also be related to greater contamination with microorganisms and viruses than with juvenile forms.

Juvenile explants are usually most readily established in vitro, grow and proliferate at a more rapid rate, and are more rootable than adult material. This is especially true with tree species where micropropagation of adult material is often difficult. Explants from mature shoots more frequently produce dark, phenolic exudates in vitro and often suffer necrosis than juvenile-source explants (Hanus and Rohr, 1987). Although seedling shoot and embryonic axis explants grew well on the same medium formulation, we observed shoot tip explant death within a few hours for adult Juglans nigra (eastern black walnut) (Preece and Van Sambeek, unpublished). It was only by changing the medium and culture conditions that we have been able to maintain adult J. nigra shoot cultures for years (Pearson and Preece, unpublished). We still cannot root adult origin microshoots of black walnut, although juvenile origin microshoots of black walnut can be rooted (Heile-Sudholt et al., 1986).

When breeding and selecting new, superior plants for clonal propagation, it is usually necessary to wait until the plant reaches maturity for full evaluation of the phenotype. This allows for assessment of important features, such as ultimate form and size, flowering and fruiting characteristics, autumnal coloration, and other traits. At the point that the mature phenotype is known, the plant is an adult and often becomes difficult to propagate clonally.

Cone of Juvenility

The majority of juvenile growth occurs when a plant is young and still exhibiting juvenile characteristics. As a plant ages, the older and lower parts retain their juvenile traits and the adult phenotype appears on the newest growth. On a tree, the trunk and lower branches that formed when the tree was young will retain their juvenile characteristics. Once the tree is older, the newest branches that form exhibit adult traits. Some temperate tree species, such as Quercus (oak), Fagus (beech), and some Acer (maple) retain their leaves throughout the winter as a juvenile characteristic. Viewing mature specimens of these species during the winter and focusing on the portion still holding its leaves, allows visualization of the shape of the tree when it was young and juvenile. This older, but more juvenile portion of the plant is called the "cone of juvenility".

A challenge is to be able to collect explants from within the cone of juvenilty. If water sprouts have formed from the lower stem, or suckers have grown from the roots on a large woody plant grown outdoors, they are frequently difficult or impossible to surface disinfest, but they can be harvested during the dormant season and forced in solution indoors. When such juvenile shoots are not available, people have even gone to the extreme measure of cutting down a tree to encourage juvenile shoots to grow from the stump or roots. When this is done, propagules often respond as well as juvenile cuttings or explants.

SHOOT FORCING

When practical, it is best to grow stock plants in a greenhouse or other controlled environment where they are isolated from the wind and rain. When outdoors, plants have microflora growing on them that are difficult to remove using surface disinfestation techniques. These microbes are often in tiny cracks and crevasses and the disinfestant may not penetrate these areas. A problem is that many woody species are either too large or are growing in the ground and cannot be moved easily to a greenhouse or growth room. Therefore, it is often necessary to remove parts of the plants such as branch tips or segments, usually during the dormant season, and force new shoot growth on them indoors or in a greenhouse environment. This new growth can then be excised, successfully surface disinfested and used for explant material. Although species specific, these explants tend to be relatively contaminant-free, they may also be more readily established in culture than explants taken during spring and summer, and can be less prone to produce polyphenolic exudates.

We have been conducting research on forcing shoots for years. Much of the focus has been on shoot tips harvested from trees and shrubs during the dormant season. Commonly, 20-25 cm long stems (Read and Yang, 1991) are harvested from deciduous trees and shrubs and then soaked for 15 min in a 0.78% NaClO solution with Tween 20 (Read and Yang, 1988, 1991; Yang and Read, 1992, 1993). Following the bleach treatment, a fresh cut is made at the base. The lower, cut ends of the stems are placed in containers with water, 200 mg l-2 8-hydroxyquinoline citrate (8-HQC) (Read and Yang, 1987), sucrose and sometimes plant growth regulators. Yang and Read (1992) reported that faster bud break and more bud and shoot elongation was promoted if the stems received the 15 min soak in bleach solution prior to forcing compared to those that did not.

A related technique is forcing terminal shoots in a greenhouse medium under greenhouse or other controlled environment. Onay (2000) harvested 3-4 cm long terminal, lignified stem sections from 30 year-old pistachio trees and, immersed the cut ends in plant growth regulator solutions, before placing them in a greenhouse medium. New, softwood shoots were forced in the greenhouse under a 24 hour photoperiod until they were sufficiently large to excise, surface disinfest and place in vitro.

Because these are excised from the tips of branches, the most adult portion of a plant, they can prove to be somewhat difficult to propagate vegetatively. A solution to this can be the use of plant growth regulators applied to the cuttings before placement in a greenhouse medium or by incorporation into the forcing solution. For example, in vitro shoot proliferation from shoot tips of Philadelphus and Dirca was increased by introducing BA into the forcing solution (Read and Yang, 1987). Yang and Read (1993) also found that more softwood explants from forced 'Vanhoutte's' spirea stems produced shoots or more shoots were produced per explant if the forcing solution contained 44.4 µM compared to 4.4 µM BA. However, as GA3 concentration in the forcing solution increased from 2.9 to 145 µM, the number of explants that produced shoots decreased. When GA3 was in the forcing solution of American chestnut or Aesculus, there was no effect on in vitro performance of shoot explants. Therefore, the effects of plant growth regulators in the forcing solution are at least somewhat species and plant growth regulator specific.

Forcing Large Stem Segments

Large branches can be excised from the juvenile portion of trees and shrubs, placed on a suitable medium in the greenhouse or other controlled environment and softwood shoots will elongate from epicormic or latent buds (Henry and Preece, 1997a,b; Van Sambeek et al., 1997a,b; Van Sambeek and Preece, 1999; Vieitez et al., 1994). This technique is very different from forcing shoot tips because large stem segments can be used and no forcing solution is used. Stem diameter is less important than stem length (Henry and Preece, 1997b), and segments must be cut to at least 30 cm long to enhance number of softwood shoots that will elongate. We usually use 40-50 cm long segments with calipers ranging from less than 1 cm to more than 80 cm, depending on the species and availability of plant material.

If the stem segments are excised from within the cone of juvenility, it is expected that the new, softwood shoots will have juvenile characteristics, especially with reference to propagation. This technique can have advantages over forcing branch tips not only because of the juvenility factor, but some species elongate poorly from stem tips, including Juglans nigra (Khan and Preece, unpublished). Larger stem segments have more stored food than shoot tips and that may contribute to shoot growth.

Softwood shoots are forced from the stems by placing them horizontally in a bed or flat of perlite. Other media can be effective, but none that we have tested is better than perlite.

The best forcing environment that we have tested has been under intermittent mist (Van Sambeek and Preece, 1999). However, if explants are to be excised for in vitro purposes, intermittent mist is not acceptable because it results in high microbial contamination. Rather, for in vitro use, irrigation must be such that water does not contact the new shoots. Then it is possible to establish explants aseptically (Van Sambeek et al., 1997a,b; Vieitez et al., 1994).

To minimize microbial contamination, watering can be done by hand, providing care is taken to avoid water contact with the large stem segments and new elongating shoots. Watering can be automated by use of drip or soaker hoses. Care must be taken to ensure that there is no spray coming from the irrigation drip or soaker hoses, or there will be excessive microbial contamination of explants. A solution that we have used is to bury soaker hoses in the perlite medium.

Forcing in partial shade, especially when mist is not used, often results in new softwood shoots that appear to be of higher quality than when grown in full sunlight. However, when we have compared full sun to shade in controlled experiments, we have seen no differences in shoot number and length between those in shade and those in full sunlight. Shoot quality is difficult to quantify.

To reduce irrigations and any splash that would contribute to microbial contamination on the new softwood shoots, we conducted a study on eastern black walnut and Acer saccharum (sugar maple) where we compared shade to no shade and flooding the shoots to watering by hand twice per day. Flooding of shoots meant that the level of the water covered no more than half the diameter of the horizontal stem segments. The shade had no effect, as mentioned above and all segments of both species that were flooded died, therefore stems of these species will not tolerate flooding (Connolly et al., 1998).

The species that appears the most adaptable to large stem forcing is Hydrangea quercifolia (oakleaf hydrangea) (Preece et al., 2001). It has exfoliating bark that peels away to reveal green bark underneath. Stem segments of this species will continue to produce softwood shoots for up to six months when maintained under intermittent mist. They also can be forced using soaker hose irrigation and explants from the softwood shoots can be used as explants. If used for rooting, compared to similar softwood shoots collected from plants grown outdoors, forced shoots root in a higher percentage and produce more roots.

Although large stem segments are not as easy to pulse with plant growth regulators as excised shoot tips that are forced using a solution, the large stem segments offer several advantages over the shoot tips. As mentioned above, the larger sized branch segments have more stored food than small stem tips and therefore softwood shoots are often more vigorous from the large stem segments. Additionally, large stem segments can be harvested from within the cone of juvenility, which can facilitate vegetative propagation. Large stem segments can be forced over an extended period of time. When tested each month over a 12 month period, eastern black walnut branch segments produced softwood shoots when collected in Illinois, USA from March, when the chilling requirements had been met through September, after which dormancy had initiated on the latent buds (Van Sambeek et al., 1997b).

ENCAPSULATION OF BUDS AND SHOOT TIPS

Artificial or synthetic seed technology has been studied for several years. The technique involves being able to encapsulate and use somatic embryos as clonal seeds for production of economically important plants. Encapsulation is essential to the process for storage of the somatic embryos and protection once they are sown in the soil.

By definition, seeds contain embryos. Although the term synthetic seeds has been applied to nonembryonic organs and tissues that are encapsulated, because they do not contain an embryo like true seeds, referring to them as "seeds" may lead to some confusion and not be the most appropriate term. Therefore, in this paper, synthetic seeds will refer to encapsulated embryos and the term "encapsulated buds" will describe axillary and apical buds that are encased in a calcium sodium alginate complex.

This technology is not new, rather, it has been reported on since the mid-1980s. However, it is novel because of the limited number of reports on encapsulation of buds and the general lack of use of this technology by commercial micropropagation laboratories.

There appear to be two important applications of this technology to micropropagation: storage and acclimatization to the greenhouse or field. Many commercial labs maintain Stage II proliferating shoot cultures during times of the year when they are not in the production phase of actively producing plants. This requires considerable time and labor expenses and increases the risk of loss of cultures to microbial contamination. Methods to store cultures, shoots, or buds for extended periods would add considerable efficiency to labs.

Losses experienced with acclimatization and establishment of micropropagated plants are great (Preece, 2001). The protection offered by encapsulation of buds has the potential to facilitate, and possibly automate acclimatization.

When buds are encapsulated in the alginate matrix, it also included the nutrient salts that are in the in vitro medium, along with sucrose (Bapat and Rao, 1990). When going to a nonsterile environment, such as soil, microbial contamination has been a problem. Incorporation of fungicides into the alginate matrix increased the formation of plantlets from encapsulated Morus indica (mulberry) buds, thus facilitating acclimatization (Bapat and Rao, 1990).

Ganapathi et al. (1992) encapsulated banana shoot tips in an alginate gel matrix containing an antibiotic mixture and activated charcoal. Shoots and roots elongated from the matrix in vitro on different substrates, including moist cotton followed by filter paper. When placed on soil, complete plantlets did not form. Plantlets that grew from the matrix on a gelled medium in vitro were successfully transplanted to soil and acclimatized.

Encapsulated apple shoot tips grew into plants at an 85% rate compared to only 25% for encapsulated axillary buds (Capuano et al., 1998). Rooting was enhanced when buds and shoot tips were treated with auxin prior to encapsulation. They were able to store the encapsulated shoot tips and axillary buds for 60 days at 4oC and this treatment reduced callus formation on the tips and buds.

Miaja et al. (2000) encapsulated axillary buds of Vitis vinifera in an alginate matrix. They immersed them in increasing concentrations of sucrose and then dehydrated the beads. The dehydrated beads were placed in liquid nitrogen for at least one day. Following thawing at +40oC, a few of the buds grew.

Therefore, encapsulation of buds and shoot tips has the potential for refrigerated storage or for cryopreservation. Additionally, when fungicides are incorporated into the matrix, they have potential for use during Stage IV, acclimatization.

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