

SPECIAL–WORKSHOP

DESIGNING A MICROPROPAGATION SYSTEM: WORKSHOP PRESENTATIONS FROM THE 1998 SIVB CONGRESS ON IN VITRO BIOLOGY

Conveners: BARBARA M. REED, USDA-ARS, National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, Oregon 97333,
and MARY ANN LILA SMITH, 1021 Plant Science Laboratory, 1201 S. Dorner Drive MC 624, University of Illinois, Urbana,
Illinois 61801

Contributors: BRENT McCOWN, University of Wisconsin, ROBERT SKIRVIN, University of Illinois, ROBERTA H. SMITH, Texas
A&M University, and WAYNE A. MACKAY, Texas A&M University

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PREFACE

Development of a micropropagation system for a new species or cultivar can be a daunting task and hundreds of decisions are involved in even deciding where to start. Dozens of basal media, growth regulators, and supplemental nutrients are available for use. Environmental conditions to be considered include light intensity and quality, photoperiod, temperature, container, amount of medium, liquid vs. semisolid, gelling agent, and container closure. This workshop was designed to assist micropropagators in determining directions to take and general tissue culture procedures to use when initiating culture of a new genotype. These contributions are from researchers with substantial backgrounds and experience in micropropagation of recalcitrant or challenging plant types and have provided some key insights into the steps taken to successfully adapt the plants into continuous micropropagation systems.

First, McCown outlines a general protocol of steps to consider in implementing commercial micropropagation of specific plant types. He introduces a 'decision tool' approach to take when considering whether or not to tackle the commercial micropropagation of a new plant with no previous history in culture. As he explains, the growth characteristics a plant typically has in nature can make an enormous difference in the ease with which it can be adapted to plant microculture.

Skirvin outlines a series of interconnected strategies aimed at improving success in establishing contaminant-free plants in culture. He discusses pretreatment and growth conditions of source plants which may minimize contaminants, selection of explant pieces, disinfestation procedures, and improving sterile technique during transfer. He discusses basic procedures and plant characteristics to consider during the explant stage.

Smith introduces the particular challenges of bulb crop culture. She describes the rationale for why in vitro strategies are particularly useful for the bulbs of *Hippeastrum* and *Zephyranthes* which are difficult to propagate by conventional means. The most compelling challenge to the introduction of explants in vitro is the successful disinfestation of the bulbs, which due to their growth underground are difficult to surface disinfest. Smith describes a twin-bulb scaling technique that has proven particularly useful for these species, and compares a series of disinfestation tactics (gleaned from the literature and internet tissue culture list-serve discussions) to determine the most efficient approach to explant preparation.

Mackay's contribution deals with a specific group of plants that are also quite challenging to micropropagate, but for unique reasons. The native species of the Chihuahuan desert are adapted to low rainfall, and due to the erratic conditions in their native habitat, are not routinely available for explant collection every yr. When they are available, the window of opportunity for collecting explants in correct physiological status is quite narrow. This group of native species is also characterized by problems with excessive exudates and is particularly difficult to acclimatize to ex vitro conditions. Mackay describes a series of procedural steps taken to successfully adapt the plant materials to in vitro culture and subsequently to make them available for use as adaptable native landscape plants for the region.

Barbara M. Reed
Mary Ann Lila Smith

WORKSHOP ON MICROPROPAGATION
A GENERAL APPROACH FOR DEVELOPING A COMMERCIAL
MICROPROPAGATION SYSTEM

B. H. McCOWN¹ and D. D. McCOWN

Department of Horticulture, University of Wisconsin-Madison, Madison, Wisconsin 53706 and Knight Hollow Nursery, Inc., 3333 Atom Road, Middleton, Wisconsin 53562

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SUMMARY

Five distinct steps can be recognized in the establishment of a plant in a commercial micropropagation system, especially if the most utilized approach (shoot culture) is the focus. Failure at any one step can make the total system commercially unworkable. When one considers a plant without extensive previous history of microculture, the first step involves an analysis of the potential market (economic reality) as well as the plant's general growth habit (biological reality). For the latter, the general growth habit of the plant can provide valuable predictive information as to the potential ease of microculture. For example, plants showing indeterminant herbaceous growth (e.g., *Chrysanthemum*, *Solanum*, *Dieffenbachia*) or continuous woody seasonal growth (e.g., *Betula*, *Ulmus*, *Thuja*) are generally much more amenable to microculture than those that are determinant herbaceous (e.g., *Panix*, *Paeonia*) or episodic woody organisms (e.g., *Quercus*, *Pinus*). At times, an episodic habitat can be overcome in microculture (*Syringa*, *Rhododendron*). The next four steps involve the actual manipulation and microculture of the plant and include the initiation, stabilization, optimization, and production phases. The most intensive analytical step is usually the optimization phase in which plant growth regulator response curves, replication, repetition through multiple subcultures, and evaluation of productivity and product quality are involved. The intent of this discussion is to help develop a decision tool to be used as a first approach to designing a potential new micropropagation system for an untested plant genotype.

Key words: shoot culture; determinant growth; episodic growth; recalcitrant; stabilization.

INTRODUCTION

Introducing a new plant to microculture requires decisions on a complex array of issues, especially if neither the plant nor any closely related allies have any history of use in micropropagation. On a research scale, many different techniques can be explored, and usually a protocol can be identified that will be adequate on a small scale. However, on a commercial scale, time and resources for general exploration are usually much more limited, and the final result will be judged by productivity and economic factors. Ideally, our past experiences should be useful in our selecting the potentially most successful approaches.

HERBACEOUS PLANT

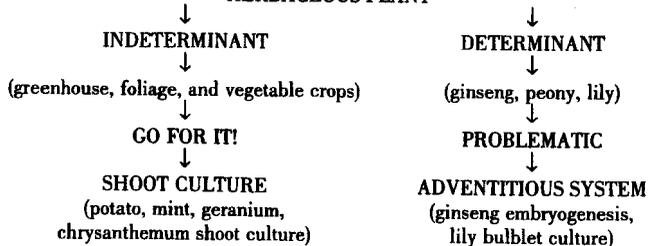


FIG. 1. A schematic depicting a logical process for discriminating alternative approaches for micropropagating a plant that is primarily herbaceous in character.

DISCUSSION

Establishment. Five distinct steps can be recognized in the establishment of a plant in a commercial micropropagation system, especially if the most utilized approach (shoot culture) is the focus: (1) Analysis of market potential, (2) Isolation of plant material under in vitro conditions, (3) Stabilization of shoot growth, (4) Optimization of multiplication/quality of microshoots, and (5) Rooting and ex vitro acclimatization of microtransplants.

Failure at any one step can make the total system commercially unfeasible. Much detail has been written on each of these steps, especially steps 2 through 5, for a myriad of plants (for example, see Debergh and Zimmerman, 1991; George, 1996). We do not intend to

WOODY PLANT

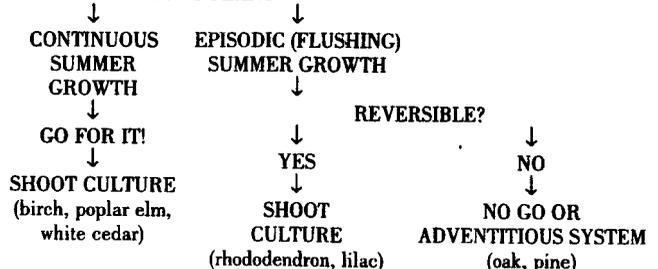


FIG. 2. A schematic depicting a logical process for discriminating alternative approaches for micropropagating a plant that is primarily woody in character.

¹To whom correspondence should be addressed.

summarize this information. However, after decades of experience in both the academic and commercial aspects of microculture, can we generalize to develop a logical decision structure that can be useful in answering the questions 'Should micropropagation be used?' and if so, 'What approaches might be most successful?' Development of such a decision tool is the overall purpose of this paper.

Analysis of market potential. When considering a plant without extensive previous history of microculture, the analysis must include both a consideration of the potential market (economic reality) and the plant's general potential to perform well under microculture conditions (biological reality). For micropropagation to be an economic success, a demand for large numbers of propagules is essential in most cases. In addition, the plant has to be valuable enough to justify the usually higher price of micropropagules. Having a high demand for higher priced units is often not reality, and thus in such cases, further consideration of micropropagation as a source of propagules should stop at this stage.

If the market situation seems favorable, then a more complex analysis of the potential feasibility of being able to supply high quality micropropagules begins. The general growth habit of the plant can provide valuable predictive information as to the potential ease of microculture. One scheme of analysis is represented in Figs. 1 and 2. The starting point is whether the plant is primarily herbaceous or woody in its growth character. If herbaceous, either annual or perennial, then the type of general shoot growth habit becomes important (Fig. 1). Herbaceous plants that are highly determinant in character (e.g., ginseng, peony) have been notoriously more difficult to handle in microculture as shoot cultures than have indeterminate plants (e.g., chrysanthemum, potato, mint, syngonium). Determinant herbaceous plants may be readily established in culture; however, they often maintain the determinant shoot character and thus cannot be stabilized as predictable and continuously growing shoot cultures. Probably the only commercial recourse for such plants is to multiply them with a true tissue/organ culture system (e.g., callus or embryogenesis) whereby shoots are generated on a one-time basis only (not subcultured as shoots) and rapidly moved into the *ex vitro* stages. As an example, this approach is now being applied to ginseng for which somatic embryogenesis is used.

If the plant under consideration is primarily woody (maintains permanent above-ground stems over multiple growing seasons), a parallel analysis is useful (Fig. 2). Woody plants that have a continuous shoot growth pattern during the growing season (e.g., birch, *Ficus*, blueberry, azalea, white cedar) are usually much more readily established as shoot cultures than are woody plants displaying episodic or flushing seasonal growth (oaks, lilac, *Rhododendron*, pines). As with determinant herbs, the episodic shoot habit leads to unpredictable growth *in vitro* which cannot sustain commercial production of high-quality micropropagules.

A redeeming feature of some episodic woody plants is that the shoot growth pattern is usually entrained in the plants when they are in a more adult phase of their life cycle. Thus, seedlings and root/collar suckers of rhododendron and lilac, for example, when grown under vegetatively invigorating conditions, will have predominantly

continuous shoot growth; however, more established or stressed plants will be highly episodic. What this means is that the episodic character of such plants may be reversible, either by stock plant manipulation or by continuous subculture *in vitro*, and thus quality shoot cultures can be generated.

As with recalcitrant herbaceous plants, an alternative micropropagation approach for woody episodic plants that cannot be stimulated into continuous seasonal shoot growth (e.g., pines, spruce, most oaks) is to resort to adventitious systems. This approach has been quite successful with spruce, loblolly pine, and Douglas fir.

Problems with somaclonal variation. Three alternative directions in developing an adventitious system may be appropriate for commercial scales: (1) organ culture (e.g., cotyledon, root, bulb scales), (2) somatic embryogenesis, and (3) nodule culture. George (1993) reviewed in detail these and other strategies. A major consideration in using an adventitious system is the potential of recovering unusually high numbers of genetic variants (somaclonal variation). In a commercial setting, this threat is often serious enough to eliminate any further consideration of micropropagation as a cloning method. This is especially true for suspension/callus-based systems which seem to generate the highest incidences of somaclonal variation in recovered propagules. Interestingly, somatic embryogenesis appears to not be as susceptible to such problems.

Somaclonal variation can also be an occurrence in shoot cultures that have been maintained by stimulation of axillary bud growth. One major source of such genetic problems, however, is the inadvertent occurrence of adventitious bud formation within the shoot cultures. Such can often be the case in situations in which cytokinin levels are maximized to maintain maximum axillary shoot proliferation.

Optimization of cytokinins. An absolutely necessary but often neglected step in the optimization of shoot cultures is the definition of a complete cytokinin response curve. In a commercial setting, it is important to know the levels of exogenous cytokinin that produce (1) only axillary buds, (2) a combination of axillary and adventitious buds, and (3) toxicity symptoms in the shoot growth. Such information is not only critical in controlling micropropagule quality but can be very influential in legal cases in which a purchaser is claiming genetic aberrations as the cause of problems in the production of a crop originally propagated by micropropagation.

CONCLUSIONS

Micropropagation is like many other horticultural undertakings in that it is a complex blend of science and art. Understanding the biology of the plant being propagated can help in many ways to discriminate the most logical approach to pursue. However, the artful application of these scientific concepts is still, and probably will always remain, a major factor in determining success.

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WORKSHOP ON MICROPROPAGATION

ESTABLISHMENT OF CONTAMINANT-FREE PERENNIAL PLANTS IN VITRO

R. M. SKIRVIN,² S. MOTOIKE, M. A. NORTON, M. OZGUR,³ K. AL-JUBOORY, AND O. M. McMEANS

University of Illinois, College of Agricultural, Consumer, and Environmental Sciences, Department of Natural Resources and Environmental Sciences, 258 ERML, 1201 W. Gregory, Urbana Illinois 61801 (R. M. S., S. M., M. A. N., M. O., K. A.-J.) and Department of Plant Pathology, Physiology, and Weed Science, 311 Fralin Biotechnology Center, Blacksburg, Virginia 24061-0364 (O. M. M.)

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SUMMARY

Perennial plant tissue cultures are established by disinfecting field or greenhouse-grown plant parts and transferring them to sterile medium in vitro. Typically, shoots harvested from field or greenhouse-grown plants are placed in water, either to force growth from dormant branches or to maintain them until ready for explanting. In spite of extreme care, 90 to 100% contamination rates in newly established in vitro cultures are not unusual. Experience has identified several routine procedures that reduce contamination, such as minimizing the amount of time a stem cutting is maintained in water before being explanted, adjusting pH of the medium to a more acidic condition, and using pH neutralized bleach to sterilize instruments during subculture. Other methods to reduce contamination include establishing field-grown plants in a greenhouse where inoculum levels can be better controlled, trellising vining plants to get them off the soil, avoiding wetting foliage, and selecting vigorous explants that are not in contact with soil.

Key words: contamination; bacteria; fungi; endogenous contamination; disinfestation; sterile technique.

INTRODUCTION

The main contaminants that affect tissue-cultured plants are bacteria and fungi. These contaminants are common on plants in vitro but have devastating effects on in vitro plants. Because of the rich media used in tissue culture, explants are very susceptible to these microorganisms in vitro (Falkiner, 1990; Leifert and Waites, 1990).

Most woody plant tissue cultures are established from branches that are brought from the field or greenhouse and stored in water, either to force growth or hold them until they are ready for explanting. The parental branches often stay in water for one to several days. Storage water and its included microorganisms can be a source of some of the internal (endogenous) contamination observed in vitro (Skirvin et al., 1993). Such organisms can live or survive in the vascular tissue of plants, tropical plants in particular. These organisms may not initially be apparent, but may eventually begin to proliferate around the base of the explant and appear as a halo of contamination. This sort of contamination is often referred to as the "white ghost." In this paper we will discuss sources of contamination and then present methods used to control these contaminants in our laboratory.

DISCUSSION

Pretreatment to reduce contamination. In spite of hard work and careful attention to procedures, contamination can still result in 100% explant loss. Researchers can improve the likelihood of obtaining clean explants by rinsing fresh explants under running water for a few hours to several days to remove debris and clean the explant. The use of a detergent with or without shaking can also be useful. The explant itself is the main source of fungal and yeast contami-

nation. Spores or other reproductive structures can adhere to explants or be hidden beneath leaf scales or other projections. Hairy plants are a particular problem because bubbles of air become entrapped in the explant and prevent good contact with the disinfecting agent. In general, fungal and yeast contamination can be seen within the first 1 or 2 wk of culture. Subsequent contamination might develop in the laboratory environment during media manipulation after autoclaving and subculturing. Leifert et al. (1994) found a direct correlation between species and genera of yeast and fungi found in the laboratory environment and those that contaminated older, previously established plant tissue cultures, suggesting that the laboratory environment is another important source of contamination.

Select explants to minimize contamination. Soil and soil particles are major sources of contamination, and trailing plants that come into direct contact with the soil can be difficult to establish in vitro. Sweet potato (*Ipomoea batatas*) shoots, as an example, have proven extremely difficult to initiate in vitro due to contamination. To reduce the amount of inoculum adhering to greenhouse-grown plants, stock plants were trellised to keep their growing point off the benches and away from soil. When watering, care was taken to avoid wetting the foliage. In addition, plants were sprayed weekly with systemic fungicide (Benomyl®) to reduce fungal growth.

Low-growing plants such as *Haworthia*, *Gasteria*, and *Hosta* can be cultured from immature inflorescences. They appear to possess latent meristems which can yield whole plants directly. We have used this system to propagate *Hosta*. Some low-growing plants can be etiolated to increase the distance between internodes. This lifts the meristem further from the soil and increases the chances of successful establishment. Gibberellic acid sprays also can be used to elongate internodes and move the growing tip away from contaminant sources.

Plants grown from a crown which is in contact with the soil, such as strawberries, iris, *Hosta*, ginger, *Haworthia*, and many carnivorous

²To whom correspondence should be addressed.

³Present address: University of Uludag, Dept. of Horticulture, Gorukle-Bursa 16384 Turkey

plants, are difficult to clean. Crowns can be scrubbed and grown on sterile potting media such as perlite, watered from the bottom to avoid wetting the meristematic region, and sprayed weekly with fungicide. Crowns removed from these plants are stripped of their outer foliage, disinfected, and rinsed. Next, another layer of leaves is peeled away and the remainder is disinfected again, with the process repeated several times. In this manner, the meristem can be extracted without inoculating it with surface contaminants. In addition, these types of plants can be grown in pure unmilled sphagnum peat, which has antimicrobial properties due to its low pH, to further inhibit incidence of surface contaminants (personal communication, M. M. Meyer, University of Illinois).

Storage water as a source of contamination. Skirvin et al. (1993) demonstrated that storage water can be a source of endogenous contamination. Stems of carnation were placed in vases of water inoculated with an unidentified rifampicin-resistant bacterium that formed black colonies. Surface-disinfected carnation stems from inoculated water yielded black colonies of bacteria on rifampicin-infused medium. The bacteria moved about 5 cm in 24 h. Control plants developed no colonies. The carnation stems were believed to have taken up the marker bacterium from the water via the xylem stream. The bacteria became trapped within the explant. This might explain some of the latent bacterial contamination observed in vitro. Trapped bacteria might begin to grow at a later date and ooze from the cut end of the explant as a "white ghost." To reduce the rate of this latent bacterial contamination, explants should not be stored in water any longer than necessary. Because acidity inhibits the growth of some bacteria, adjusting the pH of the storage solution into the acidic range may reduce bacterial contamination in storage water. Another possibility is to use commercial flower-preserving powders, such as Flor-alife®, in the holding solutions. These products contain sugar, citric acid, and a bactericide, all of which are known to be important for preservation of cut flowers (Staby et al., 1978).

We have also found that adjusting the pH of the tissue culture medium to the acidic range (4.5 to 5.2 vs. 5.6 to 5.8) also reduces the incidence of bacterial contamination (McMeans, 1997). The mechanism of this effect is unknown. It may be that the acidic conditions of the tissue culture medium mask bacterial contaminants by inhibiting their growth.

Disinfesting explants. To initiate plant tissue cultures, the explants must be disinfected to rid them of surface microorganisms. Common disinfecting agents include bleach (sodium hypochlorite, NaOCl), ethanol, and hydrogen peroxide (H₂O₂). Mercuric chloride (HgCl₂) is also a useful disinfectant, but it is so potentially toxic that it is not recommended and careful disposal procedures should be in place if it is used.

1. Bleach. The most common disinfectant is bleach (NaOCl) which is usually purchased as a commercial product. In the U.S. Clorox® or Purex® are 5.25% active ingredient (AI) and are diluted with water (vol/vol) to a concentration of 5 to 20% (0.26 to 1.1% AI). Bleach is usually more effective when a surfactant such as Tween 20® is added at about 0.01%. Explants are immersed in bleach solution for 1 to 60 min or more, usually with agitation to increase its effectiveness. Before explanting, bleach must be rinsed from the explant with two to five rinses in sterile distilled water. Bleach is also a very effective disinfectant for work surfaces but is not very stable and strength can vary from bottle to bottle. Better success with surface disinfection has resulted after purchase of bleach in 1-qt bottles (vs. 1-gal bot-

ties), most likely because the bleach loses some of its potency once opened (personal communication, R. Smith, Texas A&M University).

Hairy plants are difficult to disinfect because the bleach does not come in direct contact with plant surfaces due to surface tension and trapped air bubbles. The use of a slight vacuum during disinfection will help remove air bubbles and ensure better contact with bleach. Although vacuum infiltration can improve contact with disinfectants, it can also increase the likelihood of explant necrosis.

Some plants are quite sensitive to sodium hypochlorite, but are less sensitive to calcium hypochlorite. Unfortunately, calcium hypochlorite is not very stable so it must be mixed immediately before use (10 g of calcium hypochlorite to 140 ml of water with stirring for 15 to 20 min) and it should be used within 6 to 7 h. Five to 30 min of contact is sufficient to clean most plants. This is the standard method to sterilize orchid seeds and fern spores. Bleach was removed from the seeds and spores by centrifugation.

Recently it was reported that pH-adjusted bleach is useful for sterilization (Constantine and Cohen, 1997). Commercial bleach is diluted and its pH adjusted to 7.0 with acid. Acid should not be added to undiluted bleach because it releases chlorine gas which can be deadly. pH-adjusted bleach is so strong that it kills grape seeds, but it makes an excellent sterilizing agent for tissue culture tools. We found a marked reduction in the incidence of "white ghost" bacteria in some of our long-term tissue cultures after using pH-adjusted bleach as a tool disinfectant.

2. Hydrogen peroxide (H₂O₂). Hydrogen peroxide is used in concentrations of 3 to 12% AI with surfactant for periods of 5 to 15 min, and, like bleach, fresh product has better efficacy. Because hydrogen peroxide breaks down to yield water and free radical oxygen (O⁻), it is not always necessary that peroxide be rinsed away from the explant but a high concentration or long exposure to peroxide can "bleach" a plant free of its chlorophyll.

3. Combination disinfection. In spite of extreme care, 90 to 100% contamination of field-grown material is not unusual. Hammerschlag (1980) reported such a problem in *Prunus*. She reduced the level of fungal and bacterial contamination to as little as 3% using the following protocol. During the winter, dormant but fully vernalized scion wood was gathered from parent trees and the bases were placed in water in a laboratory. When shoots began to emerge, they were removed, the outer leaves were removed from each shoot, and the shoot length was reduced to 0.5 to 1 cm. The shoots were then soaked in 10% bleach + 0.01% Tween 20 for 15–20 min. The shoots were then placed into a 100 ppm sterile antibiotic (penicillin–streptomycin) solution for 5 min and next rinsed three times in sterile distilled water. The young shoots were then transferred to solid or liquid medium where they were grown normally.

Protecting explants from contamination during transfer. Infection with bacteria of the genus *Bacillus* (which forms alcohol- and heat-resistant endospores) can be due to inefficient sterilization of instruments used to handle plants (Leifert and Waites, 1990). *Bacillus* strains, isolated from plant tissue cultures and tentatively identified as *Bacillus macerans*, survived alcohol dips (75 and 95% for up to 5 min) and flaming over an alcohol burner but were killed by flaming over a Bunsen burner (Kunneman and Faaij-Groenen, 1988). Heat-resistant species have also been reported to survive autoclaving. *Bacillus cereus* and *Bacillus circulans*, for instance, can survive autoclaving for 20 min at 120° C (Trick and Lingens, 1985). We have found that pH-adjusted bleach is useful to prevent the spread of these organisms from infected plants to healthy plants via contaminated

alcohol or tools. With good sterile technique, contamination can be reduced to an acceptable level.

CONCLUSIONS

There are many ways to control contamination *in vitro*. Once contaminant-free cultures have been initiated it is the responsibility of the investigator to maintain them contaminant-free (Reed and Tanprasert, 1995). Each culture vessel should be examined regularly and any suspicious cultures discarded. If there are no obvious signs of contamination but growth rates are declining, indexing may be in order. James and Thurbon (1978) recommend screening cultures by transferring sample plants onto enriched bacterial medium at 1-mo. intervals.

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WORKSHOP ON MICROPROPAGATION
CHALLENGES ASSOCIATED WITH MICROPROPAGATION OF *ZEPHYRANTHES* AND
HIPPESATRUM SP. (AMARYLLIDACEAE)

ROBERTA H. SMITH,⁴ JODY BURROWS, AND KRISTEN KURTEN

Department of Soil and Crop Sciences, Texas A&M University, College Station, Texas 77843

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SUMMARY

Conventional propagation of amaryllis, *Hippeastrum* Herbert sp. hybrids by bulb offsets is slow, seasonal, and variable; additionally, some amaryllis hybrids do not produce many offsets. From seed, it takes approximately 2 yr to flower. Micropropagation of *Zephyranthes* L. sp. bulbs has challenges related to contamination of stage I cultures as well as genotype differences in culture media requirements. There are literature reports on in vitro propagation of both genera; however, the application of these reports to new cultivars leaves unanswered questions regarding surface disinfestation, explant, nutrient media, and multiplication rates. Surface disinfestation of container-grown *Hippeastrum* spp. hybrid cv. San Antonio Rose bulbs resulted in contamination rates of 20 to 100% in spite of various treatments, some of which killed the explant. Twin scale explants of San Antonio Rose bulbs responded on a Murashige and Skoog salt medium with 2 mg naphthalene acetic acid per l, and transfer to soil was not a problem. In contrast, aseptically germinated seed of *Zephyranthes* sp. served as a suitable source of clean bulb tissue.

Key words: micropropagation; in vitro bulb culture; amaryllis; rain lilies; twin scale explant.

INTRODUCTION

Amaryllis (*Hippeastrum* Herbert sp.) and rain lilies (*Zephyranthes* L. sp.) are extremely variable in flower color and foliage, and both have tremendous value in both conventional and native ornamental landscaping. *Zephyranthes* also known as zephyr, rain, or fairy lily are small bulbous herbs usually with a solitary flower, and contain alkaloids with potential medicinal value. Since it can take 2 yr from seed for a bulb to flower, hybrids are extensively propagated from bulb divisions, and hybrids vary in the numbers of offsets produced in a growing season. Micropropagation with twin scale explants (an explant with two scale bases attached to the bulb at the basal plate) can provide two to four explants per bulb depending on the size of the bulb, and organogenesis from bulb and leaf explants can produce numerous plants (Hussey, 1975; Kim and DeHertogh, 1977; Seabrook and Cumming, 1977; Huang et al., 1990; O'Rourke et al., 1991; DeBruyn et al., 1992). Each twin scale can produce from one to five bulbs in 1 to 2 mo. in culture depending on the genotype.

The major challenge associated with tissue culture of bulbs is that of contamination at Stage I. The references on micropropagation of bulb plants often mention contamination as a problem. It becomes more acute when one is dealing with a rare hybrid, and there is not enough material to examine a variety of surface disinfestation parameters. The following is a description of some of the disinfestation procedures which were examined in establishment of Stage I cultures of a unique hybrid of amaryllis called 'San Antonio Rose' (provided by Mr. Steve Lowe of the San Antonio Zoological Garden). Additionally, some rare and unique rain lilies were collected from the eastern side of the Sierra Madre Oriental mountains of northeast Mexico (by Dr. J. Fairey and Carl Schoenfeld, for Yucca Do Nursery, Hempstead, Texas) and examined for micropropagation potential.

⁴To whom correspondence should be addressed.

MATERIALS AND METHODS

Amaryllis. Bulbs were either cut in cross sections composed of leaf, bulb top, middle, and base, or lengthwise into twin scale explants (explant with two scale bases attached to the bulb basal plate). Explants were cultured under a $24 \mu\text{mol s}^{-1}\text{m}^{-2}$ light intensity for 16 h daily at $26 \pm 3^\circ\text{C}$.

The culture medium reported by Seabrook and Cumming (1977) contained Murashige and Skoog (1962) inorganic salts, and in mg/l: 100 inositol, 0.5 nicotinic acid, 0.5 pyridoxine, 0.1 thiamine, 1 2,4-dichlorophenoxyacetic acid (2,4-D), 1 N⁶-benzyladenine (BA), 30 000 sucrose, and solidified by 1.5 g Gelrite per l at pH 5.5 was used for bulb cross sections. The medium of Hussey (1975) which contained the above salts, vitamins, and sucrose with 2 mg naphthalene acetic acid (NAA) instead of BA and 2,4-D was used for twin scale explants. Medium was poured into plastic petri dishes, 25 ml/plate, after being autoclaved.

A series of disinfestation treatments designed for the amaryllis bulbs was based on previous literature and on discussions from the Plant-tc listserve (<http://www.agro.agri.umn.edu/plant-tc/listserv/listsrvh.htm>). The following steps were taken: 1. Bulbs were trimmed to remove roots, leaves, and outer scales, washed in warm soapy water, and rinsed in warm, flowing water for 30 min. The bulbs were rinsed in ethanol for 1 min and placed in 20% bleach (Clorox, active ingredient 5.25%) for 20 min followed by three rinses in distilled water. The bulbs were left in petri dishes in the hood for 2 d followed by 20 min in 20% bleach and three sterile water rinses. Bulbs were trimmed and outer scales removed.

2. Ten trimmed bulbs were pretreated in running hot water. This heat treatment was reported to work in the Netherlands for onion bulbs to prevent fungal contamination. Bulbs were dried for 2 d and then placed in a large beaker with water maintained at 52°C for either 30 or 45 min. Bulbs were cooled and dried for 24 h and rinsed in 95% ethanol for 1 min, followed by 10% bleach for 30 min. The bulbs were rinsed three times with sterile water and trimmed of burned tissue; the outer scale was removed.

3. Sixteen bulbs were washed, trimmed, and placed in a desiccator for 24 h for a chlorine gas treatment. The chlorine gas was evolved from a 100-ml beaker of bleach to which 3 ml of concentrated HCl was added. The bulbs were then surface disinfested in 20% bleach for 20 min and rinsed three times in sterile water.

4. Plant Preservative Mixture (Plant Cell Technology, Inc., Washington, DC) (PPM) was tested to reduce contamination. Foliage, roots, and the outer bulb scales were removed, and bulbs were washed in warm soapy water, rinsed, put in 20% bleach for 20 min, rinsed three times with sterile water

and then placed in various treatments of PPM. Ten explants were placed in each of the following: 0 ml PPM for 0 or 6 h; 15 ml PPM per liter of medium for 6, 12, 24, or 36 h; 30 ml PPM per liter medium for 6, 12, 24, or 36 h. PPM was added to liquid MS medium with sucrose, MS vitamins, inositol, and 2 mg NAA per l before autoclaving. The explants with the liquid medium plus PPM were cultured in petri dishes on an orbital shaker. Following the PPM treatment, the explants were placed on the same basal medium as above with 2 ml PPM per l.

5. Trimmed bulbs were rinsed in 95% ethanol for 30 sec, rinsed, placed in 20% bleach for 20 min, and rinsed three times with sterile water. The outer scale was removed and the explants were trimmed. Before being placed on the culture medium, 30 explants were dipped in 1% bleach and rinsed in sterile water; 93 explants were not dipped in bleach before placement on the culture medium.

6. Bulbs were trimmed of roots, leaves and outer scales, washed in soapy water, and placed under running tap water for 2 1/2 h. The outer scale was removed and bulbs were washed in soapy water for 15 min, rinsed in 95% ethanol for 3 min, placed in 20% bleach for 30 min, and rinsed three times in sterile water.

7. Bulbs were trimmed again, then washed and rinsed in 20% bleach for 15 min. The outer scale was removed and bulbs were again placed in 20% bleach for 15 min. The outer scale was removed, followed by placement in 20% bleach for 10 min. This step was repeated. The next outer scale was removed, followed by placement in 2% bleach for 10 min and rinsed in sterile water three times.

8. Trimmed, washed bulbs were rinsed for 1 h under flowing tap water (34°C) followed by 95% ethanol rinse for 3 min and 20% bleach for 30 min.

Zephyranthes. Seed from four species of *Zephyranthes* was available. The seed was surface-sterilized for 2 min in 95% ethanol followed by 20% bleach for 15 min and rinsed three times with sterile water. Seeds were germinated on Murashige and Skoog inorganic salts with 30 g sucrose per l. Using the aseptically germinated plants, experiments with bulb scale explants and cross sections of the bulb and leaf tissue were conducted to look at media for bulb multiplication and adventitious regeneration.

RESULTS

Amaryllis. The first three treatments were entirely contaminated while other treatments had variable results (Table 1). Treatments 4 and 5 resulted in the lowest incidence of contamination.

Zephyranthes. There was no contamination of the aseptically germinated seed. The germination of the four species of *Zephyranthes* varied with 5/6 *Z. labuffarosea*, 0/29 *Z. primulina*, 20/26 *Z. quartaro* yellow, and 22/25 *Z. handuchif* seeds germinating. There has been some initial success in twin scale explants producing bulbs and embryogenic callus has also been obtained.

DISCUSSION

Surface disinfestation of amaryllis bulbs was highly variable. One source of variation is the different bulb shipments at different times of the yr, although most material was potted plant material from the greenhouse. The size of the bulb also seems to be a factor, with larger bulbs being harder to clean. Some bulb lots appear to be more contaminated than others. However, once a clean culture is established, in vitro material can be increased by twin scale explants. Once we obtained an embryogenic culture on Seabrook and Cumming's (1977) medium, hundreds of plants were obtained. Other disinfesting agents like hydrogen peroxide may be more efficient in combination with bleach.

TABLE 1

RESULTS OF SURFACE DISINFESTATION TREATMENTS FOR AMARYLLIS BULBS

Treatment number and treatment*	#		
	Explants	Contaminated	% Clean cultures
1. 2 × w/20% bleach	80	80	0
2. Hot water	40	40	0
3. Chlorine gas	63	63	0
4. 0 PPM ^b	20	16	80
4. 15 PPM 6 hr	10	0	100 (8 D; 2 NG ^c)
4. 15 PPM 12 hr	10	4	60 (5 D, 1 NG)
4. 15 PPM 24 hr	10	0	100 (10 D)
4. 15 PPM 36 hr	10	0	100 (10 D)
4. 30 PPM 6 hr	10	7	30 (3 NG)
4. 30 PPM 12 hr	10	7	30 (3 NG)
4. 30 PPM 24 hr	10	1	90 (9 D)
4. 30 PPM 36 hr	10	0	100 (10 D)
5. 95% EtOH + bleach dip	30	23	23
5. 95% EtOH dip + bleach dip	93	28	70
6. Soapy water + 95% EtOH	56	47	16
7. 3 × bleach, scales removed	79	71	10
8. Tap water + 95% EtOH + bleach	180	164	9

*Full descriptions of individual treatments are listed in materials and methods.

^bPlant preservative mixture.

^cD, dead.

^dNG, no growth.

The *Zephyranthes* seeds were much easier to work with than bulbs. Not a lot is known regarding how true to type seeds of this species are; however, some of the yellow flowering species remain true to type in flower color. Some of the pink flowering species grown from seed can vary from white to pale pink to dark pink. It takes 2 yr for a seed-derived plant to flower. The bulbs of this genus are very small and surface disinfestation was not an option due to the very limited supply of plants and the lack of information about bulb surface disinfestation. Once a clone of plants was established and multiplied in vitro, samples were grown to flowering to verify the color.

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WORKSHOP ON MICROPROPAGATION
MICROPROPAGATION SYSTEMS FOR THE MEXICAN REDBUD (*CERCIS CANADENSIS*
VAR. *MEXICANA* L.) AND OTHER WOODY PLANTS OF THE CHIHUAHUAN DESERT

WAYNE A. MACKAY

Texas A&M University, 17360 Coit Road, Dallas, Texas 75252-6599

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SUMMARY

There are many Chihuahuan desert species that have potential as landscape plants for the arid communities of the southwestern United States [agarita, *Berberis trifoliata* Moric.; Mexican buckeye, *Unghadia speciosa* Endl.; Texas madrone, *Arbutus xalapensis* var. *texana* (Buckl.) A. Gray]. Within these plant populations, there are superior genotypes that offer even greater interest for the landscape. However, it is difficult to clonally propagate many of these species with conventional techniques, and the seed-derived populations often do not breed true. Therefore, selection of superior genotypes in wild populations coupled with clonal propagation through tissue culture may offer an attractive option. It is relatively easy to achieve disinfection of explants from desert plants due to a general lack of natural surface contamination by fungi and bacteria, even though interference from numerous trichomes can impede good contact with disinfecting agents. However, there is only a narrow window of time that is ideal for explant collection, because of the brief, periodic flushes of growth that characterize this unusual plant group. There may be years when, due to the harsh environment, the amount of suitable explant material is severely limited. Phenolics and exudates are also problematic in this group of plants, and acclimatization of ex vitro plantlets to the harsh desert environment is a particular challenge. For these reasons, specific adaptations and modifications were necessary to achieve success with micropropagation of desert plant species such as Mexican redbud (*Cercis canadensis* var. *mexicana* L.).

Key words: micropropagation; woody plants; tissue culture; propagation; desert plants; *Berberis*.

INTRODUCTION

The Chihuahuan desert is the largest desert in North America, covering approximately 175,000 square miles. The desert encompasses far-west Texas, portions of southern New Mexico, and the southeastern corner of Arizona, with the majority of the desert located in northern Mexico. It is characterized by high elevations which in turn are related to the cold winter nights (the nighttime temperatures drop below freezing at least 100 d per yr). However, despite the relatively high elevations, temperatures are high during the summer. The Chihuahuan desert averages between 7 and 12 inches of rainfall per yr with evaporation rates of more than 100 inches per yr. Most of the rain occurs during the late summer and early fall.

Introduction of plants native to the Chihuahuan desert would provide a greater diversity of trees and shrubs adapted to the urban environments of far-west Texas and the greater southwest. These native desert species, placed in landscape settings, could replace ill-adapted nonnative species currently in commercial use. For instance, eastern redbuds (*Cercis canadensis* L.) are frequently planted in the southwest, but often suffer from leaf scorch, leaf cutter bees, and leaf spot, giving them an unattractive appearance following flowering. An alternative to the eastern redbud is the Mexican redbud (*Cercis canadensis* var. *mexicana*) whose range extends into the Big Bend region of Texas (Mackay et al., 1995). Mexican redbuds exhibit good flowering characteristics, but the smaller leaves are thicker with a waxy surface. Surveys of the native populations revealed variability for flower color and leaf morphology (degree of waviness to the leaf margin and the shininess of the leaf surface), which are two important ornamental characteristics. Conventional propagation research on Mexican redbud indicated that the window of time for rootability was

narrow. Given these characteristics, coupled with the previous successful work on the closely related eastern redbud, Mexican redbud can be considered an excellent candidate for micropropagation. To successfully micropropagate and introduce these desert-adapted plants, however, specific challenges related to explant collection, tissue disinfection, culture conditions, and acclimatization must be addressed.

DISCUSSION

Explant Collection

Working with plants in a desert environment provides both challenges and opportunities. Rainfall is especially unpredictable in the desert environment. The late summer and early fall rains are primarily the result of thunderstorms, so moisture is not uniformly distributed either spatially or temporally. Since thunderstorms do not provide uniform rainfall over large areas, even areas that are relatively close to each other may have quite different rainfall amounts and distribution. The effects of this type of rainfall pattern can be quite dramatic. For example, in the course of this study one population of Big Bend Bluebonnet (*Lupinus havardii* S. Wats.), a Chihuahuan desert annual species, has not recurred in a particular location for more than 7 yr. During that same time span and less than a mile away, another bluebonnet population completed its life cycle in three different yr.

Although the effects on woody species adapted to this area are not as dramatic as those on annual species, weather still has an effect on plant growth. Flushes of growth occur during the spring but the amount of growth may be much reduced in periods of prolonged

drought. In years of normal rainfall, there will often be a second flush of growth during the summer monsoon season, but only if rainfall occurs at the location of the desired plant. Since this is unpredictable, successful collection of actively growing shoot meristems during this time can be difficult. A second factor that affects the successful collection of propagules is the distance from population centers since they are few and far apart. This makes monitoring difficult for a particular plant. For example, the closest Mexican redbud (*Cercis canadensis* var. *mexicana*) populations are approximately a 5-h drive from El Paso, Texas, the nearest large airport. Periods of active growth are often short, and arriving at the plants' location during active meristematic growth requires multiple trips. The remoteness of desired plants also adds to the expenditure of time for identification of a superior genotype. Often a preliminary scouting trip to locate populations and determine flowering time is necessary before extensive surveys of the populations can be taken. However, this can be beneficial because multiple trips provide an opportunity to learn about the plant's ecology and identify some potential problems before the plants are introduced into the urban landscapes.

Once a superior genotype has been identified in the desert wilds, another potential roadblock is the minimal numbers of propagules of a particular clone that are available to establish cultures. When the candidate species is not closely related to previously cultured species, there is no prior work on which to build. Usually, screening of microenvironmental variables at this early stage will require a larger number of available explants. The obvious solution to this situation is to do some preliminary work on culture establishment using a more readily available tissue source such as greenhouse-grown seedlings. However, in some cases the experimental results using seedlings do not produce comparable results for a wild, selected mature tree.

Explant Disinfestation

The same factors that can create barriers to the identification and selection of superior genotypes and the collection of usable propagules also have some positive effects when one considers tissue culture as the method of propagation. The low rainfall, low relative humidity, low winter nighttime temperatures, and high daytime summer temperatures minimize the number of surface contaminants on the actively growing shoot meristems and make surface disinfestation relatively simple. There are relatively few internal contaminants in the rapidly growing tissue. As a result, a high percentage of the apical shoot meristems can be successfully disinfested.

At the same time, many desert plants have numerous trichomes on the leaf surfaces and stems which can make it difficult to get good contact between the disinfesting solution and the surface of the propagule. Often in these cases, rinsing the propagule with distilled water, followed by a 60-sec rinse in 70% ethyl alcohol (ETOH), followed by 12–15 min rinse in a 10% bleach solution (0.5% active ingredient) with a surfactant added results in successful disinfestation.

Culture Conditions

Lab facilities. In a desert environment, lab facilities need to be well isolated from the ambient environment. Since maximum summer temperatures often exceed 40° C and even 45° C, an independent cooling system for the culture room is essential to control culture temperature. A secondary cooling system is essential in the event

that the primary cooling system fails. Fluorescent light ballasts should be placed outside the culture room to minimize heat. Separate isolated heating/cooling systems can eliminate many problems with contamination, especially in the spring when dust storms are common and fine dust can infiltrate even tightly enclosed spaces. Other aides include door sweeps, an outer room entry, and good filters on the air exchange units.

Exudates. Phenolics and exudates are problematic during the micropropagation of many desert trees and shrubs, which produce copious amounts of exudates during the establishment phase. Frequent transfers for some species eliminated most of the exudates, and in other cases the propagules continue to produce exudates even in combination with antioxidants (citric acid), polyvinylpyrrolidone, or activated charcoal. In the case of *Berberis trifoliata*, the cultures continually produced small quantities of a yellow exudate (presumably berberine which is produced by the stems when cut) over a 3-yr culture period (Molinar et al., 1996). The influence of these exudates on the number of shoots produced has not been assessed.

Acclimatization

The high light intensity and low relative humidity (as low as 10%) of the desert creates a difficult environment for acclimatizing micropropagated plantlets. Stomates of in vitro plants are not fully functional, the epicuticular waxes are not fully formed, and desiccation can occur rapidly even in a shaded mist system in the greenhouse (Brainerd and Fuchigami, 1981). Ex vitro rooting experiments have not resulted in high rates of rooting and survival for the native desert introductions. A more successful rooting protocol included in vitro rooting followed by growth in culture vessels containing soilless medium in the laboratory. The vessels were sealed with plastic wrap and the wrap was progressively perforated each wk during plantlet growth to gradually acclimatize the plantlet to the ambient environment. Following several wk in the laboratory the plants were taken to the greenhouse, potted, and placed in a mist chamber covered with 70% shade. The shade was gradually removed during the next 7 d and the plants were then transferred to a greenhouse bench for further growth. When the plants reached sufficient size, they were transferred to a shade structure until placement in the field. In vitro-rooted Mexican redbud plantlets (150) acclimatized in this fashion had 98% survival following the initial transfer to the mist chamber. All surviving mist chamber plants also survived transfer to the greenhouse. After 5 yr of field growth 90% of the Mexican redbud trees were still alive.

CONCLUSIONS

Modifications to a general system of micropropagation to propagate desert plants are not major but are necessary to achieve success. Certainly the challenge of finding novel material in the wild is more resource-efficient than growing large populations in field settings, selecting from these populations, and then conducting propagation studies.

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