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The Use of Shoot Tip Culture in Foundation Plant Materials Service Programs©

D.A. Golino, S.T. Sim, J. Bereczky, and A. Rowhani
Foundation Plant Materials Service, One Shields Ave., University of California, Davis, California 95616

Foundation Plant Materials Service (FPMS) is a self-supporting service department in the College of Agricultural & Environmental Sciences at the University of California, Davis, which produces, tests, maintains, and distributes disease-tested propagating material for use by California nurseries (For additional information, see the FPMS website at http://fpms.ucdavis.edu). At this time, FPMS is responsible for grape (*Vitis*), strawberry (*Fragaria*), fruit tree, nut tree, rose (*Rosa*), and sweet potato (*Ipomoea batatas*) clean-stock programs. Since its inception, scientists working with FPMS have pioneered techniques in grapevine virus disease detection and elimination (Nyland and Goheen,1969; Alley and Golino, 2000). These are the two cornerstones of the clean stock program: the ability to determine whether target virus(es) or other pathogens are present and the ability to eliminate virus from diseased stock.

Micro-shoot tip culture is the method of choice to eliminate virus(es) and other pathogens from many plant species (Baker, 1962; Langhans, 1977; Murashige, 1974). The micro-shoot tip culture technique has the advantage of regenerating a single plant from a single, minuscule (approximately 0.5 mm) shoot. The combination of low hormone levels combined with a minimum time in culture reduces the chance of mutation and regeneration of an off-type plant. At the same time, many pathogens, including viruses, are eliminated by this technique. Micro-shoot tip culture is used as the preferred method of virus elimination for our sweet potato, strawberry, and grape programs. We are also working to optimize procedures for rose shoot tip culture.

In this paper, we will provide the details of our current procedures for shoot tip therapy and briefly review the background of each crop program. Our therapy programs for sweet potatoes, strawberries, and grapes have been in place for many years. The rose effort is new and not yet fully successful.

GENERAL PROTOCOLS FOR SHOOT TIP CULTURE

Each plant species is harvested as described in the following individual crop sections. Tissue is surface sterilized by submersion in 10% commercial bleach plus 1 drop (~0.1 ml) of dishwashing liquid for 10 min. Tissue is removed under aseptic

conditions and serially transferred through three rinse containers containing sterile distilled water. Three or four sections are placed on a sterile paper plate at a time and each shoot tip is excised in a transfer hood with the aid of a dissecting scope. Individual leaf scales are removed to expose the shoot tip; after each cut, the forceps and scalpel are flame sterilized and cooled to prevent contaminating younger, inner tissues with virus particles from older tissue which might be transferred by the blade. When the meristematic dome becomes visible, a final cut is made just at the base of the last pair of leaf primordia, and the tip is gently placed on the surface of the initiation medium. If the cut was made at the correct place, the shoot tip will come off easily with a slight touch of the scalpel to the medium surface. It should not be too sticky and the dome should remain turgid and dome-shaped. One micro-shoot tip is placed in each tube. The explants are incubated in a growth chamber at 25°C, 50% relative humidity, 16-h days, and 115 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ light from cool-white fluorescent bulbs.

Media for individual plant types and stages in tissue culture are described in Table 1. A number of possible media were observed and compared experimentally through the years for each program (Golino et al, 1998). Our observation has been that there are considerable varietal differences in ease of establishment for sweet potatoes, grapes, and strawberries; therefore, a medium that works well for one variety may need modification when another variety is cultured. Much progress can be made by examining the growth of explants. For instance, if a group of explants develops vitrified tissue (stiff, distorted leaves), the salt strength was reduced; too much callus, the 6-benzylaminopurine (BAP) level was reduced; and poor rooting, indolebutyric acid (IBA) was increased. We have found that some species succeed best if an initiation medium is followed by a rooting medium after shoot development occurs.

Murashige-Skoog (MS) salts are premixed in 1 liter envelopes (Gibco/BRL Life Technologies Grand Island, NY, catalog # 11117-066); MS salts include: NH_4NO_3, H_3BO_3, KNO_3, $CaCl_2$, $CoCl_2$, $CuSO_4$, Na_2EDTA, $FeSO_4$ 7 H_2O, $MgSO_4$, $MnSO_4$ H_2O, Na_2MoO_4 2 H_2O, KI, KNO_3, KH_2PO_4, $ZnSO_4$ H_2O. MS vitamins are supplied as pre-mixed, pre-measured stock to which 200 ml of water is added before filter sterilization, producing a 500× solution (Sigma-Aldrich, St. Louis, Missouri. catalog # M7150). It includes: myo-inositol, nicotinic acid, pyridoxine·HCl, thiamine·HCl, and glycine. The following growth factors are added as a liquid from stock solutions made to 1 mg ml^{-1}: 6-BAP is 6-benzylaminopurine; IBA is indolebutyric acid; IAA is indole-3-acetic acid; and NAA is α-naphthaleneacetic acid. To make stock solutions 30 mg of the desired growth factor is dissolved in 3 ml 1M HCl for 6-BAP, or 3 ml 1M KOH for IBA, IAA, and NAA, then the total volume is brought to 30 ml with water and the mixture filter sterilized. Stock solutions are kept at 4°C for no longer than 2 months and a sterile pipette tip is used to withdraw the volume needed. To make media, agar is melted in 50% of the final volume of water in an autoclave. All other ingredients are dissolved in 40% of the final volume of water; the pH is adjusted and volume is brought to 50% final. Finally, 4 ml of each solution is added to each culture tube for a total of 8 ml per tube. The tubes are capped and autoclaved at 120 psi for 10 min.

Explants are transferred to fresh medium every 3 weeks. When the explants develop a shoot about 2 cm long and 4 to 5 well developed leaves (usually about 6 to 8 weeks after excision), they are generally transferred to a rooting medium, that contains a rooting hormone and a reduced sugar concentration. When roots are well-developed and the shoot has reached the height of the tube (anywhere from 3 to 9 weeks), the plants are ready to be introduced to soil and greenhouse conditions —

a process that takes about 3 weeks. Culture medium is rinsed off the roots, roots are trimmed if necessary, and plants are transplanted to sterilized potting mix in 1 inch pots. The pots are placed inside a clear plastic Magenta box with the lid on. One week later, the plants are gradually acclimatized to ambient humidity by leaving the box lid slightly ajar; 3 to 7 days after that, the lid is removed; and after another 3 to 7 days has passed, the plants are transplanted to 4-inch pots and taken to the greenhouse. The whole process from excision of a <0.5 mm shoot tip to a plant in a 4-inch pot in the greenhouse takes approximately 4 months or longer.

SWEET POTATOES

In the 1960s, California sweet potato growers were experiencing problems with a severe strain of russet crack disease of sweet potatoes, caused by feathery mottle virus (Campbell et al., 1974). Scientists in the Plant Pathology Department at U.C. Davis developed procedures for elimination of the disease from sweet potatoes using meristem tip culture. By combining tissue culture elimination of virus, indexing of selections on the indicator Brazilian morning glory, *Ipomoea setosa*, and maintenance of clean stock mother plants in greenhouses, California sweet potato growers have had access to healthy propagating stock for 20 years (Alconero et al., 1975; Dangler et al., 1994). In 1995, this sweet potato program was transferred from the Plant Pathology Department to FPMS with the support of the Sweet Potato Council of California, the Livingston Farmers Association, and individual sweet potato growers.

Sweet potatoes are sprouted in the greenhouse and terminal and axillary nodes down to approximately 5 or 6 nodes from the terminal node (distal end) are harvested for excision. Shoot tips are approximately 0.1 mm and may or may not include one pair of leaf primordia. Initial medium is MSIB and rooting medium is MSIP (Table 1). If a particular sweet potato variety does poorly on MSIB, we cut additional shoot tips and use MSB for the initiation medium.

GRAPES

The FPMS grape program was founded in the 1950s and is the largest of the FPMS commodity programs (Alley and Golino, 2000). At FPMS, growth chamber heat therapy was the technique of choice until the late 1980s (Nyland and Goheen, 1969), but worldwide, tissue culture techniques were being developed and used extensively for grapevines (Monette, 1988). FPMS first began applying this technology to grapes at FPMS in 1988 with support from an industry grant (Nelson-Kluk, 1988). Further work at FPMS over the 1990s has resulted in improvements in survival and the rate of virus elimination to the extent that this process is now routine and reliable (Golino et al., 1998). Molecular detection techniques for the grapevine viruses have improved, making it possible to screen young plants regenerated from tissue culture, greatly speeding up the virus screening process (Rowhani, 1992).

For grapes, we harvest terminal buds exclusively for excision for virus elimination. We have found that terminal buds are larger, easier to excise and seem to grow more vigorously than axillary buds. Shoot tips are approximately 0.4 to 0.5 mm and include 1 to 3 pairs of leaf primordia. The initial medium is MSB as described in Table 1. The rooting medium is RM (Table 1). This protocol was determined after comparing five protocols (Golino, 1998). Rapidly-growing shoots in the spring and early summer provide the best tissue for excision. Both field and greenhouse grown plants will perform well as sources of material. About 1- to 2-cm tips are harvested

Table 1. Tissue culture media used for 0.5-mm shoot-tip culture for virus elimination in various FPMS crop programs. Salts and vitamins are as described by Murashige and Skoog (MS)(1962).

Primary Use	Grape initiation	Grape rooting	Sweet potato and rose initiation	Sweet potato and rose rooting	Strawberry initiation	Strawberry maintenance
Medium name*	MSB	RM	MSIB	MSIP	MSI	MS
MS salts, pre-mixed powder	1 envelope	½ envelope*	1 envelope	1 envelope	½ envelope*	1 envelope
MS, pre-mixed sterile vitamin solution, 500X	2.0 ml	2.0 ml	2.0 ml	2.0 ml	1.0 ml	2.0 ml
Sucrose	30.0g	15.0 g	40.0 g	15.0 g	20.0 g	30.0 g
Rooting hormone	none	1.0 mg IBA	1.0 mg NAA	1.0 mg IBA	1.0 mg IAA	none
Cytokinen growth hormone	1.0mg 6-BAP	none	0.3 mg 6-BAP	none	none	none
Agar	6.0 g gum	6.0 g gum	6.0 g gum	6.0 g gum	6.0 g gum	2.0 g Phytagel™
Millipore-filtered water	1 liter	1 liter	1 liter	1 liter	1 liter	1 liter
pH	5.8	5.8	5.2	5.8	5.8	5.8

*All media adjusted to 1 liter for comparison. In practice, 2 liters of media are made with a single envelope of MS.

and brought to the lab. The procedures for surface sterilization and excision are as described above.

STRAWBERRIES

Strawberries are affected by numerous viruses and phytoplasms, many of which spread rapidly in the field (Converse, 1987). Belkengren and Miller (Belkengren and Miller, 1962; Miller and Belkengren, 1963) first reported on the use of shoot tip culture for the elimination of strawberry viruses. This technique has been useful in producing healthy strawberry planting stock.

In most cases, strawberry mother plants at FPMS are placed in heat treatment growth chambers for a minimum of 4 weeks at 37°C before explants are excised. A strong plant with well-established roots is required to survive this treatment. This heat treatment is used for both virus-infected plants and plants which have already been indexed and determined to be negative for virus diseases. Nurseries believe that even when viruses are not present, this heat treatment is valuable in reducing other pathogens and improving meristem performance (especially the total number of runners).

In strawberries, meristem tips are excised from terminal and axillary nodes on the runners of mother plants. A small stipule on the runner indicates the location of the node. There are usually 3 or 4 meristems in each node and, when cut correctly, they will separate cleanly from the surrounding tissue. Each meristem is approximately 0.3 to 0.5 mm in diameter. Each is excised aseptically, using a scalpel and forceps under a dissecting microscope. The scalpel must be flamed often to avoid contaminating the meristem with virus in the tissue and airborne fungal contaminants. Shoot tips are gently placed on MSI medium described in Table 1 on which they will usually form shoots and roots. After roots appear, explants may be transferred to MS medium for further growth or possible cold storage.

ROSES

Many viruses infect roses although two are commonly associated with rose mosaic disease, prunus necrotic ringspot virus and apple mosaic virus (Loebenstein, 1995; Horst, 1983). At FPMS, the rose virus detection program uses graft indexing onto *Rosa multiflora* and 'Shirofugen' cherry and (enzyme-linked immuno-sorbent assay) ELISA testing. Over the last two decades heat treatment has been used successfully to eliminate virus from many rose cultivars. Others, however, have repeatedly come out of heat treatment still infected. For this reason, we have started a small experimental program of tissue culture therapy for roses. At this time (Oct. 2000), only a few plants have grown out from 0.5 mm shoot tips. They will be virus-tested to see whether virus elimination was successful. The best plant material to use seems to be the axillary buds on a stem that has finished blooming and before the buds start to grow out. The initial medium we tried was MSIB and rooting medium was MSIP (Table 1). These are preliminary results and future modifications are to be expected.

CONCLUSIONS

Shoot-tip culture has become an integral part of FPMS clean stock programs for sweet potatoes, grapes,and strawberries over the last decade. As techniques and media have been optimized, shoot tip culture has proved to be reliable and is

preferred over other therapy techniques such as heat treatment, chemotherapy, and efforts to identify escapes. We look forward to expanding the number of crops to which this strategy can be applied.

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