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Meristem Micropropagation Protocols for *Vitis rotundifolia* Michx.

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Abstract. **A meristem micropropagation system was developed to produce *Agrobacterium* -free muscadine grape. Meristems were cultured on a modified Woody Plant Medium (mWPM) supplemented with 0.45 µM BAP. After 2 weeks, cultures were transferred to mWPM containing 8.92 µM BAP to enhance shoot proliferation. Propagules were subsequently subdivided and transferred to fresh medium at 2- to 4-week intervals. New shoots were excised and inserted in mWPM supplemented with 0.57 µM IAA to promote root formation. This method has been successfully used to produce *Agrobacterium* -free plants of muscadine cultivars Carlos, Doreen, Jumbo, Magnolia, and Sterling for research purposes and for a foundation planting in Mississippi. Chemical names used: benzylaminopurine (BAP); indole3-acetic acid (IAA).**

Crown gall, caused by *Agrobacterium tumefaciens* (E.F. Smith and Towns.) Conn. has been a disease of consequence in many muscadine vineyards in Mississippi (Graves et al., 1987, 1988). Recent observations of an apparently universal systemic occurrence of *Agrobacterium* spp. in symptomless muscadines and the demonstration that a large percentage of *Agrobacterium* isolates from symptomless plants are infectious and capable of inducing galls in muscadine are causes for concern (Thies et al., 1989, 1990, 1991). In a search for *Agrobacterium* -free muscadine plants, 900 rooted cuttings were screened for *Agrobacterium* presence using the methods of Tarbah and Goodman (1986). After each of three screenings, plants yielding isolates of *Agrobacterium* were discarded. However, after further screening and study, the few remaining plants were also found to be positive (C.H.G., unpublished).

Developing a means for producing *Agrobacterium* -free muscadine plants is important for research purposes and for use in studies related to the development of practical disease control methods for the muscadine industry. Meristem culture has historically been recognized as perhaps the best means of producing plants free of systemic pathogens. Production of *Vitis vinifera* grapevines free from *A. tumefaciens* biovar 3 (AT-3) by means of shoot-tip culture has been demonstrated by Burr et al. (1988).

Gray and Fisher (1985) were the first to note shoot formation in several *V. rotundifolia* cultivars when comparing species and cultivars using shoot apices on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), but *Agrobacterium* presence was not considered. In our preliminary studies, yield of shoots from the system employed by Gray and Fisher was not satisfactory (Griffin and Graves, 1988; Thies et al., 1991). Lee and Wetzstein (1990) obtained in vitro propagation of muscadine grapes by axillary shoot proliferation; however, this method does not ensure the production of disease-free plants.

We now document a meristem micropropagation protocol that has been developed and proven effective for efficient production of *Agrobacterium* -free muscadine plants. With refinements, meristem micropropagation has potential to be the most economical and efficient means for commercial propagation of muscadine.

Several media and culture regimes were evaluated in preliminary studies. Muscadines, including 'Magnolia', 'Summit', 'Carlos', 'Sterling', and 'Jumbo', were compared to 'Orlando Seedless', a Florida hybrid table grape that had previously proven to be easily propagated from meristems (Griffin and Graves, 1988). Techniques and media studied included a modified MS medium supplemented with 5 µM BAP (Gray and Fisher, 1985), a liquid MS medium sup-

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Table 1. Media used for micropropagation of muscadine grape plants.

Components	mWPM I (75% strength)	mWPM II[z] (75% strength)	mWPM III (75% strength)
WPM[y]			
Modifications			
Myo-inositol (μM)	555	555	140
Dextrose (w/v) (replacing sucrose)	2.0%	2.0%	1.0%
T.C. agar (w/v)	0.05%	0.05%	0.05%
Additional components			
BAP (μM)	0.45	8.92	---
IAA (μM)	---	---	0.57
Gelrite (w/v)	0.1%	0.2%	0.2%

[z]Modified WPM IIa was the same formula as mWPM II except that Gelrite (Scott Laboratories, Carson, Calif.) was omitted and tissue culture (T.C.) agar (KCBiological, Lenexa, Kan.) was increased to 0.6% (w/v).
[y]Woody Plant Medium (WPM) as described by Lloyd and McCown (1980) was used in each mWPM medium at 75% concentration with modifications and additional components listed.

Table 2. Yield of muscadine grape propagules and shoots from each meristem at the time of transfer, after 6 1/2 months of growth and subdivision, on modified Woody Plant Medium.

Cultivar	Replications	Propagules/ meristem (±SE)	Shoots/ meristem (±SE)
Carlos	5	60 ± 2.7	101 ± 11.4
Doreen	4	70 ± 2.2	124 ± 19.1
Jumbo	3	60 ± 5.2	79 ± 6.1
Magnolia	9	78 ± 2.7	145 ± 12.6
Sterling	3	80 ± 3.8	159 ± 7.5

plemented with 5 μM BAP (Barlass and Skene, 1978, 1983), and various other modifications of MS medium and Lloyd and McCown's (1980) Woody Plant Medium (WPM). These modifications included varying the concentration of basal salts (50% and 75%), BAP (0, 2.5, 5.0, 7.5, and 10.0 μM), and kinetin (5 μM BAP plus 2, 8, and 15 μM kinetin). Whereas 'Orlando Seedless' was easily propagated by several of these variations, acceptable shoot proliferation of muscadine was more difficult to achieve. Although a few muscadine plants were produced by some of these methods, efficient micropropagation from meristems was achieved only by using the following procedures and modifications of the WPM (mWPM):

1) Shoot tips (4 to 6 mm long) were taken from muscadine plants maintained in the greenhouse that were determined to be systemically infested with *Agrobacterium;* employing the methods of Tarbah and Goodman (1986). These shoot tips were transported to the laboratory in sealed plastic bags and refrigerated until needed. Tips were soaked for 5 min in sterile distilled water, surface-sterilized for 3 min in 1.0% sodium hypochlorite (20% commercial bleach) with 0.05% polyoxyethylene sorbitan monolaurate (Tween 20), rinsed three times in sterile distilled water for 1 min each, and transferred to sterile 1% (w/v) ascorbic acid. Meristems (apical domes 0.2 to 0.4 mm long) were excised in the ascorbic acid and aseptically transferred to appropriate medium.

2) Explants were first placed on mWMP I (Table 1) supplemented with 0.45 μM BAP using ≈16 ml medium/100 × 15-mm Falcon petri dish (Fisher Scientific, Norcross, Ga.). The dishes were incubated for 2 weeks at 28 ± 2C with an 18-h photoperiod using Gro-Lux (Sylvania, Danvers, Mass.) fluorescent lights with a light intensity of 25 ± 3 $\mu mol \cdot s^{-1} \cdot m^{-2}$.

3) After the 2 weeks, explants were rinsed in sterile distilled water and transferred to 1% (w/v) ascorbic acid where dead tissue was removed. They were then transferred to mWMP II (Table 1), supplemented with 8.92 μM BAP to encourage shoot formation. These cultures were grown under the conditions described above using 42 ml medium/100 × 25 mm Lab-Tek petri dish (Nunc, Naperville, Ill.). Subsequently, at 2- to 3-week intervals, these meristem callus cultures were rinsed under ascorbic acid, trimmed of dead tissues, when present, and transferred to fresh medium (mWPM II).

4) On the fourth or fifth transfer (2 to 3 months from the start), each callus culture was subdivided into three pieces (propagules) 5 to 15 mm in size and placed on mWPM II or mWPM IIA (Table 1). The propagules were repeatedly subdivided and transferred at 2- to 4-week intervals thereafter.

5) The first shoots of sufficient size for harvesting (5 to 9 mm long) were present beginning at 3 months. At each transfer (after the first 3 months), new shoots were excised and placed on mWPM III (Table 1) supplemented with 0.57 μM IAA to enhance root formation. For this, 25 × 150 Kimax culture tubes (Kimble Glass, Houston, Texas), with 14 ml of medium per tube, were employed. These shoots were incubated at 28 ± 2C with a 16-h photoperiod.

6) After plantlets had developed sufficient roots, they were transferred to peat pellets. Pellets were placed in polyethylene containers with StarPac (AgriStar, Sealy, Texas) containers over the top to maintain high humidity, and then were incubated at 28C with a 16-h photoperiod. Plantlets were intially fertilized with liquid mWPM III (Table 1, T.C. agar and Gelrite omitted) and subsequently, for the first week only, when plantlets required moisture.

7) Once roots emerged through the peat pellets, plantlets were transferred to 0.3-liter pots containing a peat-perlite mixture (Jiffy Mix Plus), a 1 sand : 1 soil : 1 peat (by volume) mixture, or a combination of the two.

After 6 ½ months of explant growth and subdivision following the schedule described above, individual meristems produced an average of 60 to 80 propagules (Table 2), depending on the cultivar. Propagules were yielding one to two shoots per propagule per transfer. The number of shoots varied considerably with cultivar. The total shoot count per meristem per transfer after 6 ½ months ranged from an average of 79 for 'Jumbo' to 159 for 'Sterling' (Table 2). Because of limited space and facilities, we were unable to continue culture of all propagules. However, among those that were continued, the number of shoots per propagule increased to eventually yield four to 10 shoots per propagule with each transfer, accompanied by a 2- to 4-fold increase in number of propagules beginning at 9 months. The high yields and continuing productivity of these propagules suggest that meristem systems may be superior to systems employing shoot tips, in addition to providing advantages in avoidance of systemic pathogens. Among the cultivars, Magnolia and Sterling were the most prolific, and Carlos and Jumbo were the least productive.

In the first 2 to 3 months of growth, propagules needed to be transferred every 2 weeks to maintain quality and prevent yellowing or browning. Beyond that, propagules survived up to 6 weeks without subdivision or transfer; however, tissue quality and shoot production began to deteriorate after 4 weeks. The optimum interval between transfers for maximum shoot production and quality of tissue was 3 weeks. Shoots began developing 10 to 12 weeks after initial meristem culture. The first plantlets were placed in peat pellets by the 4th month.

Root formation was evident by 1 to 2 weeks after transfer of shoots to the rooting medium, and plantlets were transferred to peat pellets after 4 to 8 weeks. At that time, plantlets ranged in length from 1.5 to 10.0 cm, and two to four roots were at least 2.5 cm long. Survival was highest when plantlets that were 2.5 to 4.5 cm long with two or more roots were transferred to peat pellets. Plantlets were transferred from peat pellets to small pots when roots emerged from the pellets, usually within 3 to 5 weeks. White's medium (White, 1943) was tried in some of our earlier studies, and good root development followed, but leaf growth was poor and plants eventually died if plantlets were not transferred within 2 to 3 weeks.

All plants (> 200) produced by meristem micropropagation and that were of sufficient size were assayed for *Agrobacterium* as described by Tarbah and Goodman (1986). Additionally, 10% of the small plantlets, representative of each cultivar, were tested by placing leaf disks or spreading surface-sterilized leaves crushed in sterile distilled water on *Agrobacterium*- selective medium (Schaacl, 1988). All were found to be free of *Agrobacterium* spp.

This meristem culture procedure has been used to produce a sufficient number of *Agro-*

bacterium -free plants of muscadine cultivars Carlos, Doreen, Jumbo, Magnolia, and Sterling for the establishment of a foundation planting to be used in crown gall control studies. Long-range projections suggest that this foundation planting, after study, may be a useful source of cuttings free of *A. tumefaciens* for the muscadine industry in establishing new plantings. The meristem culture procedures are currently being used to propagate other cultivars, including 'Summit', 'Noble', and 'Tarheel', with the goal of ultimately including all popular muscadine cultivars. The phenotypic stability of meristem-cultured muscadine cultivars must yet be proven.

The potential for production of muscadine plants by the meristem culture procedure described above suggests that the procedure may be a feasible method of economically supplying muscadine vines commercially, in which case a foundation planting of pathogen-free plants would be unnecessary. Meristem culture should be a superior propagation system because it is more amenable to development of production systems that could also eliminate the presence of other systemic pathogens in new plantings. Ensuring pathogen-free plants for new plantings is desirable, although some systemic pathogens have vectors that could reintroduce them.

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