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In vitro micropropagation and plant establishment of muscadine grape *cultivars (Vitis rotundifolia)*

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Abstract

Shoot apical meristems were used to establish regenerative axillary bud cultures of 9 muscadine grape cultivars. Meristems taken from 10 cm long shoots had less contamination (3%) and a higher survival rate (94%) than those from shorter or longer shoots. Of media tested, MS, 1/2 MS, and C_2D resulted in equivalent shoot proliferation rates, whereas, WPM produced stunted shoots. When pooling results for 3 cultivars, 5, 10 and 20 μM BA and 5 μM TDZ produced the highest average number of shoots per cultured apex (3.4–3.8). However, shoots produced with TDZ were stunted and did not root well. For rooting of shoots directly in potting mix, a rooting powder pretreatment significantly increased the number of roots per shoot but did not affect percent rooting or root length. For rooting in vitro, 1 μM NAA significantly increased all parameters measured. Although more shoots rooted in vitro than in vivo (77% vs. 46%), the latter was judged preferable since acclimatized plants were produced in less time and a major culture step was eliminated. Significant differences among cultivars were noted for measured responses in all experiments.

Abbreviations: BA – benzyladenine, Kin – kinetin, MS – Murashige & Skoog (medium), NAA – naphthaleneacetic acid, TDZ – thidiazuron, WPM – woody plant medium

Introduction

Cultivars of muscadine grape (*Vitis rotundifolia* Michx. and hybrids with *Vitis munsoniana* Simpson) represent a major fruit crop in the southeastern United States where they are used for fresh fruit, jelly, juice and wine production. Although acreage is increasing (Anonymous 1989), concern has been raised that existing vines, including nursery stock, may contain endemic infections of *Agrobacterium tumefaciens* (Graves 1987), which causes economically devastating crown gall disease. In vitro micropropagation has been shown to rid the bunch grape cultivar 'Pinot Chardonnay' of *A. tumefaciens* (Burr et al. 1988); this technique also has been shown to be effective in eliminating viruses (Barlass et al. 1982) and viroids (Duran-Vila et al. 1988) from bunch grapes. These studies suggest that use of apical meristems from rapidly growing shoots as explants might provide a method of maintaining *A. tumefaciens*-free stock of muscadine grape.

Within the genus *Vitis*, micropropagation technology is well established only for members of the subgenus *Euvitis* Planch. ($2n = 38$), which comprises the bunch grape cultivars (primarily *Vitis vinifera* L, *Vitis labrusca* L. and hybrids) (e.g. Barlass & Skene 1978; Chee & Pool 1982; Chee et al. 1984; Goussard 1981). Micropropagation technology has not been adequately developed for members of the subgenus *Mus-*

cadinia Planch. (2n = 40), which contains the muscadine grape cultivars. Muscadine grapes are botanically distinct from bunch grapes, since very low-to-no fruit set is obtained in crosses between the two subgenera and resulting hybrids are often infertile, fruit is not born in bunches, leaves lack pubescence, stem morphology differs and adventitious root development from dormant wood is so poor as to make this conventional method of bunch grape propagation unfeasible for muscadines. The differences in tissue culture responses between the two subgenera are evidenced by lack of reports concerning somatic embryogenesis for *Muscadinia*, as well as few examples of micropropagation (with plant recovery from only one cultivar, 'Summit'). Gray & Fisher (1985) studied in vitro shoot production in several muscadine cultivars ('Carlos', 'Dixie' and a numbered selection) and reported an average propagation rate of five shoots per cultured apex per month for 'Carlos', but rooting was not obtained. Sudarsono & Goldy (1988) reported that combinations of the cytokinins TDZ and Kin were more effective for establishing cultures of two cultivars whereas BA was best for another. Lee & Wetzstein (1990) obtained up to 250 shoots per axillary bud from the cultivar 'Summit' by maintaining the undivided tissue mass through 16 weeks of culture and produced greenhouse-acclimated potted plants following in vitro rooting. That study did not show that the cultures could be increased through division and it was unclear whether the methodology was suitable for other cultivars or was unique to 'Summit'. The present report details a protocol for initiating cultures, increasing culture mass through division, micropropagating shoots and producing vigorous rooted plants from nine distinct muscadine cultivars. This represents the first generally applicable and conventionally-based, micropropagation system for muscadine grape.

Materials and methods

Culture initiation

Shoot tips were excised from 5- to 10-year-old vines at the CFREC Leesburg experimental vineyard and placed between layers of moist paper towels. In the laboratory, shoot tips were further dissected to remove all extraneous leaves and tendrils, except for small appendages directly enclosing the shoot apical meristem. Shoot tips were surface disinfested for 2.5–3 min by agitation in 25% commercial bleach (1.3% NaOCl) containing a drop of Triton X surfactant, then rinsed twice and stored in sterile distilled water. The apex (approximately 1 mm in diameter) of each shoot was micro-dissected and placed, cut surface down, on autoclaved (121°C and 1.05 kg cm^{-2} for 20 min) C_2D medium (Chee et al. 1984), containing 5 μM benzyladenine (BA) as previously described (Gray & Fisher 1985; Gray & Klein 1987). Five apices were placed in each 90 × 15 mm petri dish containing 25 ml of medium. Cultures were incubated at 25°C with an 18-h cool white fluorescent light (60 μmol m^{-2} s^{-1})/6-h dark cycle.

To define and optimize a method for obtaining explants, apical meristems were obtained from shoots of 'Dixie' and 'Fry' at five distinct developmental stages. Developmental stages were subjectively categorized based on length of shoots (0.5, 1, 2, 10 and 60 cm) that developed from previously dormant vines after spring budbreak. Explants were harvested when the most rapidly growing shoots for each cultivar reached the desired lengths. Contamination percentage was scored by counting the number of meristems that had bacteria or fungi growing directly from tissue after 4 weeks of culture. Survival was scored as the percentage of meristems that remained contaminant-free and proliferated as axillary bud cultures after 6 weeks.

Shoot micropropagation

Apical meristems from cultures of 'Carlos', 'Dixie' and 'Fry' grown for 2 culture cycles on C_2D with 5 μM BA were used as explants for experiments to evaluate the effects of various medium salt formulations on micropropagation. Medium salt formulae tested were: MS (Murashige & Skoog 1962), 1/2 MS, C_2D and WPM (McCown & Lloyd 1981). C_2D differs from MS in that 709 mg l^{-1} $Ca(NO_3)_2.4H_2O$ is added, KI is omitted and $MnSO_4.4H_2O$ is reduced from 22.3 to 1.12 mg l^{-1}. WPM differs

from MS in that $CoCl_2.6H_2O$, KI and KNO_3 are omitted; NH_4NO_3 is reduced from 1650 to 400 mg l^{-1}; $CaCl_2.2H_2O$ is reduced from 440 to 96 mg l^{-1}; $CuSO_4.5H_2O$ is increased from 0.025 to 0.25 mg l^{-1}; 556 mg l^{-1} $Ca(NO_3)_2.4H_2O$ and 990 mg l^{-1} K_2SO_4 are added. Each medium contained 5 μM BA, 0.7% TC agar (Hazelton Biologics Inc., Lenexa, Kansas) and 3% sucrose. Twenty apices were placed on each medium and incubated as described above. After 6 weeks, the resulting number of shoots produced per apex was determined and an additional sample of 20 apices was harvested from the new shoots for reculture on the same respective media. This cycle was repeated a total of 3 times. Shoot proliferation data for the 3 cycles was pooled in determining average proliferation rates in order to obtain an estimate of culture responses over time.

Effect of various cytokinins at different concentrations on shoot micropropagation of the same cultivars tested above was determined using MS medium. Apical meristem explants were taken from cultures grown in the previous experiment on MS medium with 5 μM BA. Cytokinins and concentrations tested were: 5, 10 and 20 μM BA; 10, 20 and 40 μM Kin; and 0.5, 1 and 5 μM TDZ. Differential activity ranges for each cytokinin were determined in preliminary experiments. A no-cytokinin control treatment was also included. Twenty apices were plated on each cytokinin-concentration treatment and the number of shoots produced per apex was determined after 6 weeks. The experiment was repeated 3 times using new shoots from each previous culture cycle and the data were pooled as above.

Subsequently, shoot propagation rates of nine cultivars ('Carlos', 'Dixie', 'Fry', 'Jumbo', 'Nesbitt', 'Welder' and University of Florida experimental selections, AA5-37, AA6-48 and AA7-44) were compared using MS medium with 5 μM BA.

Rooting

In vivo rooting was compared with in vitro rooting, using shoots of 'Carlos', 'Jumbo' and 'Welder' obtained from MS medium with 5 μM BA. For in vivo rooting, shoots obtained from 0.5 μM TDZ were also tested. In vivo rooting utilized shoots with four-to-six nodes excised from actively growing cultures and inserted either directly into 3.5 cm dia. × 5.5 cm deep plastic liners containing Pro-mix commercial potting mix or first dipped into Rootone-F commercial rooting powder, which contains an array of the auxins NAA (0.033%), 1-naphthaleneacetamide (0.067%), 2-methyl-naphthaleneacetamide (0.013%) and 1H-indole-3-butyric acid (IBA) (0.057%). Seventy-two liners were placed in each 52 × 27 cm planting flat that was covered with a clear plastic dome and placed in a greenhouse mist chamber. This arrangement promoted a maximum humidity environment but eliminated the damaging effects of water when applied directly to in vitro-derived shoots. Planting flats were removed from the mist chamber after 2 weeks and covers were removed after an additional 2 weeks. For in vitro rooting, shoots of the same cultivars were placed in MS medium with or without 1 μM NAA. Plants rooted in vitro were acclimated to greenhouse conditions as above. The number of: rooted shoots, roots/shoot as well as average root length were determined after 6 weeks.

All data were subjected to an analysis of variance and mean separation was by the Duncan's New Multiple Range Test.

Results and discussion

Culture establishment

Relative developmental stage of shoots (as measured by length) from which apical meristems were dissected had a profound effect on bacterial and fungal contamination rate as well as culture survival. Meristems taken from 10 cm long shoots had the least contamination (3%) and resulted in more surviving cultures (94%) (Table 1). Meristems from longer and shorter shoots had more contamination and lower survival. Meristems taken from 0.5 cm shoots, which were at the bud break stage, exhibited the poorest response. These results demonstrate that a distinct growth phase occurs that is optimal for initiating cultures from field-grown muscadines. This phase occurs soon after bud burst (approxi-

Table 1. Effect of shoot length and cultivar on contamination and survival of cultured muscadine grape apical meristems.[1]

Factors	Apices tested (no.)	Contamination (%)	Survival (%)
Shoot length (cm)			
0.5	126	62a[2]	16e
1	131	27b	37d
2	118	17bc	60c
10	119	3d	94a
60	97	13c	77b
Cultivar			
'Dixie'	304	17b	58a
'Fry'	287	34a	52a
Main effects			
Shoot length		**[3]	**
Cultivar		**	NS
S × CV		NS	**

[1] Apical meristems were cultured on C_2D medium with 5 μM BA for 4 weeks to score contamination and 6 weeks to determine survival.
[2] Means with the same letter are not significantly different at $\alpha = 0.01$ according to Duncan's New Multiple Range Test.
[3] NS, ** Nonsignificant or significant at 1% level, respectively.

mately 15 days) and coincides with early rapid vegetative growth.

Presumably, apical meristems contain less endophytic contamination and are most vigorous during such rapid growth. Support for this hypothesis comes from our preliminary experiments with slower growing, potted muscadine grapes, where proliferating shoot cultures were difficult to obtain. Often, cultures obtained from greenhouse-grown material proliferated as a hard, green callus that grew from the base of meristem explants and shoot propagation did not occur. When meristems were excised from the callus and recultured, a new callus developed and the cycle was repeated. It was not determined whether or not endophytic *A. tumefaciens* may have caused these symptoms reminiscent of crown gall disease. Our methodical dependence on apical meristem explants for culture initiation (i.e. Gray & Fisher 1985; Gray & Klein 1987, 1989) differs from the protocol of Lee & Wetzstein (1990) where axillary bud explants were used.

A cultivar effect occurred with contamination where significantly more non-contaminated cultures were obtained from 'Dixie' meristems,

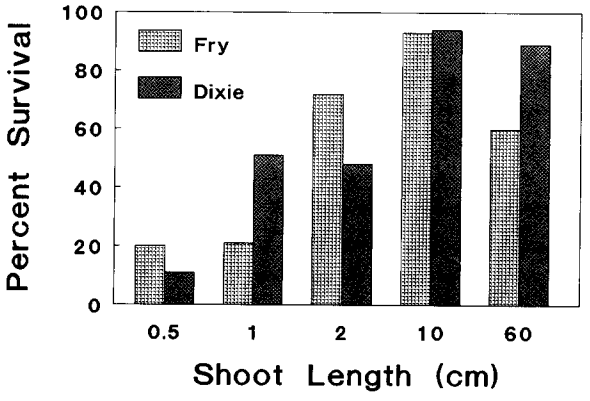

Fig. 1. Effect of length of shoots from which apical meristem explants were harvested on subsequent survival of 'Fry' and 'Dixie' muscadine grapes during in vitro culture.

whereas both 'Dixie' and 'Fry' had equivalent survival rates. A significant cultivar × shoot length interaction occurred for measurements of survival. This interaction was due to differences between the two cultivars in ranking of responses obtained from 2 and 60 cm shoots (Fig. 1). Although the 10 cm length was best for both cultivars, the 60 cm length was second best for 'Dixie' compared to 2 cm for 'Fry'.

Shoot micropropagation

A derivative of MS medium, C_2D, was determined to be superior to MS for micropropagation of *V. labrusca* cultivars (Chee et al. 1984), which led us to utilize C_2D in preliminary experiments. Subsequently, C_2D was compared directly with MS, 1/2 MS and WPM for effect on shoot production from three cultivars over three culture cycles. Shoots produced on WPM became vitrified and suffered from leaf abscission; thus, its use was discontinued. Shoots produced on the remaining three media grew from a hard mass of tissue formed by the original apex (Fig. 2). Some shoots possessed up to 8 nodes within 6 weeks, although smaller shoots were also present in such cultures. Equivalent shoot numbers of 3.3-to-3.4 per apex were obtained by culture on these three media. However, MS was judged to be the best overall choice due to its common usage in industry.

As before, the test cultivars differed in shoot production rates with 'Dixie' significantly per-

Fig. 2. Proliferating shoot culture of 'Welder' muscadine grape produced 6 weeks after plating an apical meristem on MS medium with 5 μM BA.

forming the poorest ($p > 0.01$) on all media (2.8 shoots per apex). However, 'Carlos', which rated below 'Fry' in the previous experiment, was significantly better ($p > 0.01$) here (3.8 vs. 3.3 shoots per apex). Since a medium × cultivar interaction was not present, these results suggest that observed cultivar differences were not related to the test media.

Study of the effect of three cytokinins on shoot development of three cultivars showed that BA at 5–20 μM concentrations and 5 μM TDZ were equivalent when considering shoot numbers pooled from 'Carlos', 'Fry' and 'Dixie' (Table 2). However, shoots produced on TDZ-containing medium were stunted and distorted compared to those from medium with BA. Kinetin was ineffective and was comparable to medium with no cytokinin at all. Therefore, BA was superior to both Kin and TDZ. Previously, 5 μM BA was used to micropropagate shoots of both *Muscadinia* and *Euvitis* species, hybrids and cultivars (Gray & Fisher 1985). The present findings were in contrast to a previous study (Sudarsono & Goldy 1988) that found TDZ to be superior to BA in initiating cultures for two out of three cultivars tested.

When compared to the previous medium evaluation experiment, genotypic effects appeared to be reduced regarding responses to cytokinins since 'Dixie' and 'Fry' produced statistically equivalent numbers of shoots (Table 2). A cytokinin × cultivar interaction is shown in Fig. 3. This interaction was due to differential responses of cultivars to cytokinin-concentration treatments. For example, 5 μM BA was best for 'Carlos', whereas 20 μM BA was best for 'Fry'. This suggests that optimization of cytokinin concentration for each cultivar is needed to maximize shoot production.

Table 2. Effect of cytokinins on shoot micropropagation of 3 muscadine grape cultivars.[1]

Factors	Apices tested (no.)	Shoots per apex
		'Carlos', 'Fry', 'Dixie'
Cytokinin (μM)		
0	103	0.4d[2]
5 BA	190	3.8a
10 BA	190	3.4ab
20 BA	175	3.6a
0.5 TDZ	190	3.1bc
1 TDZ	190	3.0c
5 TDZ	182	3.7a
10 Kin	118	0.4d
20 Kin	123	0.5d
40 Kin	147	0.7d
Cultivar		
'Carlos'	547	3.0a
'Fry'	485	2.4b
'Dixie'	576	2.2b
Main effects		
Cytokinin		**[3]
Cultivar		**
C × CV		**

[1] Apices were cultured on MS medium.
[2] Means with the same letter are not significantly different at $\alpha = 0.01$ according to Duncan's New Multiple Range Test.
[3] ** Significant at 1% level.

Rooting

For in vivo rooting, the rooting responses of

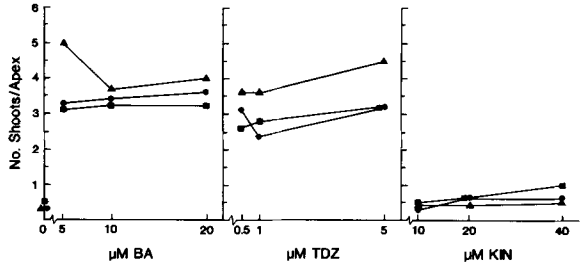

Fig. 3. Comparison of 3 cytokinins at 3 concentrations on shoot proliferation of 'Carlos' (▲), 'Dixie' (■) and 'Fry' (●) muscadine grapes during in vitro culture.

Table 3. In vivo rooting of 3 muscadine grape cultivars.[1]

Factors	No. shoots tested	Rooted (%)	Roots/shoot (No.)	Rootlength (mm)
Rooting pretreatment				
None	96/28[2]	46a[3]	3.2b	18a
Rootone	96/30	43a	4.3a	14a
Cultivar				
'Welder'	72/20	56a	3.9b	14b
'Carlos'	48/18	44ab	5.2a	20a
'Jumbo'	72/20	33b	2.3c	13b
Main effects				
Rooting pretreatment	NS[4]	*	NS	
Cultivar	*	**	**	
R × CV	**	NS	NS	

[1] Shoots were harvested from cultures grown on MS medium with 5 μM BA and placed in potting mix.
[2] Number of shoots evaluated for 'rooted'/number of shoots evaluated for 'roots/shoot' and 'rootlength'.
[3] Means with the same letter are not significantly different at $\alpha = 0.01$ according to Duncan's New Multiple Range Test.
[4] NS, *, ** Nonsignificant or significant at 5%, 1% level, respectively.

shoots harvested from cultures grown on MS medium containing either 5 μM BA or 0.5 μM TDZ were compared. When considering the pooled response of 3 cultivars ('Carlos', 'Jumbo' and 'Welder'), significantly more shoots ($p > 0.01$) were obtained from medium with BA rooted (34%) than with TDZ (8%). Thus, only shoots produced with BA were further analyzed.

Treatment of BA-produced shoots with Rootone resulted in significantly more roots per shoot than without Rootone but percentage of shoots that rooted and average root length were unaffected (Table 3). Of the cultivars tested, more 'Welder' shoots produced roots when compared to 'Carlos'; however, 'Jumbo' was statistically equivalent to both. An interaction between cultivar and Rootone treatment occurred for the percentage of shoots that rooted because 'Carlos' and 'Welder' responded better without Rootone, whereas 'Jumbo' exhibited the opposite response (Fig 4).

In vitro rooting studies demonstrated that 1 μM NAA incorporated into the medium significantly increased percentage of shoots that rooted, number of roots per shoot and root length for all three cultivars (Table 4). For both root initiation and number, 'Carlos' exhibited the highest root initiation rate and produced more roots per shoot. 'Welder' was better than 'Jumbo' and equivalent to 'Carlos' when considering root length.

The stimulative effects of the auxin NAA on adventitious rooting of in vitro-produced bunch grape shoots have been previously described (Chee & Pool 1982; Chee et al. 1984; Gray & Klein 1987). It appears that exogenously supplied auxins stimulated the number of root primordia to increase during in vivo treatments because more roots developed from Rootone-treated shoots; however, the other parameters were not affected (Table 3). Similarly, auxin apparently accelerated all aspects of in vitro rooting (Table 4). Previous studies of in vitro rooting in micropropagated shoots of 'Summit' demonstrated that IBA alone was effective (Lee & Wetzstein 1990). Although in the present study, in vivo rooting resulted in a lower rooting

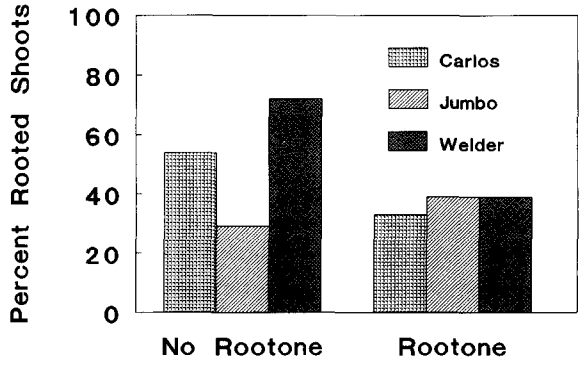

Fig. 4. Effect of Rootone on percent rooting of 'Carlos', 'Jumbo' and 'Welder' muscadine grapes.

Table 4. In vitro rooting of 3 muscadine grape cultivars.[1]

Factors	No. shoots tested	Rooted (%)	Roots/shoot (No.)	Rootlength (mm)
Auxin treatment				
None	110/89[2]	55b[3]	0.8b	11b
1 μM NAA	167/60	77a	2.9a	19a
Cultivar				
'Carlos'	183/140	77a	2.6a	13ab
'Welder'	62/36	58b	1.2b	16a
'Jumbo'	32/13	41c	0.8b	12b
Main effects				
Auxin		**[4]	**	**
Cultivar		**	**	*
A × CV		NS	NS	NS

[1] Shoots were harvested from cultures grown on MS medium with 5 μM BA.
[2] Number of shoots evaluated for 'rooted'/number of shoots evaluated for 'roots/shoot' and 'rootlength'.
[3] Means with the same letter are not significantly different at $\alpha = 0.01$ according to Duncan's New Multiple Range Test.
[4] NS, *, ** Nonsignificant or significant at 0,5%, 1% level, respectively.

percentage than that obtained in vitro (compare Tables 3, 4), the former method was more efficient because a major tissue culture step was eliminated. This resulted in production of vigorous plants (Fig. 5) in less time and with less effort.

Fig. 5. 'Fry' muscadine grape plant produced by in vivo rooting method 6 weeks after a 4-node micropropagated shoot was placed in potting mix.

The best shoot proliferation medium (MS with 5 μM BA) in conjunction with in vivo rooting was subsequently tested on nine cultivars in order to determine general applicability of the optimized protocol. 'Carlos', 'Fry', 'Jumbo', 'Welder' and AA6-48 exhibited the best shoot production rates (2.9–3.7 shoots per month), whereas 'Nesbitt' and AA5-37 were poorest (1.7–2.2 shoots per month) (Table 5). Similar differential responses in shoot development have been noted previously in both *Muscadinia* and *Euvitis* species and cultivars (Gray & Fisher 1985). These results suggest a genotypic effect on

Table 5. Comparison of shoot propagation and in vivo rooting rates for nine muscadine grape cultivars.[1]

Variety	Apices tested/ shoots per apex	Shoots tested/ percent rooted
'Carlos'	60/3.7a[2]	72/33b[3]
'Fry'	60/3.7a	36/11cd
'Welder'	60/3.3ab	72/56a
AA6-48	60/3.0ab	24/13cd
'Jumbo'	55/2.9abc	48/44ab
'Dixie'	45/2.7bc	72/46ab
AA7-44	35/2.7bc	30/27bc
'Nesbitt'	60/2.2cd	36/3d
AA5-37	45/1.7d	48/10cd

[1] Apical meristems were cultured on MS medium with 5 μM BA for 6 weeks prior to determination of shoot number. Data reflects pooled results from 3 successive culture cycles.
[2&3] Means with the same letter are not significantly different at $\alpha = 0.01$ and 0.05, respectively, according to Duncan's New Multiple Range Test.

shoot production that could perhaps be attenuated by modification of culture conditions. Cultivar differences in shoot production between this and previous experiments (compare Table 5 with 2) were due to pooling results from several media in the first experiments. Rooting rates similarly varied, with best response from 'Dixie', 'Jumbo' and 'Welder' and poorest from 'Nesbitt' and AA5-37. The low rooting response of the latter two cultivars was likely related to overall poor performance in culture.

Thus far, proliferating shoot cultures have been maintained for over 24 months. With a 6 week proliferation rate of up to 3.7 shoots (Table 5), it is possible to produce 690 shoots per apex in 30 weeks by culturing only the new apical meristems formed during each transfer cycle. However, this proliferation rate can be dramatically increased by using both nodal segments and apices as explants. For example, with the above proliferation rate and transferring nodal segments plus the apex, 3-node shoots would yield over 710,000 new shoots in 30 weeks. In comparison, propagation by division of shoot-bearing clumps resulted in up to 250 shoots during a 16-week proliferation scheme (Lee & Wetzstein 1990). The effectiveness of micropropagation in eliminating *A. tumefaciens* will be determined in our next study. If successful, a generally applicable muscadine micropropagation system, providing a source of *Agrobacterium*-free plants for the industry, will result.

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