Micropropagation of 'Norton' Winegrape

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SUMMARY. A method has been developed for micropropagation of the difficult-to-root winegrape cultivar 'Norton' (*Vitis aestivalis*). Plants were established in vitro from axillary bud cuttings of field-grown plants. Four levels of 6-benzylaminopurine (BA) and three levels of naphthaleneacetic acid (NAA) were tested in a factorial arrangement for their effectiveness in promoting multiplication of shoots from single-node explants. Three levels of NAA and two concentrations of Murashige and Skoog (MS) basal medium were tested for their effectiveness in promoting rooting of shoot tips. The greatest number of shoots per axillary bud in combination with the greatest shoot length were produced with 4 $\mu\text{mol}\cdot\text{L}^{-1}$ [0.90 $\text{mg}\cdot\text{L}^{-1}$ (ppm)] BA. NAA had no effect on shoot multiplication. NAA was not required for in vitro rooting. All rooted plants survived the transition to soil.

The American winegrape 'Norton' (syn = 'Cynthiana', 'Virginia Seedling') is an old cultivar, introduced into cultivation before 1830. Although its exact parentage is not known, it is

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believed to be predominantly *Vitis aestivalis* (Tarara and Hellman, 1991). 'Norton' has several desirable traits: it is highly disease resistant, more cold hardy than *V. vinifera* cultivars, adaptable to many different soil types, and has excellent winemaking qualities (Hedrick, 1908). It is a popular regional cultivar in Missouri, Arkansas, and southern Illinois where demand for plants far exceeds supply due to difficulty of propagation. 'Norton' is grown on its own roots and is normally propagated as hardwood or softwood cuttings. These cuttings root poorly, with 40% to 50% rooting considered excellent.

Micropropagation is a viable alternative method of propagating winegrapes. Deloire et al. (1995) studied the viticultural and enological characteristics of micropropagated 'Chardonnay' and 'Pinot noir' (*V. vinifera*) over 10 years. They reported some morphological differences in micropropagated plants characteristic of juvenility (deeper lobing in leaves, presence of a blush on shoots, and villosity on the undersides of leaves), lower production rates for the first 4 years after planting, and excessive vigor. These differences had disappeared after seven years. No differences were apparent in winemaking qualities.

The purpose of this study was to establish effective culture conditions for the in vitro multiplication and rooting of 'Norton' grape.

Materials and methods

ESTABLISHMENT OF STERILE CULTURES: Shoot tips from semihardwood shoots of field-grown 'Norton' were collected in mid-September 1999. The shoots were cut into single nodes which were surface sterilized by agitation for 35 min in 25% bleach (1.3% v/v NaOCl) with 50 drops/L (47 drops/qt) of the surfactant Triton (0.5% t-octylphenoxypolyethoxyethanol, Sigma-Aldrich Co., St. Louis, Mo.). Explants were rinsed twice in sterile distilled water. Buds were excised from the attached stem portion, and a 2-mm (0.08-inch) growing point was removed through the cut surface. Growing points were placed in 25-mm culture tubes containing 10 mL 75% C₂D salts, a basal medium developed specifically for grapes (Chee and Pool, 1983), supplemented with Staba vitamins (Staba, 1969), 100 $\text{mg}\cdot\text{L}^{-1}$ (ppm) myoinositol, 30 $\text{g}\cdot\text{L}^{-1}$ sucrose, 4 $\mu\text{mol}\cdot\text{L}^{-1}$ (0.90 $\text{mg}\cdot\text{L}^{-1}$) BA, and

6 $\text{g}\cdot\text{L}^{-1}$ agar, with the pH adjusted to 5.6 with 1 N potassium hydroxide (KOH) before autoclaving for 20 min at 121 °C (250 °F) and 1.27 $\text{kg}\cdot\text{cm}^{-2}$ (18 lb/inch²). Tubes were incubated in a growth room at 25 °C (77 °F) under cool-white fluorescent lights with a 16-h light/8-h dark photoperiod and a photon flux density of 131 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the agar surface. The resulting plantlets were maintained on the same medium in 200 mL (6.75 fl oz) glass jars (babyfood jars) and subcultured at 6-week intervals.

MULTIPLICATION: To determine effective concentrations of auxin and cytokinin for in vitro multiplication of 'Norton', the cytokinin BA and the auxin NAA were tested in a factorial arrangement. BA was added at 0, 2, 4, or 8 $\mu\text{mol}\cdot\text{L}^{-1}$ (0, 0.45, 0.90, or 1.80 $\text{mg}\cdot\text{L}^{-1}$) and NAA at 0, 0.5, or 1 $\mu\text{mol}\cdot\text{L}^{-1}$ (0, 0.09, or 0.19 $\text{mg}\cdot\text{L}^{-1}$). A completely randomized design was used, with 10 culture tubes per treatment. The experiment was repeated twice.

The basal medium consisted of 75% C₂D salts, Staba vitamins, 100 $\text{mg}\cdot\text{L}^{-1}$ myoinositol, 30 $\text{g}\cdot\text{L}^{-1}$ sucrose, and 6 $\text{g}\cdot\text{L}^{-1}$ agar. The pH was adjusted to 5.6 with 1 N KOH before autoclaving. Growth regulators were dissolved in dimethylsulfoxide (DMSO) and added before autoclaving. The control treatment contained DMSO only. Each treatment contained 0.37% DMSO. Media were dispensed into 25-mm culture tubes, 10 mL per tube, and autoclaved for 20 min at 121 °C and 1.27 $\text{kg}\cdot\text{cm}^{-2}$.

Shoots from in vitro stock plants were cut into single node cuttings with one attached leaf and planted one per tube with the axillary bud at the surface level of the medium. Tubes were incubated in a growth room as described above. Four weeks after planting, the explants were removed from the culture tubes and divided into individual shoots. The number of shoots that formed and the length of each individual shoot were recorded. Data were analyzed using the PROC GLM statement of PC-SAS for analysis of variance (SAS, 1996).

ROOTING: To determine an effective concentration of auxin for in vitro rooting of 'Norton', in vitro shoot tips were planted in culture tubes containing either full-strength or half-strength modified MS medium (Murashige and Skoog, 1962) with 0, 1, or 10 $\mu\text{mol}\cdot\text{L}^{-1}$ (0, 0.19, or 1.86 $\text{mg}\cdot\text{L}^{-1}$) NAA in a factorial

Table 1. Effect of 6-benzylaminopurine (BA) concentration on the quantity and length of shoots multiplying from a single node of 'Norton' grape in vitro.

BA level [$\mu\text{mol}\cdot\text{L}^{-1}$ (mg·L ⁻¹ or ppm)]	Shoots/ node (no.)	Shoot length (mm ²)
0 (0)	0.1 c ^y	0.4 c
2 (0.45)	0.8 b	4.4 b
4 (0.90)	1.8 a	7.6 a
8 (1.80)	1.5 a	5.3 b

^z25.4 mm = 1.0 inch.

^yMeans within columns with the same letter are not significantly different according to Student-Newman-Kuels at the 0.05 level.

arrangement. A completely randomized design was used, with 10 culture tubes per treatment. The experiment was repeated twice.

The full-strength basal medium consisted of MS salts supplemented with Staba vitamins, 100 mg·L⁻¹ myo-inositol, and 30 g·L⁻¹ sucrose. Half-strength medium consisted of this full-strength medium diluted 50% with distilled water. NAA was dissolved in DMSO. The control treatments contained DMSO only. The total DMSO per treatment was 0.019%. Growth regulators were added and pH was adjusted to 5.6 with 1 N KOH before autoclaving. Media were gelled with 6 g·L⁻¹ agar and 10 mL were dispensed into 25-mm culture tubes, which were autoclaved for 20 min at 121 °C and 1.27 kg·cm⁻².

Shoot tips about 15 mm (0.6 inch) long were taken from in vitro stock plants and planted one per tube. Tubes were incubated in a growth room as described above. Three weeks after planting, explants were removed from the tubes, the agar was rinsed

gently from roots, and the number of primary roots and the length of each root were recorded. Data were analyzed using the PROC GLM statement of PC-SAS for analysis of variance (SAS, 1996). Rooted explants were transplanted to soil and acclimated to ex vitro conditions under mist.

Results

MULTIPLICATION. There was a highly significant effect of BA on both the number and the length of shoots multiplying from a single bud of 'Norton' grape ($P < 0.0001$). There was no significant effect of NAA on shoot multiplication. The number of shoots forming per bud and the mean length of these shoots increased up to 4 $\mu\text{mol}\cdot\text{L}^{-1}$ BA (Table 1). At 8 $\mu\text{mol}\cdot\text{L}^{-1}$ BA the number of shoots per bud was the same as at 4 $\mu\text{mol}\cdot\text{L}^{-1}$ BA, but shoot length decreased significantly. We also observed hyperhydricity and fasciation of shoots at 8 $\mu\text{mol}\cdot\text{L}^{-1}$ BA, cultural disorders that have been linked to excessively high cytokinin levels in vitro

(Heloir et al., 1997).

ROOTING. Half-strength MS salts were far superior ($P < 0.0001$) to full-strength MS salts for both rooting and plant vigor (Table 2). On half-strength MS, more explants formed roots, more roots formed per explant, and these roots were longer than those forming on full-strength MS. In addition, the shoot portion of explants cultivated on half-strength MS continued to grow, resulting in healthy, vigorous plantlets, all of which survived transfer to soil and acclimation to greenhouse conditions. Explants grown on full-strength MS, even if they formed roots, died in vitro within 3 weeks.

NAA had a significant effect on root formation of 'Norton' in vitro on half-strength MS salts (Table 3). More roots formed at the highest level of NAA tested (10 $\mu\text{mol}\cdot\text{L}^{-1}$); however, NAA was not necessary for root formation in vitro. Roots forming on explants treated with NAA were shorter than those forming on untreated explants. NAA did not affect shoot elongation.

Discussion

In designing a micropropagation system for any plant, multiplication rate is only one factor to consider. Of equal importance is plant quality, including health and vigor of explants, ease of transition to soil, and lack of somaclonal variation. Often the highest multiplication rates are correlated with poor shoot quality.

One measurable indicator of shoot quality is shoot length. Although multiplication rates for 'Norton' did not dif-

Table 2. Effect of Murashige and Skoog (MS) salts on rooting of 'Norton' grape in vitro.

MS salt	Explants rooted (%)	Roots/ explant (no.)	Root length (mm)	Shoot length (mm ²)
Full strength MS salts	28 b ^y	0.6 b	5.4 b	4.5 b
Half strength MS salts	88 a	5.2 a	28.7 a	16.8 a

^z25.4 mm = 1.0 inch.

^yMeans within columns with the same letter are not significantly different according to Student-Newman-Kuels at the 0.05 level.

Table 3. Effect of naphthaleneacetic acid (NAA) concentration on rooting of 'Norton' grape in vitro on half-strength Murashige and Skoog (MS) salts.

NAA treatment [$\mu\text{mol}\cdot\text{L}^{-1}$ (mg·L ⁻¹ or ppm)]	Explants rooted (%)	Roots/ explant (no.)	Root length (mm)	Shoot length (mm ²)
0 (0)	85 ^{ns}	2.3 b ^y	35.0 a	36.0 ^{ns}
1 (0.19)	80 ^{ns}	2.9 b	29.7 ab	35.9 ^{ns}
10 (1.86)	100 ^{ns}	10.4 a	21.5 b	31.5 ^{ns}

^z25.4 mm = 1.0 inch.

^yMeans within columns with the same letter are not significantly different according to Student-Newman-Kuels at the 0.05 level.

^{ns}Nonsignificant at $P = 0.05$.

fer between 4 and 8 $\mu\text{mol}\cdot\text{L}^{-1}$ BA (Table 1), shoot length did significantly decrease at the highest level of BA. This indicates that although 8 $\mu\text{mol}\cdot\text{L}^{-1}$ BA promotes a high multiplication rate, this rate is less effective overall than 4 $\mu\text{mol}\cdot\text{L}^{-1}$ because the quality of the resulting shoots is low. Our data understate this effect slightly because we did not record shoots less than 0.5 cm (0.2 inches) in length. We observed numerous shoots of less than 0.5 cm in the 8 $\mu\text{mol}\cdot\text{L}^{-1}$ BA treatments.

Hyperhydricity (vitrification) and fasciation of tissues are cultural disorders that decrease the vigor and quality of explants. These disorders are thought to be caused in part by excessive cytokinins in the multiplication medium and are often associated with high multiplication rates (Heloir et al., 1997). We observed little hyperhydricity and fasciation of shoots in the 4 $\mu\text{mol}\cdot\text{L}^{-1}$ BA treatments; however, both disorders were common in the 8 $\mu\text{mol}\cdot\text{L}^{-1}$ BA treatments (data not shown). High multiplication rates are also associated with an increased incidence of somaclonal variation (Karp, 1989; Skirvin et al., 1994). 'Norton' is an old cultivar which has been vegetatively propagated for nearly two centuries during which time it has had ample opportunity to accumulate somatic mutations and maintain them as chimeras of varying complexity. Rapid multiplication in vitro can cause chimeras to break down, presumably due to instability of the meristem's layered structure (Skirvin et al., 2000). While our micropropagated 'Norton' plants appear to be uniform, we will observe them for several years under field conditions for the appearance of somaclonal variation.

Root quality of 'Norton' decreased at the highest level of NAA tested. Although more roots formed at 10 $\mu\text{mol}\cdot\text{L}^{-1}$ NAA compared with 0 and 1 $\mu\text{mol}\cdot\text{L}^{-1}$ NAA (Table 3), these roots were shorter. We also observed greater amounts of callus on explants rooted on the highest level of NAA, and we noted that the roots were brittle and broke easily when the in vitro plantlets were transferred to soil. At 0 and 1 $\mu\text{mol}\cdot\text{L}^{-1}$ NAA, explants formed similar numbers of roots, and these roots did not differ significantly in length between the two treatments, indicating that NAA can be omitted from rooting medium for 'Norton' grape.

Many commercial micro-propaga-

tion laboratories save time and money by eliminating an in vitro rooting step altogether. Instead, they transfer shoots directly from multiplication medium to soil, utilizing a mist bed or fogging system to keep humidity high until the shoots form roots and acclimate to ex vitro conditions. However, while in vitro 'Norton' shoots formed roots spontaneously on half-strength MS medium without NAA, they failed to root directly in soil under mist with or without NAA treatment (data not shown).

Once tissue culture-propagated plants were acclimated to ex vitro conditions and were growing vigorously, we took softwood cuttings to root conventionally under mist. One-hundred percent of these cuttings rooted with no NAA treatment. This may be a result of plant rejuvenation in tissue culture. Rejuvenation in vitro has been observed in several woody fruit crops, including plum (*Prunus insititia*), pear (*Pyrus communis*), apple (*Malus* ^[XimesX] *domestica*), and grape (*Vitis vinifera*) (Mullins et al., 1979; Howard et al., 1989a; Jones and Webster, 1989; Webster and Jones, 1989). Subsequently, some of these ex vitro plants demonstrated improved rooting. For example, more cuttings rooted from stock plants established from micropropagated plums and pears than from stock plants established by conventional propagation methods (Howard et al., 1989b; Jones and Webster, 1989). Further experiments are planned to determine the extent and duration of improved rooting from cuttings taken from micropropagated 'Norton'.

To summarize, micropropagation is a feasible method for propagating 'Norton' winegrape. 'Norton' is easily multiplied in vitro on 75% C₂D medium supplemented with 4 $\mu\text{mol}\cdot\text{L}^{-1}$ BA, rooted in vitro on half-strength MS medium without growth regulators, and acclimated to soil under mist. In addition, micropropagated 'Norton' plants may be useful as stock plants for conventional propagation.

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