12-2010

MICROPROPAGATION AND ACCLIMATIZATION OF 'NORTON' GRAPEVINE (*VITIS AESTIVALIS*)

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by

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A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Horticulture

Under the Supervision of Professor Paul E. Read

Lincoln, Nebraska

December, 2010

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University of Nebraska, 2010

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Propagation of the *Vitis aestivalis* cultivar 'Norton' (syn='Cynthiana') through traditional woody cuttings has been difficult. Rooting of woody cuttings has been a major hindrance in propagating this cultivar and providing enough plants to meet grower needs. *In vitro* propagation offers another method of increasing plant material. Cultures were established and maintained on Murashige and Skoog (MS) medium supplemented with 4 μM 6-benzyladenine (BA) and thiamine at 0.5 mg•L^{-1} and solidified with Difco-Bacto agar at 7.5 g•L^{-1}. The objectives of this study were to determine optimal methods for *in vitro* production and *ex vitro* establishment of 'Norton' plantlets. A factorial treatment with 4 concentrations of BA (0, 2, 4 & 8 μM) and 2 concentrations of naphthaleneacetic acid (NAA) (0 & 0.5 μM) was used for the multiplication study. Plantlets were acclimatized to *ex vitro* conditions without *in vitro* rooting. Plantlets were rooted *ex vitro* either with or without a 1000 ppm (0.1%) indolebutric acid (IBA) basal dip for 5 seconds. Auxin did not have a significant effect on explant growth or plantlet rooting. This demonstrates an improved method of *in vitro* propagation and acclimatization for efficient multiplication of 'Norton' grapevines.

I dedicate this thesis to my wife, Tami Bigger, whose love, encouragement, and support has helped me achieve my goals.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to his advisor, Dr. Paul E. Read, for his guidance, support, advice, encouragement, and most of all his patience. Thanks are also extended to the members of the graduate committee, Dr. Jay B. Fitzgerald and Dr. Scott J. Josiah, for their valuable comments and advice.

Appreciation is also extended to Virginia Miller-Roether and Hristina Stamenova-Berberova for their technical assistance, Dr. Steve Kachman for his statistical assistance, Vaughn Hammond, Sanjun Gu, and fellow graduate students from the Department of Agronomy and Horticulture for their friendship and support, especially Nick & Becky Iwig, Jeff & Jamie Fassett, and the late Bryan Terrell.

All my gratitude is extended to my wife, Tami; my daughters, Hailey and Hannah; my son, Blake; my parents, Garry and Betty Bigger; and the rest of my family & friends who have supported me throughout my life and have helped me achieve my goals. Special thanks are extended to my past research advisors and teachers (Dr. Paul E. Read, Dr. John J. Weiland, Dr. Zong-Ming Cheng, Dr. James Walla, Greg Ogard, Jim Schaffer and Woody Blasing) who inspired my curiosity in science and encouraged my pursuit of a fulfilling career in research.

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LITERATURE REVIEW

Grapevines (*Vitis sp.*) are grown worldwide for a variety of purposes including fresh fruit, juice, jams, jellies, wine, raisins and other processed products. The cultivation of grapevines dates back several thousand years and remains economically and socially important today. *Vitis* species are the most widely planted fruit crop in the world with an annual production of over 65 trillion tons valued at over $31trillion. The United States rank third in the world for both grape production and the value of the crop (Food and Agriculture Organization, 2009).

As members of the family Vitaceae, grapevines are perennial, woody vines that produce edible fruit. There are over a dozen genera with over 500 species. Of these, over 50 species belong to the genus *Vitis*. They have winding tendrils that generally arise opposite a leaf and their inflorescences are generally located in place of a tendril. Most members of this genus can be found in either warm or temperate regions of the world. *Vitis* species are diploid (2n=38). Most commercially important cultivars have perfect flowers, while unisexual male and female plants do exist. (Bailey, 1924; Mullins et al., 1992; Columbia Encyclopedia, 2008)

'Norton' is a historic cultivar that remains economically important to the Central Appalachian Mountain region and South-Central Great Plains area of the United States (Morton, 1985) and appears to have potential as far north as southern Iowa and Nebraska (Read et al, 2004; Saenz, 2001). It is the most widely planted cultivar in Missouri accounting for over 15% of the total acreage planted to grapevines (Missouri Grape Growers Assoc., 2001). Among its desirable characteristics are 1) exceptional fungal

disease resistance (Hendrick, 1908), 2) it is a vigorous plant that 3) its grapes produce a dark, full-bodied, red wine (Herald and Herald, 1998) 4) displays tolerance to Pierce's Disease (Kamas et al, 2000) and 5) and is very resistant to phylloxera (Hedrick, 1908).

The origins and history are vague, but recent historical and scientific research has helped remove some of that uncertainty. 'Norton' is believed to have resulted from a lost *Vitis vinifera* cultivar 'Bland' being pollinated by stray pollen, possibly from *Vitis aestivalis* (Ambers and Ambers, 2004). It was first described by Prince in 1830. Appropriately described by the Latin "aestival: of or relating to summer" since "summer grape" is one of its common names of which it has many: Norton, Cynthiana, Virginia's Seedling, and Norton's Virginia Seedling.

Since the Civil War era there has been controversy regarding who was responsible for discovering, breeding or selecting 'Norton'. At that time, F. W. Lemosy came forward with a story attributing the discovery of the 'Norton' grape to his father, Dr. F. A. Lemosy in 1835 or 1836 on Cedar Island in the James River. His father then told Dr. Daniel Norton about this grape, and Norton dug up the plant and took it away. This vine was supposedly the one that became known as 'Norton' (Ambers and Ambers, 2004; Lemosy, 1861). As Hedrick (1908) explained, every aspect of this story is in direct conflict with the discussion of the 'Norton' grape provided by Prince (1830) particularly since the discovery of the wild grape occurred in the mid-1830s. Yet this unlikely version of actual events was perpetuated by Munson in *Foundations of American Grape Culture* (1909) normally a reliable source, he stated that 'Norton' was "found wild on Cedar Island, in James River, near Richmond, Va., in 1835, by Dr. F. A. Lemosque [sic],

and named and introduced by Dr. D. N. Norton of Richmond." This error has unfortunately been propagated to the present day, as recounted by Roberts (1999). A short review of the debates surrounding the history of 'Norton', its common names and the quality of its wines was written by Tarara and Hellman (1991). A more detailed investigation into the origins of 'Norton' and its possible parentage is presented by Ambers and Ambers (2004).

'Norton' has a bit of a celebrity status in the popular culture of grapes and wines as evidenced by two non-technical, leisure-reading books (Kliman, 2010; Roberts, 1999) and magazine articles about either the wine or the grapevine too numerous to list. 'Norton'/'Cynthiana' grape (*Vitis Aestivalis*) was adopted as the official grape for the state of Missouri on 11 July 2003 (Missouri Code of State Regulations, 2010).

'Norton' and 'Cynthiana' were previously considered distinct cultivars or 'Cynthiana' possibly being a sport of 'Norton'. But recent isozyme and genetic analysis has shown they are the same cultivar. Minor differences in ripening times, ampelologic characteristics and other perceived differences that have been noted between these two cultivars are most likely attributed to the multitude of biotic and abiotic influences acting upon individual plants and is no more prevalent or dramatic than what is observed with some other cultivars. (Parker et al, 2005; Reisch et al, 1993; Stover, 2009)

One of 'Norton's' significant downfalls is how recalcitrant this cultivar is to the traditional method of grapevine propagation through dormant woody cuttings. The difficulty in propagating this cultivar led Nicholas Longworth, one of Ohio's most prominent viticulturists, to declare in 1850, "'Norton' is worthless as a wine grape

because of the vine's difficult propagation…" (Hasmann, 1867). In fact, propagation rates below 15% have been reported (Avery, 1999). Recent progress has been made in this area using bottom heat or synthetic auxin either alone or in combination. Success rates of dormant woody cuttings lag behind most other grapevine cultivars and continue to result in limited stocks available for growers to plant. (Keeley & Preece, 2000; Keeley et al, 2004; Matsui et al, 2003; Portz et al, 2005)

Other methods for propagating grapevines are softwood cuttings, layering and *in vitro* plant tissue culture. Each of these methods has advantages over the others depending on specific conditions and desired outcomes. Softwood cuttings are similar to dormant cuttings, except the cuttings are made from actively growing shoots and often require misting or other method of maintaining increased humidity while the cuttings develop their own roots (Avery, 1999). Propagation by the layering method involves burying a short length of a shoot until it sprouts and shows root growth from the buried section. At that point the short vine section that has sprouted is severed from the mother plant and replanted (Hartmann et al, 2002). *In vitro* propagation (micropropagation or tissue culture) requires specialized facilities, equipment and expertise, but can potentially yield exponentially more plants (Terregrosa et al, 2001).

The first report of *in vitro* culture of grapevines was by Morel (1944). Since that early study, culture of callus, production of protoplasts, development of somatic embryos, regeneration via organogenisis with or without a callus phase, and multiplication through axillary bud or nodal culture have all been attempted with varying results and

achievements. Several reviews of the topic have been published (Krul and Mowbray, 1984; Lumsden et al, 1994; Read, 2007; Sharp et al, 1979; Torregrosa et al, 2001).

Rapid multiplication of grapevine plantlets has been achieved through nodal cuttings containing a single axillary bud (Buyukdemirci, 1997; Ikten, 2000; Minal-Mhatre et al, 2000; Nas et al, 2005; Tapia and Read, 1998). This method consistently yields true-to-type plants by avoiding potential somaclonal variation associated with hyperhydricity (vitrification) caused in part by excessive cytokinins in the multiplication medium (Heloir, et al, 1997). High rates of multiplication have also been associated with increased incidence of somaclonal variation (Karp, 1999; Skirvin et al, 1994)

Nodal tissue culture of grapevines can propagate elite or scarce varieties much quicker than traditional methods (Torregrosa et al, 2001). Grapevines established from micropropagated plants may display signs of plant juvenility or excessive vigor for a few years. These characteristics diminish until the plants are indistinguishable from traditionally propagated plants after several years (Deloire et al, 1995; Martinez and Mantilla, 1995; Mullins et al, 1979). This method has also proved useful for other *Vitis* species such as Muscadine (*Vitis rotundifolia)* that were recalcitrant to propagate through dormant woody cuttings (Lee and Wetzstein, 1990).

Micropropagation of ‘Norton’ has previously been described. Qiu et al (2004) manipulated several culture conditions devised for *Vitis vinifera* species to facilitate the propagation of ‘Norton’ microshoot tips for virus elimination. Norton and Skirvin (2001) reported success initiating, proliferating and rooting cultures *in vitro* and establishing the plants *ex vitro*. They were unable to root the plantlets directly in potting medium.

Rooting plantlets directly into potting medium is preferable because: 1) it eliminates the time, material and labor required for an *in vitro* rooting step 2) roots developed *in vitro* can be difficult to manipulate and are easily broken (Norton and Skirvin, 2001) 3) in other woody species *ex vitro* formed roots are anatomically and morphologicly superior to those formed *in vitro* (McClelland et al, 1990) . The removal of *in vitro* formed roots before planting in soil has shown to improve the *ex vitro* growth of micropropagated grapevines (Thomas and Ravindra, 1997). One conflicting report suggests that roots developed *in vitro* contribute to plantlet growth during acclimatization (Gribaudo, 1995).

An important, and often limiting, part of any micropropagation system is the transition from *in vitro* to *ex vitro* growth or acclimatization (Lewandowski, 1991; Pierik, 1988). Several factors affect survival of microcuttings and successful transition from a largely heterotrophic state to a complete autotrophic plant (Thomas, 1998). Size of the microcutting often dictates its vigor and ability to withstand the stress of acclimatization (Mohammed and Vidaver, 1990). Relative humidity of the culture environment influences the severity of desiccation to the microcutting while the cuticular structure of *in vitro* leaves develops further and thickens (Marin and Gella, 1998; Marin et al, 1998).

During successful acclimatization, relative humidity levels gradually decreases from the high humidly of *in vitro* culture vessels to the lower humidity of greenhouse or field conditions (Preece and Sutter, 1991; Read and Fellman, 1985). Methods employed to manipulate relative humidity to the desired levels for acclimatization include: mist bed, fogging system, enclose microcuttings in plastic bags (i.e.: sachet system), and plastic

containers (such as sundae cups) (Nas and Read, 2003; Norton and Skirvin, 2001; Ravindra and Thomas, 1985).

Success of any micropropagation system to propagate ‘Norton’ grapevines is not only determined by the number of plants that are multiplied *in vitro* or established in the field, but if those plants maintain trueness to type and continue genotypic and phenotypic traits of the donor plant.

INTRODUCTION

The American hybrid *Vitis* spp. 'Norton' is a premium wine grapevine for use in the central Midwest (Reisch et al., 1993). It has several desirable characteristics, but difficulty associated with propagation has limited its use in vineyards (Tarara and Hellman, 1991). Grapevines are traditionally propagated from cuttings of dormant one-year-old canes (Hartmann et al., 2002). Propagation of 'Norton' through this method has proven difficult because cuttings root poorly (Avery, 1999). *In vitro* propagation offers another method of increasing plant material for this cultivar. Micropropagation of 'Norton' has previously been reported (Norton and Skirvin, 2001). The goal of this project was to develop a system for improved *in vitro* plantlet quality and acclimatize plantlets without an *in vitro* rooting step. Potential benefits of this research include increased plant material available for growers at a lower cost and an increase in availability of this grape for wineries.

MATERIALS AND METHODS

In Vitro Culture Establishment and Maintenance

Greenhouse-grown 3-year-old potted plants with actively growing shoots (30-50 cm in length) were used as source material. Axillary buds (0.5 X 0.5 cm) were excised, then surface disinfested for 15 minutes in a 10% commercial bleach solution and washed three times for 5 minutes in sterile water. Single explants were placed in 25mm culture tubes containing 10 ml Murishige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 4 μM 6-benzyladenine (BA) and thiamine at 0.5 $mg \bullet L^{-1}$ and solidified with Difco-Bacto agar at 7.5 $g \bullet L^{-1}$ (Buyukdemirci, 1997). Established cultures were transferred monthly to fresh medium before experiments were begun. Explants were propagated placing individual single-node segments containing an axillary bud, with leaves removed, horizontally on the medium in a culture tube. Cultures were maintained at 23±1^{o}C for 16 hours per day under cool white florescent light (28 $\mu mol \bullet s^{-1} \bullet m^{-2}$) in the laboratory culture room.

In vitro Propagation and Multiplication

MS medium supplemented with BA (0, 2, 4, or 8 μM), NAA (0 or 0.5 μM), thiamine at 0.5 $mg \bullet L^{-1}$ and solidified with Difco-Bacto agar at7.5 $g \bullet L^{-1}$ (pH 5.6±0.1) was used in all multiplication experiments. Explants were incubated as described above.

Ex vitro Acclimatization

Microcuttings excised from four to six week old cultures with basal portions (callus, roots, tissue from previous subcultures) removed at approximately the level of the growth medium were used. Microcuttings, either with or without a five second basal (0.5 cm) dip in 1000 ppm (0.1%) indole-3-butyric acid (IBA), were transferred to hydrated peat pellets (Jiffy 9, Jiffy Products Ltd., Shippagan, Canada) within plastic sundae cups with lids (cup: DSD8X & lid: LD8-58, Sweetheart Cup Company, MD, USA) for rooting and acclimatization under the same temperature and light intensity used for culture establishment and multiplication (Nas and Read, 2003). The planting medium was gently packed around the basal portion of the microcutting after it was inserted into the pre-made hole of the peat pellet. After four days, plantlets and peat pellets were planted in potting medium (34% peat, 31% perlite, 31% vermiculite, 4% soil) in 1 L plastic pots and transferred to the greenhouse under the partial shade of heavy white cloth. Supplemental lighting was not used because these experiments were conducted in late spring and early summer. Sundae cup lids were used to maintain the relative humidity surrounding the microcutting. Relative humidity under the sundae cup lids was gradually reduced to ambient levels by lifting one side and resting the tilted lid against the side of the pot. Lids were initially tilted to ~15^{o} angle. Each week, the lids were tilted an additional ~15^{o} until after four weeks the plantlets were fully acclimatized. Each microcutting was inspected at least every two days for signs of wilting or excessive desiccation. For those microcuttings, the acclimatization process was slowed by temporarily adjusting the

sundae cup lid to a lower angle and increasing the relative humidity surrounding that microcutting.

Experimental Design

Single explants placed in culture tubes were cultured as described above. Ten replications were assigned to each treatment in a factorial arrangement. The experiment was a completely randomized design and conducted twice. Data were collected after four weeks and statistically analyzed using SAS V8 GLM (SAS, 1999). Separation of treatment means was done by LSD at alpha 0.05 level.

RESULTS AND DISCUSSION

Establishment

In three different attempts, a total of ninety-three cultures were initiated with a contamination rate of 4.3 % (4 of 93) and 61.3 % (57 of 93) of the cultures became fully established and growing *in vitro* after five weeks as shown in Figures 1 and 2. The relatively low amount of contamination in initiated cultures may be attributed to harvesting tissue from healthy, actively-growing, greenhouse-grown stock plants and use of an effective disifestation procedure. For the cultures that did not become established, the disinfestations treatment may have been too harsh.

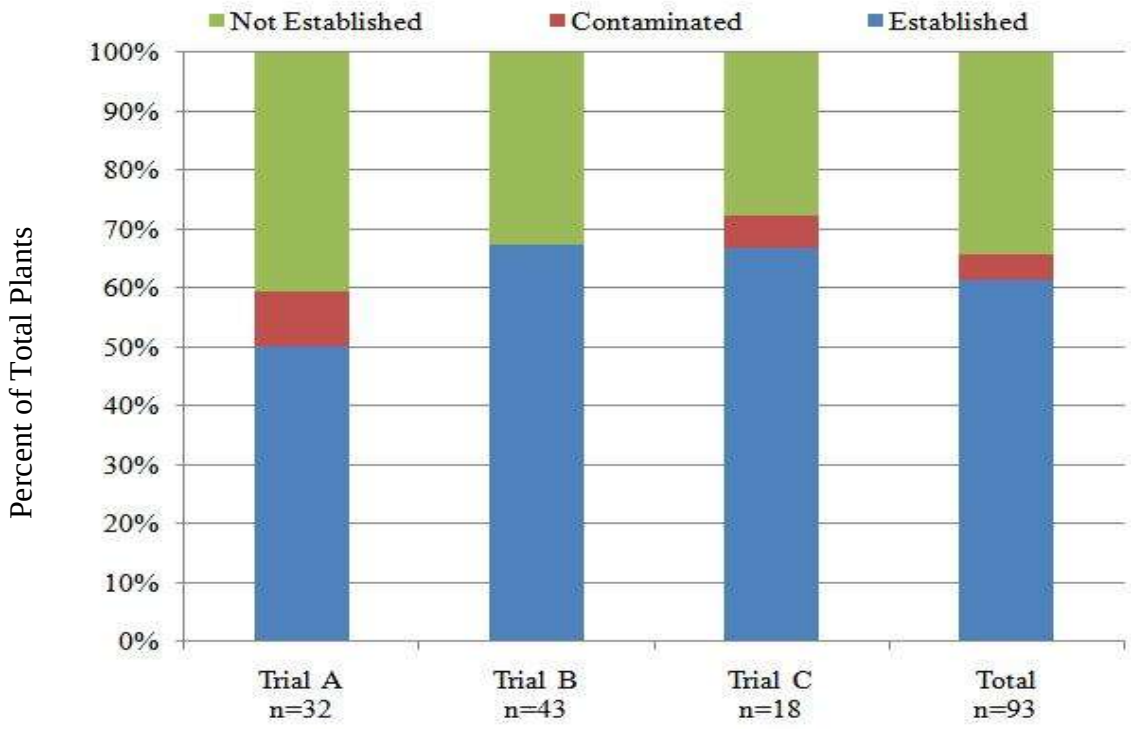

Figure 1

Establishment of *in vitro* cultures from excised axillary buds of 'Norton' grapevine on modified MS medium

Figure 2

In vitro cultures of ‘Norton’ grapevine plantlets two weeks (culture on the left) and four weeks after subculture on modified MS medium

Multiplication

The effects of cytokinin, auxin, and interaction of cytokinin x auxin on number of shoots per explant and number of axillary buds per shoot are presented in Figure 3 and Table 1. Auxin did not have an effect on either the number of shoots per explant or the number of nodes per shoot. The interaction between cytokinin and auxin was not significant.

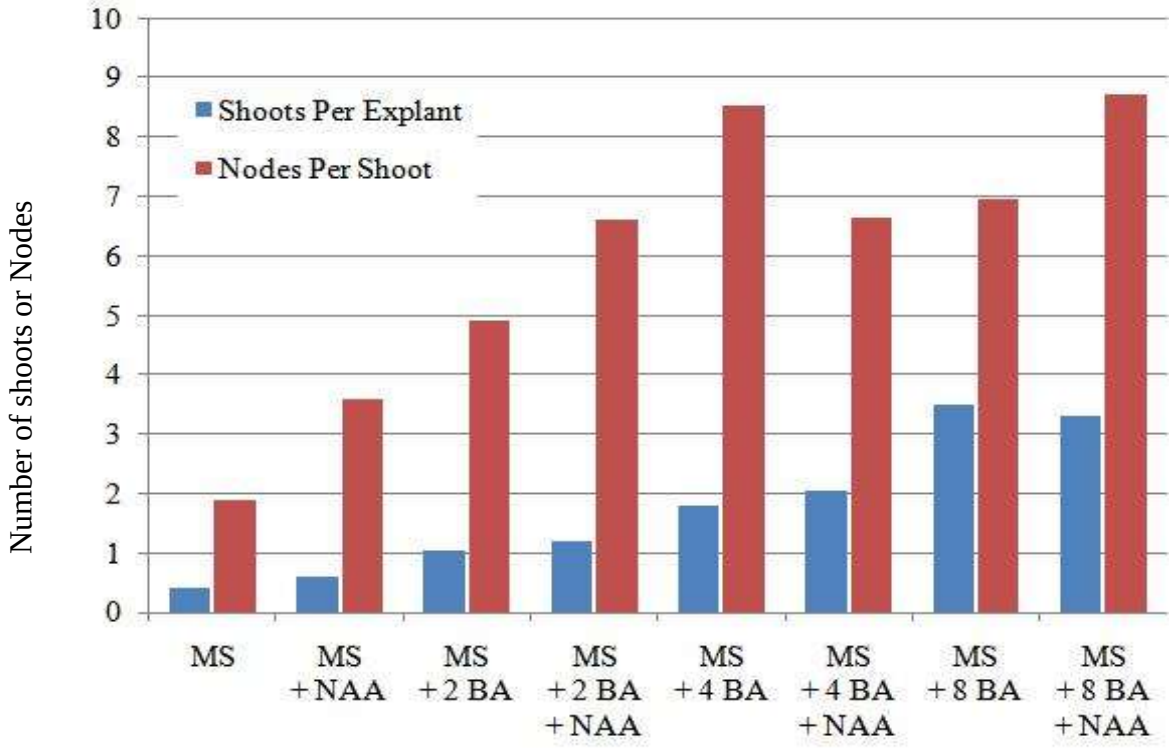

Figure 3

Effect of various concentrations of benzyladenine (BA) and naphthaleneacetic acid (NAA) on growth of 'Norton' grapevine cultured *in vitro* on modified MS medium.

Table 1

Generalized linear model for dependent variables: number of shoots per explant and number of axillary buds per shoot of 'Norton' grapevine cultured *in vitro* on modified MS medium with various concentrations of 6-benzyladenine (BA) and naphthaleneacetic acid (NAA)

		number of shoots per explant	axillary buds per shoot
Source	DF	F value	F value
BA	3	63.96****	22.58****
NAA	1	0.41 ^{NS}	2.75 ^{NS}
BA x NAA	3	0.42 ^{NS}	3.34**

, ** significant F-value at P<0.05 and 0.0005 level
^{NS} nonsignificant at P>0.05

Acclimatization

Although treatment of microcuttings with a basal dip in IBA resulted in a slightly higher rate of acclimatization and *ex vitro* establishment, the difference is not statistically significant. Ninety-eight percent of microcuttings with basal dip in IBA survived and were growing vigorously versus 92% without the treatment (Figure 4). The improvement is marginal and is not enough to justify the added cost of treatment. Plantlets cultured under the relatively low light conditions of the culture room can produce endogenous levels of auxin and contribute to the rooting of plantlets (Economu and Read, 1986). Plantlets rooted quickly and produced 3-7 strong, vigorous roots and shoots (Figures 5 and 6). The high establishment rate of the microcuttings to *ex vitro* conditions may be attributed to the plantlets regaining some juvenile characteristics while in culture and/or endogenous auxin levels in the microcutting.

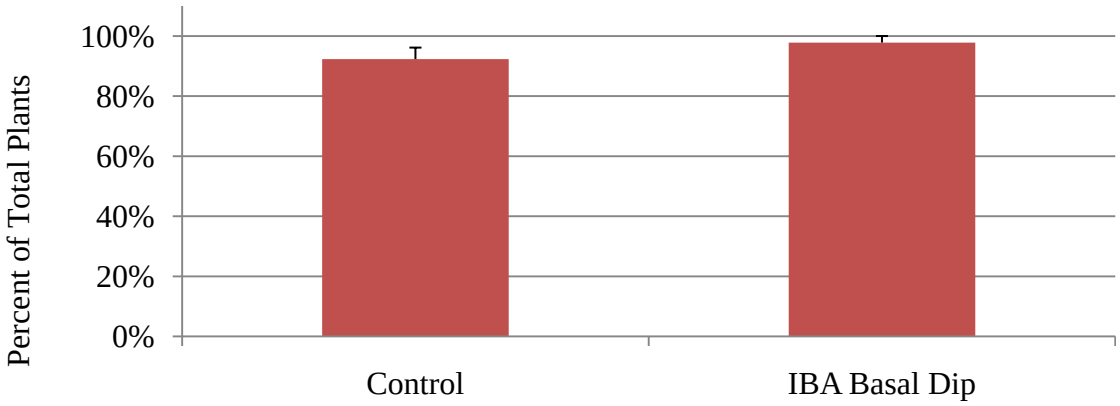

Figure 4

Effect of 0.1% indole-3-butyric acid (IBA) basal dip on establishment of *in vitro* microcuttings of 'Norton' grapevine to *ex vitro* conditions

Figure 5

Ex vitro rooting and acclimatization of 'Norton' microcuttings

Microcutting planted in a hydrated peat pellet (left) and after 7 days in enclosed sundae cup (right). Note new root growth emerging from pellet (arrow).

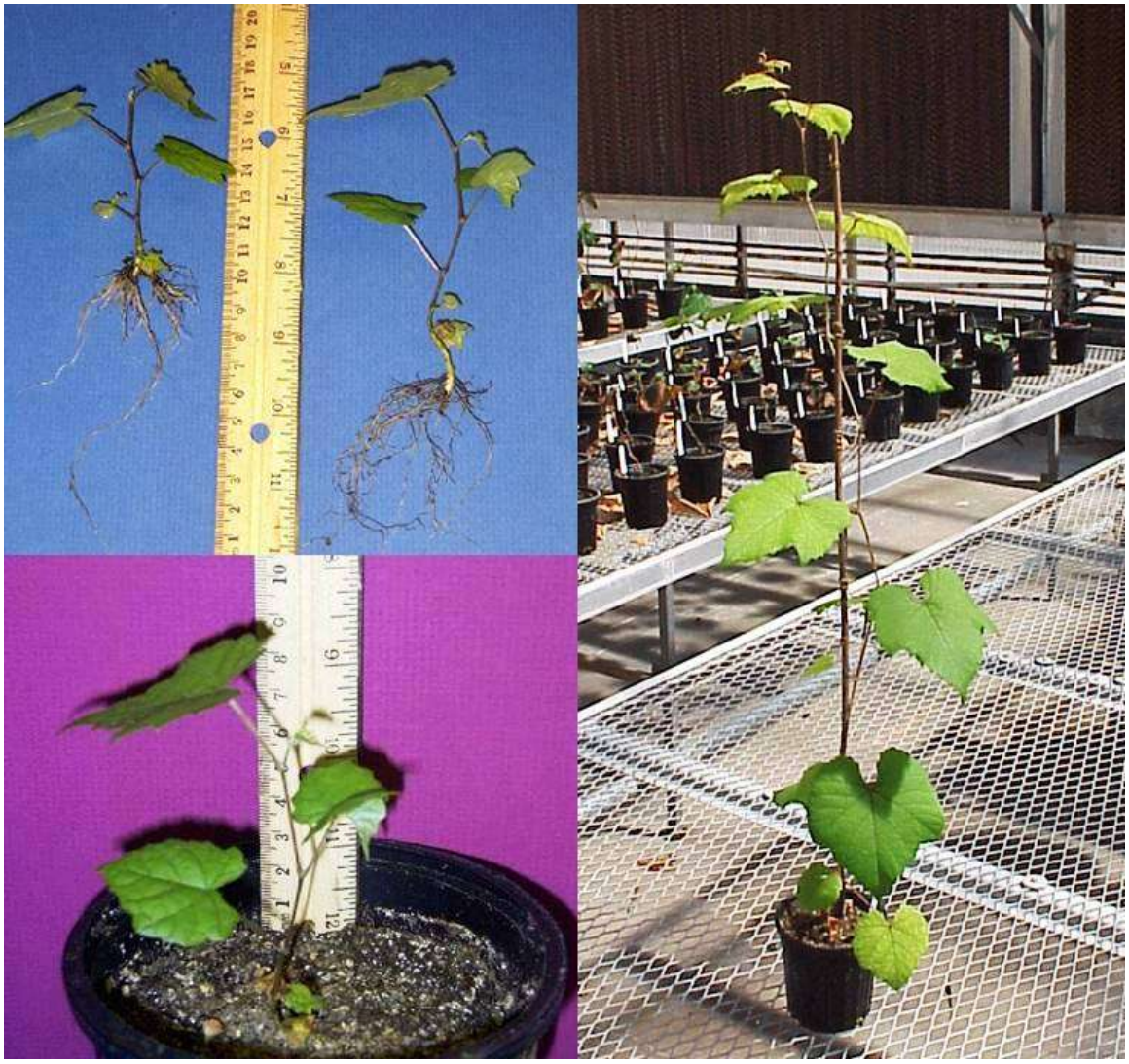

Figure 6

Shoot and root growth of established ‘Norton’ microcuttings

Root growth (top left) and fully acclimatized microcutting after 4 weeks (bottom left).

Established grapevine three months after *ex vitro* rooting of microcutting (right).

CONCLUSION

Tissue culture presents a method of efficient propagation of 'Norton' grapevine. Explants respond with more shoots and more nodes per shoot with increasing concentrations of cytokinin. High concentrations of cytokinin (8 μM BA) produce plantlets of lower quality than lower concentrations (2 & 4 μM BA). Auxin did not play a significant role in explant growth or microcutting rooting at the concentrations tested. Although Norton and Skirvin (2001) were unable to directly root microcuttings in potting medium without an *in vitro* rooting step, we successfully acclimatized microcuttings directly to peat pellets. The high rate of acclimatization and establishment of the microcuttings is likely due to several factors: 1) endogenous levels of auxin in the microcuttings allowed them to root easily, 2) rejuvenation of the cultured plantlets, and 3) attention to detail during the acclimatization steps. Producing acclimatized plantlets is quick and efficient with simple rooting procedures that does not include an *in vitro* rooting step. Although continued research is needed to evaluate field and grape characteristics of tissue culture derived 'Norton' grapevines, micropropagation has the potential to lower establishment costs for growers and increase grape availability for wineries.

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APPENDIX

Appendix 1

Propagation of softwood and woody cuttings of 'Norton' grapevine

Softwood and woody cuttings of 'Norton' grapevine were collected from field grown plants at the University of Nebraska Viticulture Program Research Vineyard located on the Kimmel Orchard near Nebraska City, NE in June 2002. One set of softwood cuttings was prepared from distal portions of the actively growing shoot that included the shoot tip and two additional nodes. Another set of softwood cuttings was prepared from the next three proximal nodes of the shoot. For both sets of softwood cuttings, the bottom two leaves and petioles were removed. The top leaf was trimmed to approximately 100 cm^2 to reduce water loss to transpiration. Woody cuttings were prepared from current season growth that had hardened and formed bark. All leaves and petioles were removed from the woody cuttings. All cuttings were treated with a 5 second basal dip (1 cm) of NAA (0, 0.05% or 0.2%) prior to planting in a soilless mix (50% perlite : 50% vermiculite) on a mist bench (1 minute mist every 10 minutes) in the greenhouse. Cuttings were allowed to root for 4 weeks before shoot and root growth was evaluated. Greenwood cuttings from the proximal portion of growing shoots had a higher percentage of signs of growth (shoot growth, root growth, or callus formation) and more roots than either woody cuttings or greenwood cuttings from the shoot tip (Figures 7 and 8).

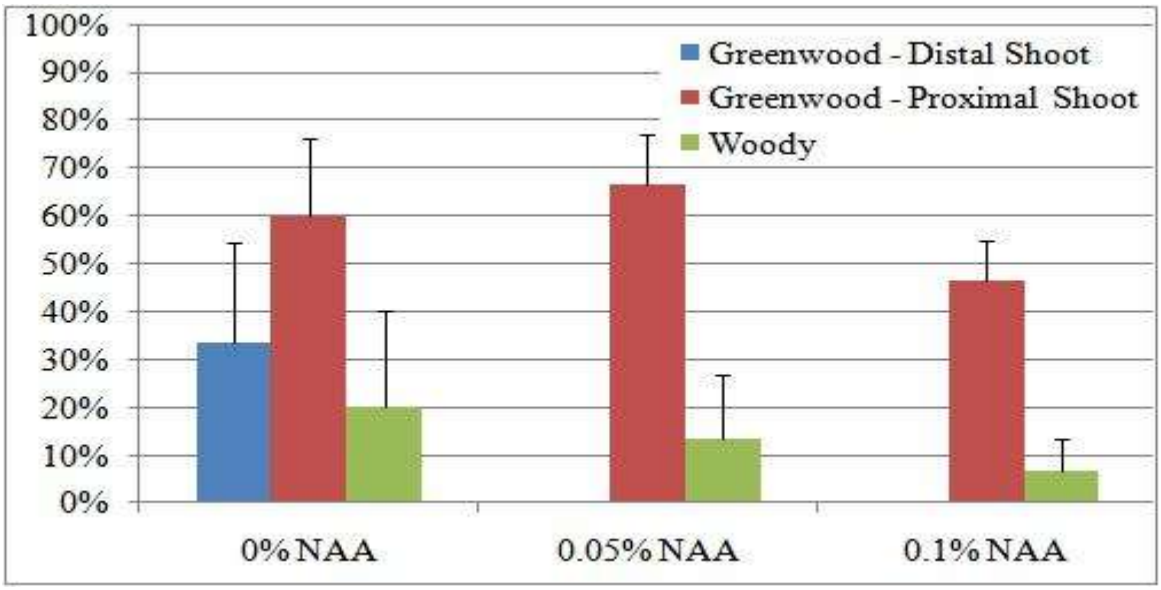

Figure 7

Growth of cuttings from actively growing greenwood and woody tissue treated with basal dip of naphthaleneacetic acid (NAA) and rooted in a mist bench

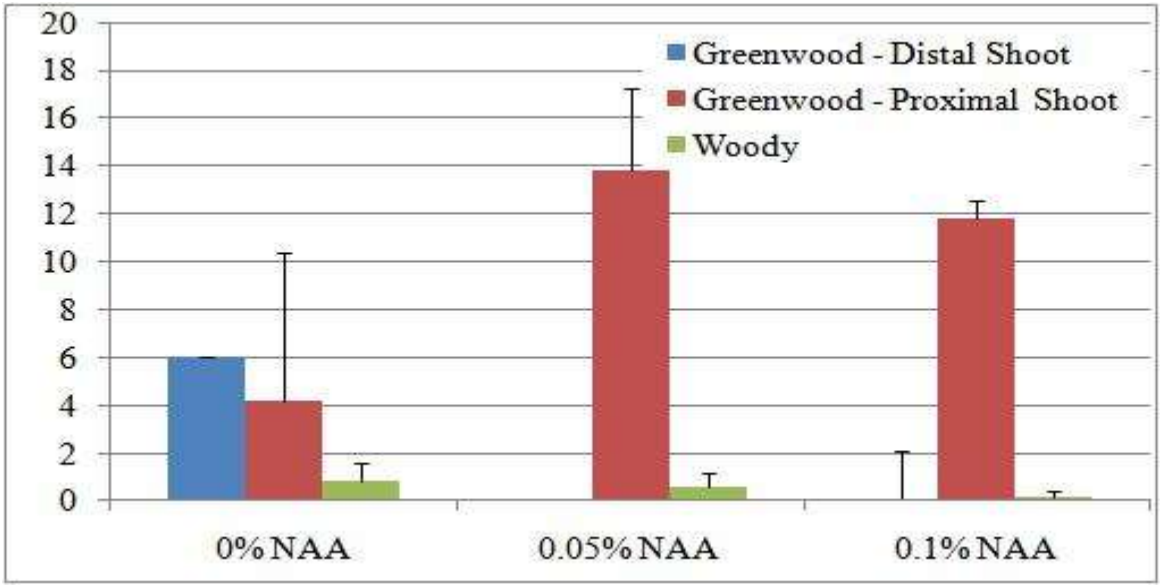

Figure 8

Rooting of cuttings from actively growing greenwood and woody tissue treated with basal dip of naphthaleneacetic acid (NAA) and rooted in a mist bench

Treatment with NAA also increased average root length that grew from the proximal greenwood cuttings. While relatively high rates of root and callus growth were achieved from these cuttings, none had produced new shoot growth after one month. While propagating 'Norton' through rooting cuttings from actively growing shoots on a mist bench may produce a small number of additional plants, the high input costs of a greenhouse and a misting system does not make it a viable option. While Avery (1999) was able to achieve limited success using similar treatments and growing conditions, we were not as successful in this attempt. These results are from a single field collection and need to be repeated over several more years. Additional work to determine optimal auxin concentrations, timing of harvesting cuttings and allowing longer for growth of the cuttings may increase the success of rooting cuttings of actively growing 'Norton" shoots.

Appendix 2

Micropropagation of Several Hybrid *Vitis* Species

Several cultivars of hybrid *Vitis* species were established in tissue culture and maintained *in vitro* on modified MS medium for up to two years. These red or white wine cultivars (Table 2) are either currently being grown in Nebraska or show promise as suitable for Nebraska's variable climate (Read et al, 2004). Methods for establishing and maintaining these cultivars were described earlier in the MATERIALS AND METHODS. The described method was employed with similar rates of success as for 'Norton' and shows the flexibility of the method and its utility in a range of hybrid *Vitis* species including: *V. vinifera*, *V. aestivalis*, and *V. riparia*. The described methods may also work equally well on other *Vitis* species.

Table 2

Cultivars of hybrid *Vitis* grapevine species initiated and maintained in *in vitro* culture on modified MS medium

Red Wine Cultivars	White Wine Cultivars
'Chancellor'	'Frontenac Gris'
'Frontenac'	'La Crescent'
'Marechal Foch'	'Lacrosse'
'Valiant'	MN 1162 (Univ. of MN unnamed selection)
	'Prairie Star'

Appendix 3

Proposed Future Research

Future areas of research involving micropropagation and acclimatization of 'Norton' grapevine should focus on following *in vitro* derived plants from the culture container, to the greenhouse and finally to field to study the long-term field performance of these plants. Several groups (Deloire et al, 1995; Martinez and Mantilla, 1995; Mullins et al, 1979) have previously shown that while mature grapevines can and do regain some juvenile characteristics *in vitro*, those juvenile traits are quickly lost once the plant is growing in field conditions. While the same trend would be expected of 'Norton', it should be confirmed through replicated trials. Trials that evaluate the quality of plants, growth characteristics, fruit quality and wine from *in vitro* derived 'Norton' grapevines should alleviate growers' and winemakers' concerns about putting these plants and fruit in their vineyards and their wine bottle.