# *IN VITRO* CULTURE OF 'DOG RIDGE' GRAPEVINE

A Senior Scholars Thesis

by

KAH-YAT ISAAC WONG

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Horticulture

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Approved by:

Research Advisor: R. Daniel Lineberger
Associate Dean for Undergraduate Research: Robert C. Webb

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# ABSTRACT

*In Vitro* Culture of 'Dog Ridge' Grapevine. (April 2009)

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*Vitis champini* 'Dog Ridge' grapevine is a potentially desirable rootstock for Texas grapevines because of its disease resistance. This selection is reported to be difficult to root through hardwood cuttage as is often practiced for grapevine. A study was undertaken to establish a protocol to propagate 'Dog Ridge' grapevine *in vitro* by comparing combinations of explant type, basal salts, and benzyladenine (BA) concentration to proliferate shoots followed by *in vitro* and *ex vitro* rooting. Shoot tip and axillary bud explants were harvested from actively growing stock plants, disinfested with 10% v/v Clorox, rinsed in sterile distilled water and cultured on either Murashige and Skoog (MS) or Woody Plant Medium (WPM) containing 0, 4.4 and 8.8 μM BA for 12 weeks. Axillary bud explants cultured on MS medium proliferated better than shoot tips. Axillary bud explants cultured on media containing 4.4 or 8.8 μM BA proliferated better than shoot tip explants regardless of the BA concentration in the medium. Tissue cultured shoots rooted in either WPM medium without BA *in vitro* or in Redi-earth®

potting mix *ex vitro*. Shoots developed roots *in vitro* better than under *ex vitro* conditions.

# DEDICATION

For Dr. Lineberger and the Department of Horticultural Sciences, TAMU.

Thank you for providing me with more than just an education in horticulture.

# ACKNOWLEDGMENTS

First, I must thank Dr. R. Daniel Lineberger, who served as my research advisor, student organization advisor, work supervisor, teaching professor, mentor and friend. No one person has influenced the course of events during my college career as much as he has. I am blessed to have the support of such a dedicated advisor.

I must acknowledge Dr. George Ray McEachern, who inspired the project out of his desire to proselytize 'Dog Ridge' grapevine as a rootstock for Texas vineyards, Dr. David Reed and Matt Kent for providing me with greenhouse space and assistance in the maintenance of my stock grapevines, Dr. Michael Arnold who provided expertise and patience in the statistical analysis of my data, and Dr. Creighton Miller's lab who continues to be a good neighbor from whom I have borrowed many supplies. Also, I thank the College of Agriculture and Life Sciences Quality Enhancement Program Council for their financial support to present this research at ASHS conferences.

My gratitude also goes to Alicia Ford, my former co-worker, and my friend, Terry Felderhoff, who have always been gracious in assisting me with my research. Also, the faculty and staff at the Department of Horticultural Sciences, and my friends, especially the members of the TAMU Horticulture Club, past and present, must be acknowledged for their support. Finally, I thank my family for their unconditional love and support, and God, through whom all things have been made possible.

# NOMENCLATURE

| | |
|---|---|
| BA | Benzyladenine |
| MS | Murashige and Skoog media |
| WPM | Woody Plant Media |

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# CHAPTER I

# INTRODUCTION

## An overview of 'Dog Ridge' grapevine

'Dog Ridge' grapevine is a seedling selection of *Vitis champini*. It is a native rootstock discovered by T. V. Munson in Bell County, Texas near its namesake, the Dog Ridge mountains (Mcleroy and Renfro, 2004; Pongrácz, 1985). 'Dog Ridge' grapevine shows potential as a Texas rootstock (G.R. McEachern, personal communication) because it produces great vigor in the grafted vine, and is noted to produce quality fruit in sandy, nematode-infested soils (Winkler, 1974). It also has reported resistance to Pierce's Disease and phylloxera (Loomis, 1965; Pearson and Goheen, 1988). However, 'Dog Ridge' grapevine is not reproduced easily in Texas by the current propagation technique of cuttage because it roots with great difficulty (Pongrácz, 1985; Winkler, 1974). Numerous growers in the Texas vineyard industry have failed in their attempt to propagate this selection successfully (G.R. McEachern, personal communication).

## *In vitro* propagation – an alternative method

*In vitro* propagation is an alternative method to propagate grapevines. Numerous methods for grapevine *in vitro* propagation have been described

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This thesis follows the style of HortTechnology.

(Chee et al., 1984; Gray and Fisher, 1985; Harris and Stevenson, 1982). "*In vitro* propagation can be used to rapidly produce large numbers of disease-free clones, and is economically feasible if the demands for a species or cultivar are enough to justify cost" (Gray and Fisher, 1985). *In vitro* propagation also promotes rejuvenation; and an effect of rejuvenation is an increase in rooting capabilities (Howard et al., 1989a; Howard et al., 1989b; Webster and Jones, 1989). Rejuvenation has been reported in grapevines (Mullins et al., 1979), and *in vitro* propagation has been successfully used to rejuvenate difficult-to-propagate grapevines. 'Norton' grapevine cuttings, taken from conventionally propagated stock plants, only achieve 40 to 50% rooting. However, cuttings taken from *in vitro* propagated stock yielded one hundred percent rooting. Similarly, 'Norton' microcuttings rooted *in vitro* have achieved one hundred percent rooting (Norton and Skirvin, 2001).

**Media type**

A factor that must be considered when propagating a plant species *in vitro* is the type of medium to use. The medium is comprised of basal salts and essential nutrients that a plant requires for proper growth and development. *In vitro* culture techniques involving the use of high- and low-salt media, such as Murashige and Skoog (Murashige and Skoog, 1962) (MS) medium and Woody Plant medium (Lloyd and Mccown, 1981) (WPM), have been described. MS is a widely used high-salt medium, and has been successfully used to propagate *Vitis* cultivars (Chee et al., 1984; Gray and Fisher, 1985).

By comparison, WPM is an alternative low salt medium developed to propagate cultures of birch, rose, rhododendron, oak and other species (Lloyd and Mccown, 1981).

**Benzyladenine concentration**

Cytokinins are often used in tissue culture media to help induce shoot growth and proliferation. Benzyladenine (BA) is a commonly used cytokinin to propagate *Vitis* species (Chee et al., 1984; Gray and Fisher, 1985; Norton and Skirvin, 2001). However, different concentrations are often required to yield the optimum shoot proliferation. "Optimum cytokinin concentration is cultivar dependent and should be determined on a case-by-case basis" (Gray and Klein, 1989). 0, 4.4 µM, and 8.8 µM (0, 1 mg/L, and 2 mg/L) are commonly used levels in the experimental determination of optimum cytokinin concentration (Maxwell, 2004).

**Explant type**

Many different explants sources can be used successfully in tissue culture. Shoot tips are widely used explants in *Vitis* tissue culture (Chee et al., 1984; Li and Eaton, 1984). Protocols to propagate 'Dog Ridge' grapevine described by Gray exclusively involve the use of shoot tips (Gray and Fisher, 1985). However axillary buds (nodal cuttings) have also been used in the propagation of plant species such as walnut (Driver and Kuniyuki, 1984), apple (Dutcher and Powell, 1972), pecan (Hansen and Lazarte, 1984), and grapevines (Novak and Juvova, 1983).

**Rooting**

Rooting the shoots produced *in vitro*, or microcuttings, has been achieved through *in vitro* and *ex vitro*, or non-sterile, conditions (Briggs and Mcculloch, 1984). In some cases, microcuttings root better *in vitro* environments. *In vitro* rooting was superior to *ex vitro* rooting for *Prunus* x 'Hally Jolivette' (Lineberger, 1983). Also, in some cases *in vitro*, it is beneficial to make changes to the medium. Li and Eaton (1984) reported that rooting 'Marechal Foch' grapevine in half-strength MS salts was superior to rooting in full strength MS salts. But in other cases, superior rooting can result under *ex vitro* conditions. Rooting of *Halesia Carolina* L. non-sterile conditions was reported to be fart superior to rooting in sterile conditions (Brand and Lineberger, 1986). Also, microcuttings of *Syringa vulgaris* rooted more successfully under non-sterile conditions than under *in vitro* environments (Hildebrandt and Harney, 1983).

**'Dog Ridge' grapevine *in vitro* propagation**

Although several studies have described methods for grapevine *in vitro* propagation, only Gray and Fisher (1985) have attempted to propagate the selection 'Dog Ridge' grapevine. The method described the use of modified MS medium, and a 5 μM concentration of BA to proliferate shoots *in vitro* (Gray and Fisher, 1985). However, a range of cytokinin concentrations was not used to determine the effective concentrations for shoot proliferation. Furthermore, there are no studies describing a procedure to root 'Dog Ridge' grapevine *in vitro*.

The objective of this study is to establish an effective *in vitro* propagation protocol to proliferate shoots and establish roots of ‘Dog Ridge’ grapevine.

# CHAPTER II

# METHODS

## Shoot proliferation

Two experiments were conducted to evaluate whether shoot tip or axillary bud explants of 'Dog Ridge' grapevine resulted in better shoot proliferation *in vitro*. Each experiment used a factorial combination of two media; MS and WPM, with different concentrations of the growth regulator BA (0, 4.4, and 8.8 μM) for a total of 12 treatment combinations. Six repeated observations were in each experiment, and three repetitions of each experiment were conducted in time.

### *Explant collection and disinfection*

Vegetative shoots from six greenhouse-grown 'Dog Ridge' grapevines were collected from March to August, 2007. Leaves, flowers, and tendrils were removed from the shoots. Shoot tips and axillary buds of approximately 1 cm in length were excised and used as explants.

Explants were sterilized in a 10% solution of Clorox bleach (0.6% sodium hypochlorite) containing one drop of Tween 20 for 15 minutes, and then rinsed twice in sterile distilled water.

*Medium preparation*

Full strength MS medium was supplemented with 30 g/L sucrose, and solidified with 6 g/L Gibco Phytagar (Gibco Laboratories, Grand Island, N.Y.). The appropriate BA concentrations of 0, 4.4, and 8.8 μM were added. The pH was adjusted to 5.8. Full strength WPM medium was supplemented with 30 g/L sucrose, and solidified with 6 g/L Gibco Phytagar. The appropriate BA concentrations of 0, 4.4, and 8.8 μM were added. The pH was adjusted to 5.3. Media were sterilized in an autoclave at 121 °C for 20 minutes.

*Shoot initiation*

Clorox damaged tissue was removed from the explant in a laminar flow hood. Individual explants were then placed in 25 x 150 mm culture tubes containing 12.5 mL of medium. Cultures were incubated for 12 weeks at 28 ± 2°C under a 16 hour photoperiod (20 $\mu mol^{-2} \cdot s^{-1}$) provided by cool-white fluorescent tubes.

*Data collection*

The number of shoots that proliferated on each explant was counted. Data for three repetitions in time were pooled for analysis using the General Linear Models procedure in SAS (version 9.1; SAS Institute, Cary, NC). Means were compared using the least squares means procedure.

**Rooting**

An experiment was conducted to determine whether rooting *in vitro* with WPM media or *ex vitro* in non-sterile conditions resulted in higher rooting percentages. Two treatment combinations, 36 repeated observations, and three repetitions of the experiment were conducted.

*Microcutting collection*

Microcuttings of approximately 1 cm in length were excised from the explants that had been in continuous culture with 8.8 μM BA for 4 weeks. Microcuttings were selected for uniformity in terms of size and degree of expansion.

*In vitro rooting*

Full strength WPM medium was supplemented with 30 g/L sucrose, and solidified with 6 g/L Gibco Phytagar. The pH was adjusted to 5.3. Media were sterilized in an autoclave at 121 °C for 20 minutes. Six microcuttings were placed in a Magenta GA-7 vessel (Magenta Corp., Chicago, IL) containing 25 mL of medium. Microcuttings were rooted for 4 weeks at 28 ± 2°C under a 16 hour photoperiod (20 $\mu mol^{-2} \cdot s^{-1}$) provided by cool-white fluorescent tubes.

*Ex vitro rooting*

Twelve microcuttings were placed into plastic deli trays filled with moist Redi-earth® (Sun Gro Horticulture, Bellevue, WA) potting mix. Microcuttings were rooted for 4

weeks at 28 ± 2°C under a 16 hour photoperiod (20 $\mu mol^{-2} \cdot s^{-1}$) provided by cool-white fluorescent tubes.

*Data collection*

The number of explants that rooted was counted. Data for three repetitions in time were pooled for analysis using the General Linear Models procedure in SAS. Quantitative means were compared using the least squares means procedure. Categorical data were analyzed using frequency analysis and Chi-square procedure.

# CHAPTER III

# RESULTS AND DISCUSSION

## Shoot proliferation

*Shoot development and contamination*

In total, approximately 66% of the explants successfully developed shoots (data not shown). Of the 34% that did not develop shoots, 14% were contaminated with fungi or bacteria (data not shown). The remaining 20% became necrotic and did not develop shoots (data not shown). One repetition of the experiment was disregarded due to an abnormal amount of contamination, and was not included in the data analysis. The experimental repetition was later repeated.

Overall, enough explants successfully developed in order to sufficiently observe and record conclusive data on the experiments. The main effects from the factors analyzed were not statistically significant (Table 1). However, the results from the two- and three-way interactions were significant at the 0.05 level (Table 1).

**Table 1. Procedure GLM analysis of treatment effects on shoot proliferation.**
GLM analysis and sources of variation for comparing the treatment effects (media type, BA concentration, and explant type) and their interactions on *in vitro* shoot proliferation in 'Dog Ridge' grapevine.

| Source of variance | df | Type III SS | Mean Square | F Value | PR > F |
|---|---|---|---|---|---|
| MEDIA | 1 | 53.0046296 | 53.0046296 | 2.76 | 0.0983 |
| BACONC | 2 | 660.7777778 | 330.3888889 | 17.19 | <.0001 |
| MEDIA*BACONC | 2 | 206.2592593 | 103.1296296 | 5.37 | 0.0054 |
| EXPLANT | 1 | 330.0416667 | 330.0416667 | 17.17 | <.0001 |
| MEDIA*EXPLANT | 1 | 105.5601852 | 105.5601852 | 5.49 | 0.0201 |
| BACONC*EXPLANT | 2 | 229.3333333 | 114.6666667 | 5.97 | 0.0030 |
| MEDIA BACONC*EXPLANT | 2 | 62.7037037 | 31.3518519 | 1.63 | 0.1983 |

MEDIA = media type
BACONC = BA concentration
EXPLANT = explant type

*Medium and explant interaction*

Axillary bud explants cultured on MS medium produced an average of 5.7 shoots per explant (Fig. 1). This is significantly greater than the average of 3.3 shoots produced by axillary buds on WPM and the 1.8 and 2.2 shoots proliferated by shoot tip explants on MS and WPM media, respectively (Fig. 1). It is suspected that axillary buds explants respond favorably to the higher concentration of basal salts in MS than to the comparably lower basal salts in WPM. The shoot tip explants did not yield a significant response to either medium.

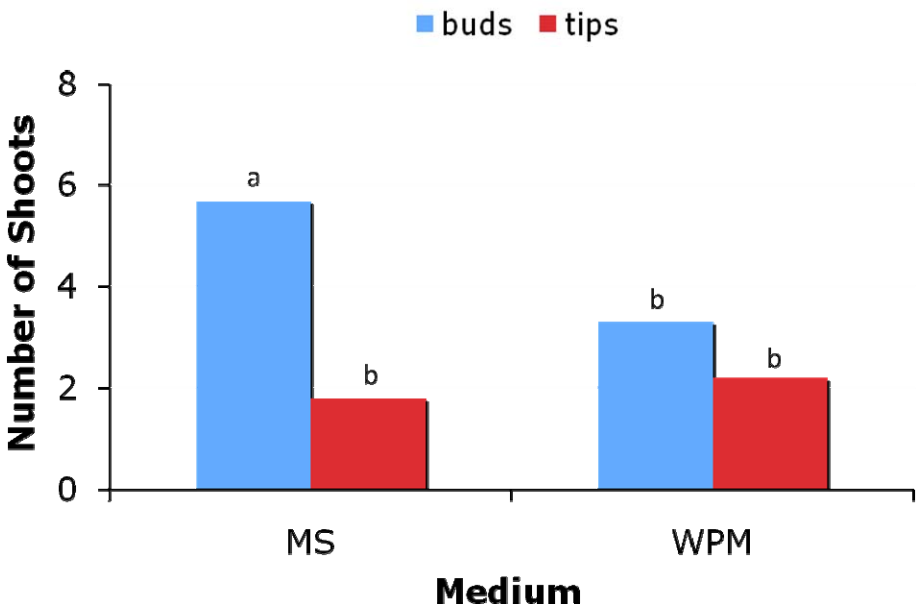


**Figure 1. Response of shoot tip and axillary bud explants to basal salts in the medium.** Number of shoots produced per explant after 12 weeks of culture on the indicated media. Means labeled by same letter are not significantly different at the 0.05 level.

*BA concentration and explant interaction*

Axillary bud explants in 4.4 µM BA produced an average of 6.2 shoots per explant (Fig. 2). This value is not statistically different from 6.7 shoots produced by axillary bud explants in 8.8 µM BA (Fig. 2). However, these two values are statistically different from the other explants and BA concentration combinations; axillary bud explants without BA produced an average of 0.6 shoots, shoot tip explants without BA produced an average of 1 shoot per explant, shoot tip explants with 4.4 µM BA produced an average of 2.6 shoots per explants, and shoot tip explants produced an average of 2.4 shoots per explants (Fig. 2). The axillary bud explants appear to respond better to the

higher concentrations of BA than the shoot tip explants, and proliferate a greater number of shoots.

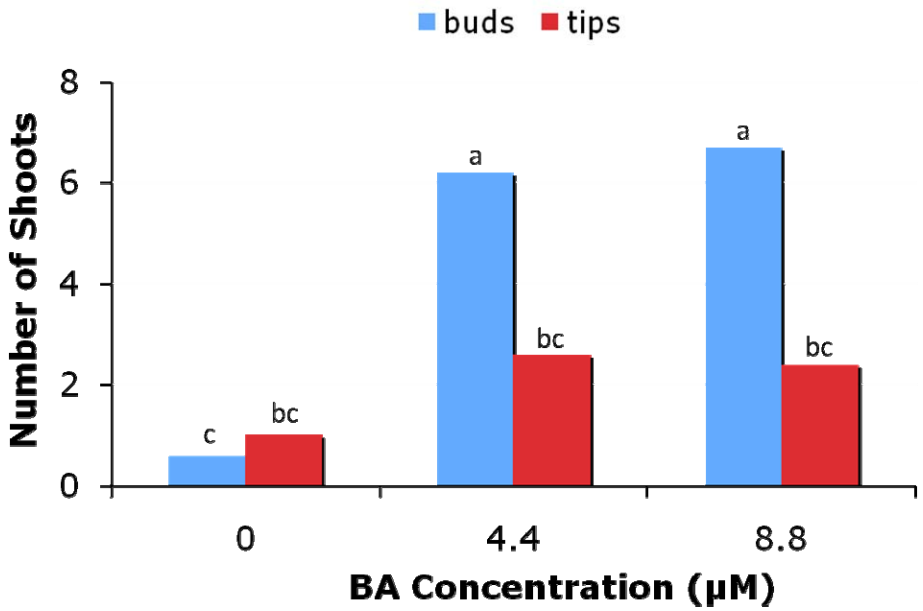


**Figure 2. Response of shoot tip or axillary bud explants to media containing different BA concentrations.**
Number of shoots produced per explant after 12 weeks of culture on the indicated media. Means labeled by same letter are not significantly different at the 0.05 level.

*BA concentration and medium interaction*

Explants in the combination of WPM and 4.4 µM BA produced an average of 4.5 shoots, and explants in MS media and 8.8 µM BA produced an average of 6.4 shoots (Fig. 3). Although the difference between the averages is 1.9, the difference is not statistically significant (Fig. 3). However, these two averages are significantly different from the other medium and BA concentration combinations; explants in MS and 0 µM BA

produced an average of 0.5 shoots, explants in WPM and 0 µM BA produced an average of 1shoot, explants in MS media and 4.4 µM BA produced an average of 4.3 shoots, and explants in WPM and 8.8 µM produced an average of 2.4 shoots (Fig. 3). The interaction displays that the greatest number of shoots were produced by a combination of either MS with 8.8 µM BA, or with WPM and 4.4 µM BA (Fig. 3).

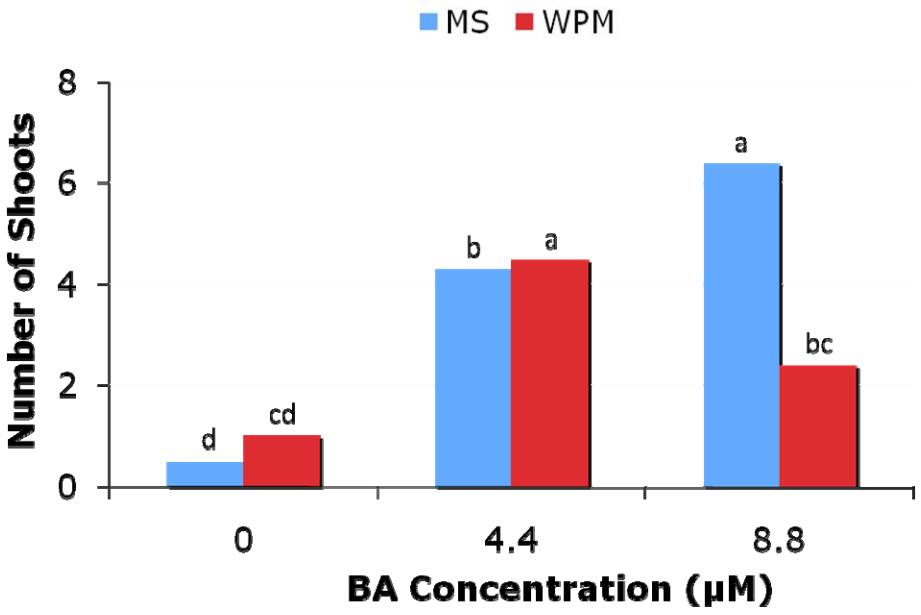


**Figure 3. Response of explants to MS and WPM media containing varying concentrations of BA.** Number of shoots produced per explant after 12 weeks of culture on the indicated media. Means labeled by same letter are not significantly different at the 0.05 level.

*Optimal proliferation*

A previous study by Dr. D.J. Gray and L.C. Fisher investigated the *in vitro* propagation of 'Dog Ridge' grapevine, and determined that 4 shoots/apex could be obtained using

MS modified by Chee et. al. with 5 μM BA (Gray and Fisher, 1985). The results of the three interactions from this study determined that even more shoots could be obtained with the use of unmodified MS and higher BA concentrations. The combination of axillary bud explants with MS medium and 8.8 μM BA produced an average of up to 10 shoots per explants (Fig. 5).

*Leaf expansion*

Explants cultured with BA produced the most shoots. The low number of shoots developed from explants without BA demonstrates that growth regulator is necessary for shoot proliferation. However, as the concentration of BA increases, leaf expansion decreases (Figs. 4,5). If expanded leaves are necessary for proper growth and develop of the mature plant, further research needs to be conducted to establish the proper BA concentration that results in acceptable balance of leaf expansion and shoot proliferation.

*Preliminary rooting*

Shoot tip explants cultured in WPM containing no BA rooted frequently (Fig. 4), suggesting that changing basal salts during *in vitro* rooting phase might be beneficial.

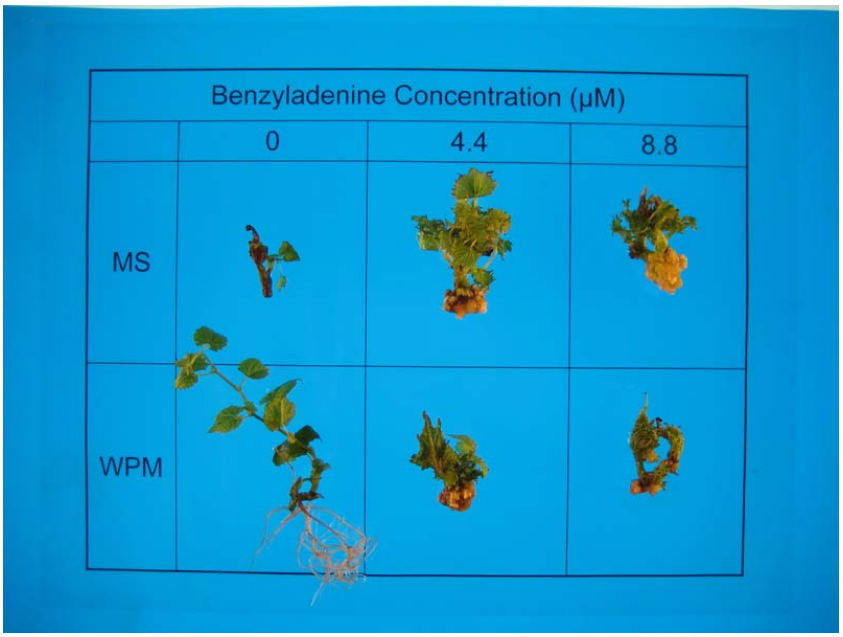


**Figure 4. Representative 'Dog Ridge' grapevine explants.**
Appearance of representative 'Dog Ridge' grapevine explants at the end of 12 weeks culture on MS or WPM medium containing the indicated concentration of BA.

**Figure 5. Shoot proliferating culture of 'Dog Ridge' grapevine**
Shoot proliferating culture of 'Dog Ridge' grapevine photographed after 20 weeks growth, and 4 subcultures *in vitro* on MS medium with 8.8 μM.

## Rooting

*Shoot development and contamination*

Microcuttings rooted *in vitro* with 76% success (data not shown). Microcuttings under non-sterile conditions rooted with 38% success (data not shown). The remaining microcuttings did not develop roots or died. Most microcuttings developed one or two roots 2-3 cm long. Of those that rooted, 67% rooted under *in vitro* conditions, 33% rooted in non-sterile environments (Fig. 6). Additional analysis of rooting data is provided in the Appendix.

*In vitro* rooting proved to be a superior method of rooting 'Dog Ridge' grapevine microcuttings. The hypothesis of the effects of the *in vitro* rejuvenation increasing the success of rooting may be correct; higher levels of rooting have been obtained from *in vitro* microcuttings. However, even 67% percent rooting may not be high enough for commercial endeavors. The majority of the microcuttings that did not root *in vitro* remained as rootless microcuttings. More factors must be examined in order to determine optimal rooting conditions for 'Dog Ridge' grapevine microcuttings. The use of auxins and many other factors and changes in the rooting environment have been described in order to enhance the rooting of microcuttings (Brand and Lineberger, 1986).

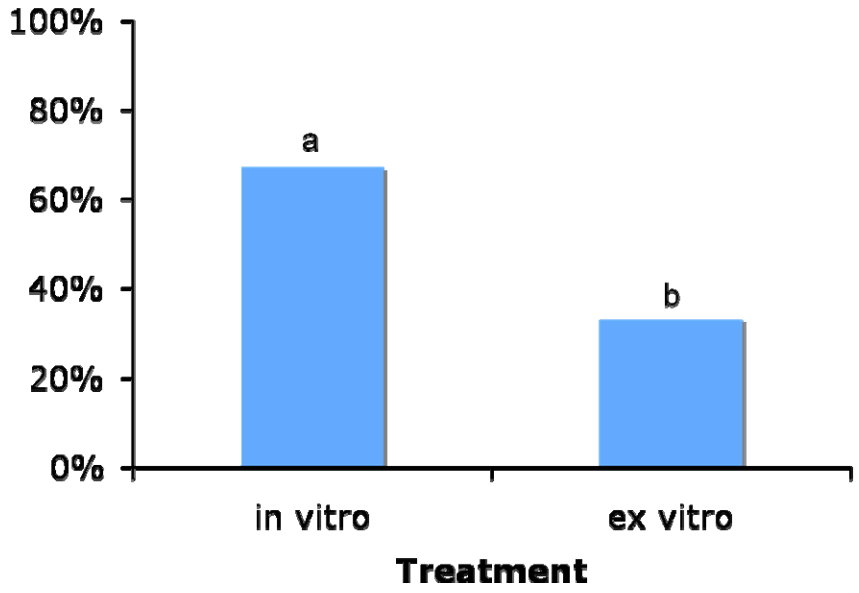


**Figure 6. Comparison of rooting methods for microcuttings of 'Dog Ridge' grapevine.**
Percentage of microcuttings rooted *in vitro* in WPM and *ex vitro* in Redi-earth® after 4 weeks. Percentages labeled by same letter are not significantly different at the 0.05 level.

# CHAPTER IV

# SUMMARY AND CONCLUSIONS

Further refinements to the protocol to culture 'Dog Ridge' grapevine *in vitro* have been made. The use of high concentrations of BA and axillary buds explants was shown to increase the number of shoots proliferated *in vitro*. A combination of axillary bud explants with MS medium and 8.8 μM BA could produce an average of up to 10 shoots per explants in 12 weeks. Also, successful rooting of 'Dog Ridge' grapevine microcuttings has been described. *In vitro* microcuttings rooted with 76% success, an *ex vitro* microcuttings rooted with 38% success.

'Dog Ridge' grapevine is responsive to *in vitro* culture. A tissue culture protocol using axillary bud explants with unmodified MS medium and 8.8 μM BA for shoot proliferation, and rooting microcuttings with WPM *in vitro* can be recommended as an alternative production method to growers of 'Dog Ridge' grapevine.

The rooting success may be a limiting factor for production. Further refinements to the shoot proliferation protocol to produce optimal shoot proliferation while maintaining adequate leaf expansion and changes in the rooting conditions to produce greater success should be examined in order to create an profitable *in vitro* culture protocol for the large-scale production of 'Dog Ridge' grapevine.

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# APPENDIX

A1. **Procedure Chi-square analysis of treatment effects on rooting.**

| | |
|---|---|
| Chi-Square | 13.6667 |
| DF | 1 |
| PR > ChiSq | 0.0002 |

sample size = 123

A2. **Frequency analysis of treatment effects on rooting.**

| Condition | Frequency | Percent | Cumulative Frequency | Cumulative Percent |
|---|---|---|---|---|
| ExVitro | 41 | 33.33 | 41 | 33.33 |
| In Vitro | 82 | 66.67 | 123 | 100 |

A3. **Procedure GLM analysis of treatment effects on rooting.**
GLM analysis and sources of variation for comparing the treatment effects (condition and date) and their interactions on the rooting of 'Dog Ridge' grapevine microcuttings.

| Source of variance | df | Type III SS | Mean Square | F Value | PR > F |
|---|---|---|---|---|---|
| CONDITION | 1 | 11.11574074 | 11.11574074 | 28.91 | <.0001 |
| DATE | 2 | 6.287037014 | 3.14351852 | 8.18 | 0.0004 |
| CONDITION*DATE | 2 | 2.06481481 | 1.03240741 | 2.68 | 0.0706 |

CONDITION = rooting condition
DATE = date of experimental repetition

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