A Protocol for Endophyte-Free Callus Tissue of the Grape *Vitis aestivalis* 'Norton/Cynthiana' (Vitaceae)

by
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ABSTRACT

Vitis aestivalis is used in commercial wine production. Propagation rates of *V. aestivalis* are low, therefore, plant tissue culture may be a viable method for propagation. However, a fungal endophyte exists within the plant which complicates tissue culture methods. This study reports a protocol for obtaining fungus-free callus from *V. aestivalis*. Explant tissue was chosen from healthy plants grown in a plant growth room and in a vineyard. Tissues were sterilized with isopropanol, bleach, and chlorine dioxide gas and plated onto media containing chlorothalonil. Successful results showed endophyte-free callus using explants taken from plants grown in a plant growth room, sterilized in alcohol, bleach, and chlorine dioxide, and plated on selection media containing an antifungal agent. This technique can be used with plants that have endophytes or other contamination problems to establish callus tissue for research and/or commercial propagation.

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INTRODUCTION

The grape, *Vitis aestivalis* Michx. 'Norton' (syn: 'Cynthiana') is a hardy vine native to North America that has been used in commercial wine production. This particular cultivar of *Vitis* was developed by Dr. Daniel Norton from hybridization efforts involving the now-lost 'Bland' cultivar with other native North American species (Ambers and Ambers, 2004). The resulting hybrid 'Norton' has been shown to produce a high quality wine (Ambers, 2013) with highly favored traits (Stover *et al.*, 2009; Norton and Skirvin, 2001; Ambers and Ambers, 2004; Parker *et al*., 2009). The cultivar's resistances have allowed it to tolerate infectious pathogens (Krivanek and Walker, 2005) without succumbing to disease far better than several European varieties (Polesani *et al*., 2010; Espinoza *et al*., 2007; Fung *et al*., 2007). These traits allow for growers to use less pesticides (20-25% of the current rate of pesticide use in equivalent European varieties) when growing 'Norton' and 'Cynthiana' grapevines (Ambers, 2013).

While *V. aestivalis* possesses several traits that make it attractive for agricultural production, the vines are recalcitrant to standard *Vitis* vegetative propagation techniques of using cuttings (Hartmann *et al*., 2002; Norton and Skiven, 2001). Modifications to standard vegetative propagation protocols have shown some promise (Qiu *et al.*, 2003; Norton and Skiven, 2001); however, *V. aestivalis* is still difficult to vegetatively propagate with the previously mentioned techniques (Bigger, 2010; Qiu *et al*., 2003; Norton and Skiven, 2001).

When an agricultural crop cannot be reliably propagated via seed, and vegetative propagation has proven to be difficult, tissue culture techniques can be employed as an

alternative method of propagation (Murashige, 1974; García-Gonzáles *et al*., 2010). For example, micropropagation techniques relying on tissue culture have been employed for orchids (Chugh *et al*., 2009; Murashige, 1974), and several agricultural products ranging from peaches to avocados (García-Gonzáles *et al*., 2010). In plant tissue culture, small explant samples of the plant in question are taken, sterilized both externally and internally depending on the species (Akin-Idowu *et al*., 2009), and then treated with plant regulatory compounds (PRCs) (Constabel, 2012; Akin-Idowu *et al*., 2009; García-Gonzáles *et al*., 2010). PRCs can be utilized for a number of purposes, the induction and regulation of callus being one of them. Callus is a de-differentiated, disorganized, and totipotent mass of parenchyma tissue that can be used for a variety of plant tissue culture applications (Constabel 2012).

Upon achieving stable callus cultures, researchers gain a tool that they can employ to achieve one of many goals. Callus can be used to rapidly produce somatic embryos (Akin-Idowu *et al*., 2003) or to create virus-free cultivars (Harris and Stevenson, 1982). Research into genes related to crop resistances or production traits benefits from callus cultures as well (García-Gonzáles *et al*., 2010). Additionally, callus can be deployed in attempts to rapidly produce daughter plants from a relatively small pool of explant donors (Harris and Stevenson, 1982; Preece, 2003).

V. aestivalis has several traits that make it favorable for production as discussed earlier (Ambers, 2013; Stover *et al*., 2009; Polesani *et al*., 2010; Espinoza *et al*., 2007; Fung *et al*., 2007), but vines are impractical to reproduce via seed due to their hybrid nature, and this cultivar is recalcitrant to standard vegetative methods (Hartmann *et al*., 2002; Norton and Skiven, 2001). As such, plant tissue culture

techniques allow an alternative route for mass vegetative propagation of plantlets (Preece, 2003). Effort to initiate aseptic cultures of *V. aestivalis* is complicated by the vine's ability to tolerate low levels of pathogenic infection (Krivanek and Walker, 2005). Internal tissues in the explant must be sterilized in initial decontamination (Akin-Idowu *et al*., 2009), or cultures will yield contamination from the growth of internal pathogens. Sterility protocols that are designed to kill internal pathogens are difficult because deep penetration of the explant by sterilizing agents leads to stress and loss of viability in the sample (Murashigi, 1974).

To overcome the issue of internal contamination of explant material, an alternative technique to solely relying on surface sterilization was devised. Explant materials infected with either fungi or bacteria can be viewed as a colony consisting of multiple different cell types. Surface sterilization can be used to lessen the number of different cell types. Then, the explant material can be plated on a medium that selectively kills the cell types that are not desired, while allowing the desirable portion of the colony (the plant tissue) to continue to grow. With this approach, it was hypothesized that one could use less severe sterilization techniques to reduce stress on the explant material, while still producing a viable plant callus culture for research and development. The combination of less severe surface sterilization techniques with a selection pressure could, therefore, be used on tissues known for recalcitrance resulting from internal cohabitation with fungi or bacteria for the production of aseptic plant tissue cultures.

An exploratory study to test the viability of such a program was devised. A small set of tissues were sourced from a local vineyard, and an experimental protocol was implemented. Of the initial small sample size, a promising amount of explants were

successfully induced into callus. Results were promising enough to warrant a full study of the viability of the novel sterilization technique.

METHODS

I. Explant Tissue - Explant tissues (fresh, new shoot growths) were chosen from healthy, actively-growing mature plants from the Rutherford County Agricultural Extension Service/ MTSU Vineyard. Tissue explants were also chosen from fresh shoots of growth room plants sourced from the previously identified vineyard. Growth room lighting and temperature conditions were set to spring conditions (for Tennessee, USA), with 12 hours of daylight and 12 hours of darkness. Light intensity (PAR) in the growth room was on average 513 μmol m^{-2} s^{-1} with day temperatures at 22°C and night temperatures at 15°C. All samples were selected from September 21, 2015 to November 13, 2015.

II. Media Preparations - Callus growth media (1 L) consisted of Lloyd and McGown (1980; McGown and Lloyd 1981) basal salt mixture with 0.9% (w/v) agar, 3% (w/v) sucrose, 0.01 g/l Thiamine, and 0.1 g/l Casein. Media was set to a final pH of 5.6-5.8 and autoclaved for 20 minutes at 121°C. After autoclaving PRCs were added to the solution to achieve 13.57 mM 2,4-D and 9.29 mM Kinetin concentrations. Selection media included 1450 uL (per 1 L) of the antifungal agent Daconil® (chlorothalonil), non-selection media did not include the antifungal agent.

III. Tissue Sterilization - Explants were first washed with warm water and 1% (v/v) dish detergent. Tissues were rinsed thoroughly and transferred to a sterile hood. Tissues were then surface sterilized in 70% isopropanol for 30 seconds followed by a 20 minute

wash in 2.5% NaOCl + 1% (v/v) dish detergent with 0.1% antibiotic. Clean explants were washed three times with sterile deionized water. After the rinse, tissues were subjected to a chlorine dioxide gas treatment with a concentration of 1500 ppm for one hour. Explants were divided into two treatment groups; one plated onto media containing chlorothalonil, and the other plated onto similar media without chlorothalonil as a control. Plates were sealed with parafilm and incubated at 25ºC for 4 weeks. The study was repeated three times using tissues from both vineyard-grown plants and also growth room-grown plants. Plates were sealed with parafilm and incubated in the dark at 25 degrees C at almost 100% humidity for one week.

IV. Dropout Method - In order to determine the effect of each sterilization agent on the explant tissues, a "dropout method" was devised where explants were set aside after each stage of the protocol. These tissues were then plated on both selection and non-selection media. This "dropout method" was executed in conjunction with each repetition of the experiment. Tissues were then observed for 4 weeks. Contamination and callus rates were recorded. Calli generated on antifungal-containing media were later transferred onto media without the added antifungal agent in order to verify that the fungus was not just suppressed, but completely eradicated.

Three separate trials were conducted in the fall of 2015 for each of the following conditions: growth room plants/non-selection media; vineyard plants/non-selection media; growth room plants/selection media; and vineyard plants/selection media. Ninety explant samples were used for each trial.

V. Statistical Analysis of Tissue Culture Results – A one-way analysis of variance (ANOVA) was performed using successful plate data (plates containing callus formation with no infection). The ANOVA analysis was preformed using SigmaStat (Version 3.10) software.

RESULTS

I. Successful Callus: The success for each treatment was determined as callus growth without the fungal endophyte. Successful endophyte-free grape callus is shown in Figure 1.

Figure 1: Petri plate (100mm diameter) showing successful production of endophyte-free callus from V*itis aestivalis* 'Norton/Cynthiana.'

II. Non-selection / Growth Room*:* Trial one resulted in a 100% success rate (callus formation with no infection) in all three levels of sterilization. Trial two resulted in 100% success using alcohol and bleach and 100% success using alcohol, bleach, and chlorine dioxide gas as well. Trial three resulted in 90% success using all three methods of sterilization (Figure 2).

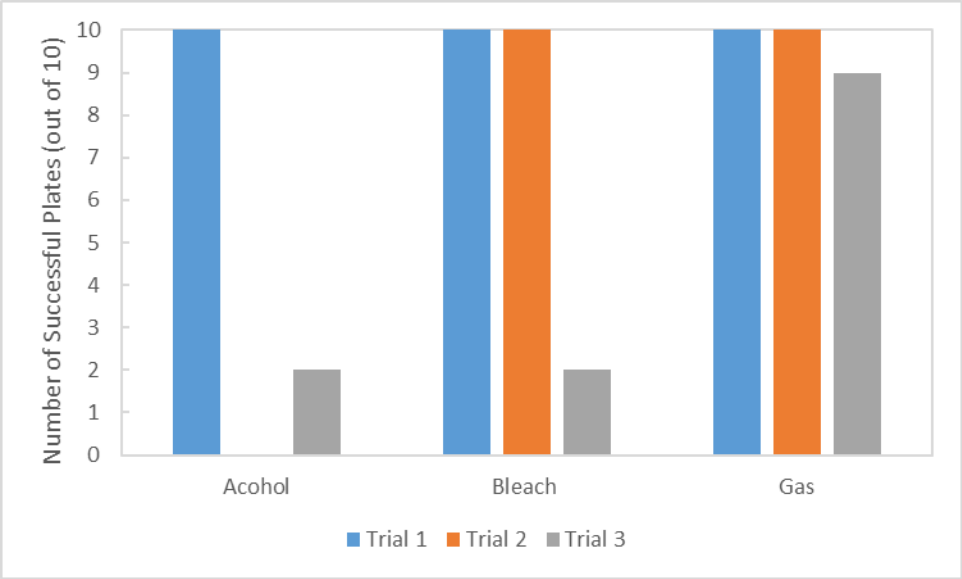

Figure 2: Results from three trials using samples taken from plants in the plant growth room and placed on non-selection media after sterilized. There were significant ($p = 0.022$) differences between the Non Selection Growth Room A+B+C treatments and the Vineyard A treatments, both with Selection and Non Selection.

III. Non-selection / Vineyard - Trial one resulted in a 40% success rate using alcohol and bleach and a 40% success rate using alcohol, bleach, and chlorine dioxide gas as well. Trial two resulted in a 40% success rate using only alcohol, and a 30% success rate using alcohol, bleach, and chlorine dioxide gas. Trial three resulted in a 20% success rate using alcohol and bleach (Figure 3).

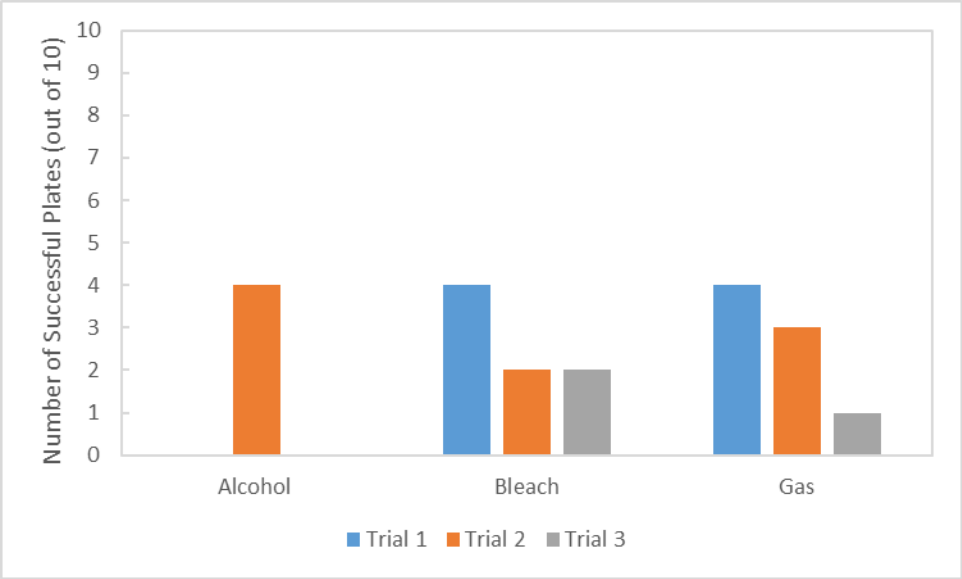

Figure 3: Results from three trials using samples taken from plants in the vineyard and placed on non-selection media after sterilization. There were significant differences between the Non Selection Vineyard A and the Selection Growth Room A ($p = 0.031$), A+B ($p = 0.015$), and A+B+C ($p = 0.015$) treatments and the Non Selection Growth Room A+B+C ($p = 0.022$) treatments.

IV. Selection / Growth Room - Trial one resulted in an 80% success rate using alcohol alone and a 100% success rate using alcohol and bleach and a 100% success rate using alcohol, bleach, and chlorine dioxide gas. Trial two resulted in a 100% success rate in all three levels of sterilization. Trial three also resulted in a 100% success rate in all three levels of sterilization (Figure 4).

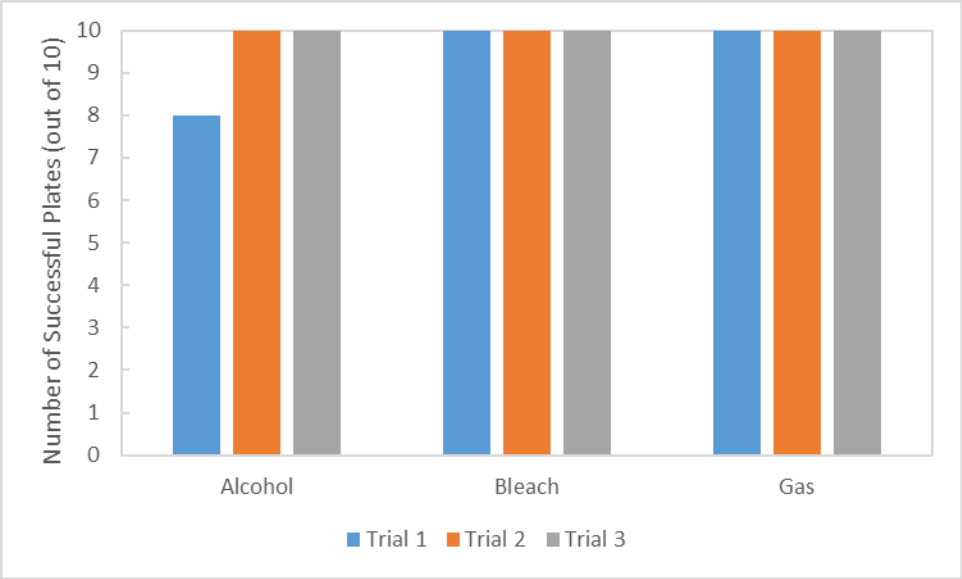

Figure 4: Results from three trials using samples taken from plants in the plant growth room and placed on selection media after sterilization. There were significant differences between the Selection Growth Room A and Selection Vineyard A ($p = 0.031$) treatments; the Selection Growth Room A+B treatments and the Selection Vineyard ($p = 0.015$) treatments; the Selection Growth Room A+B treatments and the Non Selection Vineyard A ($p = 0.015$) treatments; the Selection Growth Room A+B+C treatments and the Selection Vineyard ($p = 0.015$) treatments; and the Selection Growth Room A+B+C treatments and the Non Selection Vineyard ($p = 0.015$) treatments.

V. Selection / Vineyard - Trial one resulted in a 60% success rate using alcohol and bleach and a 90% success rate using alcohol, bleach, and chlorine dioxide gas. Trial two resulted in a 40% success rate using alcohol and bleach. Trial three resulted in a 40% success rate using alcohol and bleach and a 40% success rate using all three methods of sterilization (Figure 5).

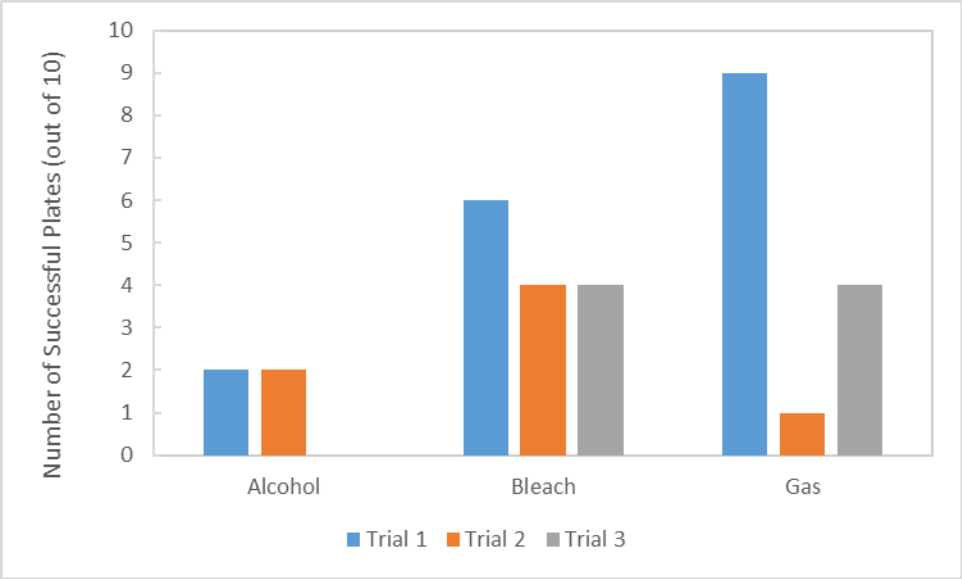

Figure 5: Results from three trials using samples taken from plants in the vineyard and placed on selection media after sterilization. There were significant differences between the Selection Vineyard A treatments and the Selection Growth Room A+B+C ($p = 0.015$) treatments, the Selection Growth Room A+B ($p = 0.015$) treatments, the Non Selection Growth Room A+B+C ($p = 0.022$) treatments, and the Selection Growth Room A ($p = 0.031$) treatments.

VI. Statistical Results for Tissue Culture – ANOVA results, followed by Tukey All Pairwise Multiple Comparison Procedure showed significant differences between the growth room plants/selection media and the vineyard plants/non-selection media (P=0.007) and between growth room plants/selection media and vineyard plants/selection media (P=0.014). Power of this test (with alpha = 0.05) was 0.924 (Table 1).

Table 1. Number of successful plates (out of 30) for explants from plant growth room (GR) or

vineyard (Vin) plants on selection (S) or non-selection media (NS). Sterilization treatments were: alcohol (A); alcohol + bleach (A + B); alcohol + bleach + chlorine dioxide (A + B + C).

Treatment	GR – NS	GR – S	Vin – NS	Vin – S
A	12	28	4	2
A + B	22	30	10	14
A + B + C	29	30	8	14
Mean $\pm$ SD	21.0 $\pm$ 8.5	29.3 $\pm$ 1.2[a,b]	7.3 $\pm$ 3.1[a]	10.0 $\pm$ 6.9[b]

Significant differences: [a]P=0.007; [b]P=0.014. Power = 0.924.

DISCUSSION

In all instances, explants taken from vines grown in controlled plant growth room conditions more readily dedifferentiated into callus than explants taken from vineyard plants. This discrepancy could be partially explained by the time of year at which the field samples were taken. *V. aestivalis* vines become dormant in the late fall in Northern latitudes and become considerably more recalcitrant to tissue culture efforts. The simulated spring-time conditions in the plant growth room allowed the vines to continue growing vigorously into the fall; These tissues proved much more amenable to tissue culture than the vineyard-sourced explants.

Results from the sterilization dropout trials suggest that the alcohol and bleach sterilization steps are necessary to adequately surface sterilize tissues and acquire a sufficient amount of callus. However, the third sterilization (with chlorine dioxide gas) showed mixed results in regards to callus formation. In the non-selection/growth room trials, as sterilization steps were added, the rate of successfully initiating a contamination-free callus culture increased. In the non-seletion/vineyard trials, the tissues that were sterilized only with alcohol had a much lower success rate than the other tissues. However, the tissues that underwent the alcohol and bleach treatments were shown to have the same percentage of callus formation as the tissues that underwent all three sterilization steps. This observation suggests that alcohol sterilization alone is not enough to suppress and eliminate the fungal endophyte present in *V. aestivalis*. Explants that were sterilized in only alcohol had a lower success rate than tissues which were sterilized with all three steps (alcohol, bleach, and chlorine

dioxide gas). The tissues that were sterilized with alcohol and bleach also had the same success rate as the tissues that underwent all three sterilization protocols. Therefore, along with using tissues from plants grown in a growth room, sterilizing with at least alcohol and bleach (and chlorine dioxide, if available) increases the success rate of callus production from *V. aestivalis.*

Without penetration and exposure to sterilizing agents, reserves of contamination can be

transferred to the tissue culture, leading to sample loss. Fungal endophytes kept in check in natural conditions can overwhelm and outcompete the plant tissues in the nutrient-rich, high humidity conditions favorable for tissue culture production. Therefore, to suppress both endophyte and surface contamination, treatments more intensive than alcohol surface sterilization should be employed.

While thorough surface sterilization, small explant size, and repetition are recommended for sterile tissue culture work (Murashige, 1974), the use of selection media can be implemented as well. A selection pressure in the media can be taken up by the tissues during the initial phase of culture establishment, preventing the fungal contaminant from surviving the procedure and establishing colonies during the culture process. Tissue cultures that initiated into callus on selection media did not show fungal contaminants after passaging onto non-selection media, supporting the idea that once exposed to a selection pressure for a short time, endophyte contamination can be eliminated.

Plant tissue culture offers a viable alternative to problems associated with propagating *V. aestivalis.* However, the fungal endophyte associated with *V. aestivalis*

frustrates tissue culture efforts. This study was intended to provide a viable protocol to overcome this issue. The results from this study suggest that in order to obtain callus tissue that is endophyte-free, vine explants are to be taken from plants grown in a plant growth room simulating springtime conditions, sterilized in alcohol, bleach, and chlorine dioxide, and plated on a selection medium containing an antifungal agent, such as chlorothalonil. Tissues from such treatments can, therefore, be used in somatic reproduction techniques for plant propagation. These methods help maintain traits valued by the industry, namely pest, disease and drought tolerance, by overcoming the difficulty of using current reproduction methods. Therefore, this technique could potentially be used with plants that have associated endophytes or other contamination problems in order to facilitate research and commercial propagation efforts.

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