

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

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Abstract

Vitis aestivalis is used in commercial wine production. Vegetative propagation of *V. aestivalis* has shown a low success rate. Although plant tissue culture has been a successful method to propagate many species, *V. aestivalis* has not yet been reliably grown as pure callus culture due to a fungal endophyte that exists within the plant. This study reports a viable protocol for obtaining fungus-free tissue culture callus from *V. aestivalis*. Explant tissue was chosen from healthy, actively growing plants grown in a growth room and in a vineyard. Tissues were sterilized with a combination of isopropanol, bleach, and chlorine dioxide gas and plated onto media containing chlorothalonil. The results from this study suggest that in order to obtain endophyte-free callus tissue, vine explants are to be taken from plants grown in a growth chamber simulating springtime conditions, sterilized in a combination of alcohol, bleach, and chlorine dioxide, and plated on selection media containing an antifungal agent, such as chlorothalonil. This technique could potentially be used with plants that have associated endophytes or other contamination problems to establish callus tissue for research and/or commercial propagation efforts.

Keywords

Chlorothalonil, Endophyte, *Vitis aestivalis*, Norton, Cynthiana

1. 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 [1]. The resulting hybrid “Norton” has been shown to produce a high quality wine [2] with highly favored traits [1] [3]-[5]. The cultivar’s resistances have allowed it to tolerate infectious pathogens [6] without succumbing to disease far better than several European varieties [7]-[9]. 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 [2].

While “Norton/Cynthiana” possesses several traits that make it attractive for agricultural production, the vines are recalcitrant to standard *Vitis* vegetative propagation techniques of using cuttings [4] [10]. Modifications to standard vegetative propagation protocols have shown some promise [4] [11]; however, “Norton/Cynthiana” is still difficult to vegetatively propagate with the previously mentioned techniques [4] [11] [12].

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 [13] [14]. For example, micropropagation techniques relying on tissue culture have been employed for orchids [13] [15], and several agricultural products ranging from peaches to avocados [14]. In plant tissue culture, small explant samples of the plant in question are taken, sterilized both externally and internally depending on the species [16], and then treated with plant regulatory compounds (PRCs) [14] [16] [17]. 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 [17].

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 [16] or to create virus-free cultivars [18]. Research into genes related to crop resistance or production traits benefits from callus cultures as well [14]. Finally, callus can be deployed in attempts to rapidly produce daughter plants from a relatively small pool of explant donors [18] [19].

“Norton/Cynthiana” has several traits that make it favorable for production as discussed earlier, but vines are impractical to reproduce via seed due to their hybrid nature, and this cultivar is recalcitrant to standard vegetative methods [4] [10]. As such, plant tissue culture techniques allow an alternative route for mass vegetative propagation of plantlets [20]. Effort to initiate aseptic cultures of “Norton/Cynthiana” is complicated by the vine’s ability to tolerate low levels of pathogenic infection [6]. Internal tissues in the explant must be sterilized in initial decontamination [16], 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 [13].

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 dif-

ferent 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 co-habitation 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.

2. Methods

Explant tissues (fresh, new shoot growths) were chosen from healthy, actively-growing mature plants from the Rutherford County Agricultural Extension Service/MTSU Vineyard. Average temperatures for the vineyard in September through November were 12.1°C - 22.4°C with 13.0 - 14.8 cm of total precipitation per month. Tissue explants were also chosen from fresh shoots of growth chamber plants sourced from the previously identified vineyard. Growth chamber conditions were set to spring conditions (for Tennessee, USA), with 12 hours of daylight and 12 hours of darkness and 70% relative humidity. Light intensity (PAR) in the growth chamber was on average 513 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with day temperatures at 22°C and night temperatures at 15°C. All samples were selected from September 21, 2015 to November 13, 2015.

Tissue Culture Procedures: Callus growth media (1 L) consisted of basal salt mixture [19] 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 μL (per 1 L) of the antifungal agent Daconil® (chlorothalonil), non-selection media did not include the antifungal agent.

Explants were first washed with warm water and 1% (v/v) dish detergent. 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 concentrations 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. One explant was applied to each plate and there were 10 replicate plates per treatment. Plates were sealed with parafilm and incubated at 25°C for 4 weeks. The

study was repeated three times using tissues from both vineyard-grown plants and also growth chamber-grown plants. This gave a total of 30 explants per treatment.

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. Treatments in the “dropout method” were: alcohol (A); alcohol + bleach (A + B); alcohol + bleach + chlorine dioxide (A + B + C). 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 chambered plants/non-selection media; vineyard plants/non-selection media; growth chambered plants/selection media; and vineyard plants/selection media. Ninety explant samples were used for each trial.

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 performed using SigmaStat (Version 3.10) software.

Scanning Electron Microscopy (SEM) Preparations—Explant Sourcing—Cuttings were taken from the Rutherford County Agricultural Extension Service/MTSU Vineyard and propagated in a growth chamber (conditions described above). After establishment, explants were harvested, surface-sterilized, underwent a fungal incubation protocol and then fixed and analyzed.

Sterility protocol—Tissues were washed in deionized water with a detergent containing Chloroxlylenol 0.3% and L-lactic acid 2%. Tissues were rinsed thoroughly and transferred to a sterile hood. Tissues were washed in 70% Isopropyl alcohol [21] [22]. Modifications to sterilization protocols were for explants to be visualized via SEM to make them compatible with the tissue fixation stage. Sterile tools were used to dissect tissues and plate them on callus--inducing non-selection media for the fungal incubation stage.

Fungal incubation—Plates were sealed with parafilm and placed in an incubator. Tissues were incubated in the dark at 25 degrees C at almost 100% humidity for one week. At the end of the incubation period, tissues observed to have fungal growths were selected and fixed for SEM investigation.

SEM fixation protocol—Tissues were fixed following protocols developed from Bhwana *et al.* [23] and Anderson and Miller [24]. Modifications to the protocols were as follows: tissues selected from the fungal incubation plates were fixed with 2.5% glutaraldehyde and 2.0% osmium. After fixation, tissues were dehydrated in a series of four acetone washes and the critical point drying using a Polaron E3000 critical point dryer. Dried materials were then mounted with 12-mm adhesive carbon conductive tape attached to a 15-mm aluminum specimen mount and coated with gold and palla-

dium using a Hummer VI sputter coater.

SEM imaging protocol—Fixed tissues were imaged for visual analysis with a Hitachi 3400N scanning electron microscope, using imaging protocols modified from Bhawana *et al.* [23] and Anderson and Miller [24]. Accelerating voltages of 8, 10, 12 and 15 kV were used in the imaging procedure. Regions of the explant samples near the dissection cuts were visualized to illuminate the vascular regions. Special attention was paid to explant regions with high levels of hyphal development and interactions between the hyphae and the plant tissue.

3. Results

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

Non-selection/ Growth Chamber. 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**).

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**).

Selection/ Growth Chamber—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



Figure 1. Petri plate (100 mm diameter) showing successful production of endophyte-free callus from *Vitis aestivalis* “Norton/Cynthiana”.

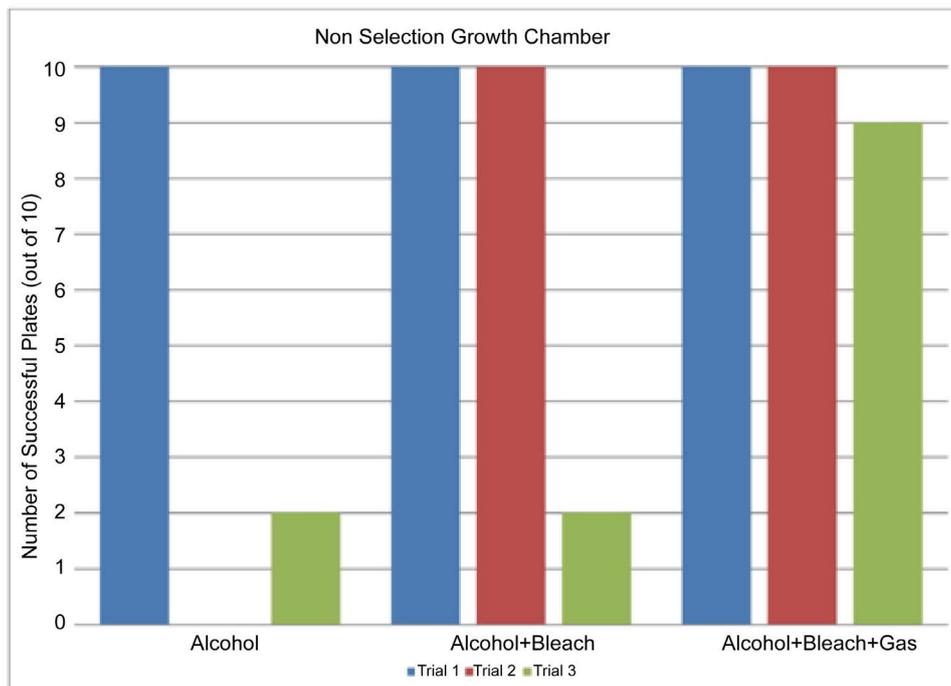


Figure 2. Results from three trials using samples taken from plants in the growth chamber and placed on non-selection media after sterilized. There were significant ($p = 0.022$) differences between the Non Selection Growth Chamber A + B + C treatments and the Vineyard A treatments, both with Selection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

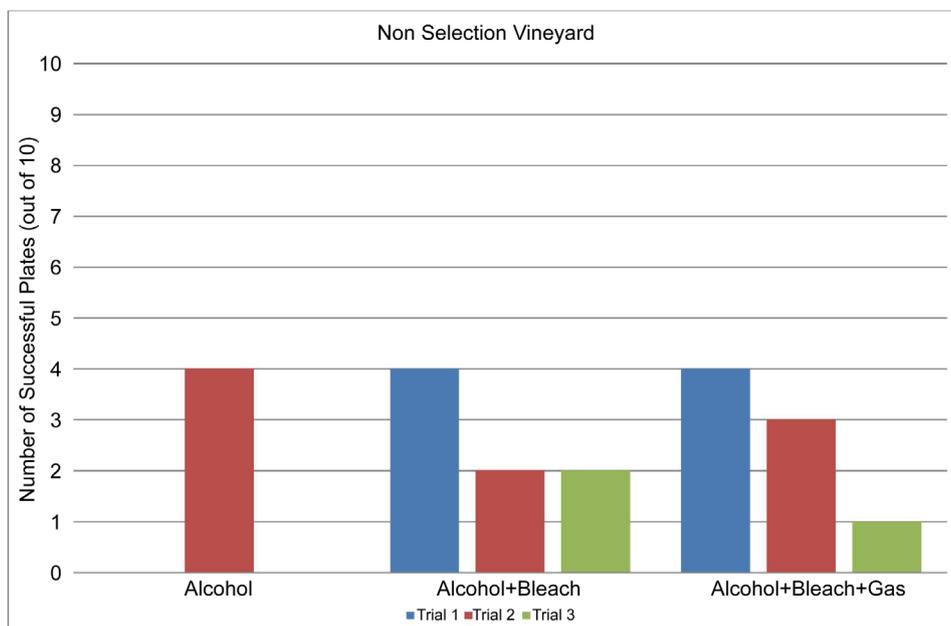


Figure 3. Results from three trials using samples taken from plants in the vineyard and placed on non-selection media after sterilization. There were significant differences between the Non Selection Vineyard A and the Selection Growth Chamber A ($p = 0.031$), A + B ($p = 0.015$), and A + B + C ($p =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

three levels of sterilization. Trial three also resulted in a 100% success rate in all three levels of sterilization (Figure 4).

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).

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

SEM Results—Fungal hyphae were visualized in the prepared samples. Fungal growths were detected erupting from vascular tissues in the samples at dissection sites (Figure 6 and Figure 7). Hyphae were also shown to erupt from uncut regions, pushing aside plant tissue to reach the surface of the explant (Figures 8-11). Fungal tissue was shown to originate from within the explant samples, with fully developed fungal tissue

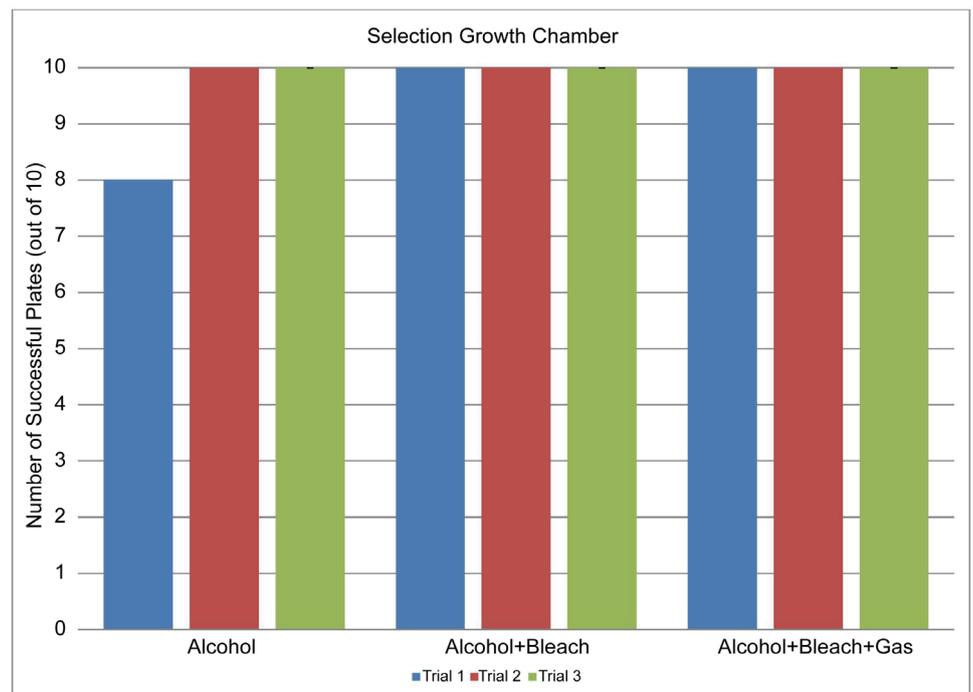


Figure 4. Results from three trials using samples taken from plants in the growth chamber and placed on selection media after sterilization. There were significant differences between the Selection Growth Chamber A and Selection Vineyard A ($p = 0.031$) treatments; the Selection Growth Chamber A + B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

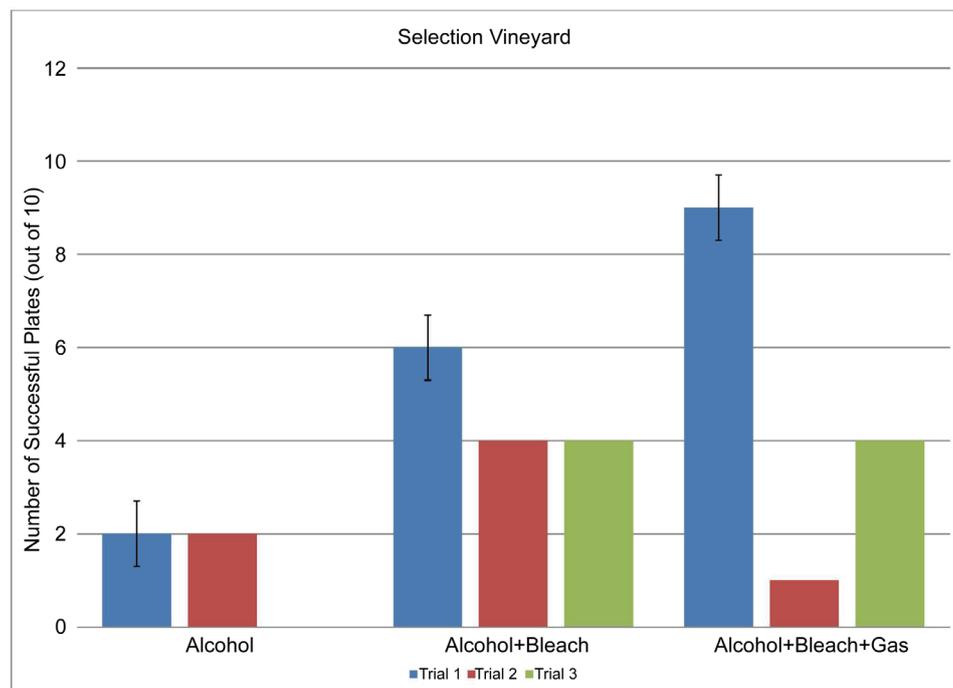


Figure 5. Results from three trials using samples taken from plants in the vineyard and placed on selection media after sterilization. There were significant differences between the Selection Vineyard A treatments and the Selection Growth Chamber A + B + C ($p = 0.015$) treatments, the Selection Growth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

Table 1. Number of successful plates (out of 30) for explants from growth chambered (GC) 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	GC – NS	GC – 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 ^{ab}	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.

having grown out of the internal portions of the explant, and not from the surface.

4. Discussion

In all instances, explants taken from vines grown in controlled growth chamber 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. The *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 growth chamber allowed the vines to continue

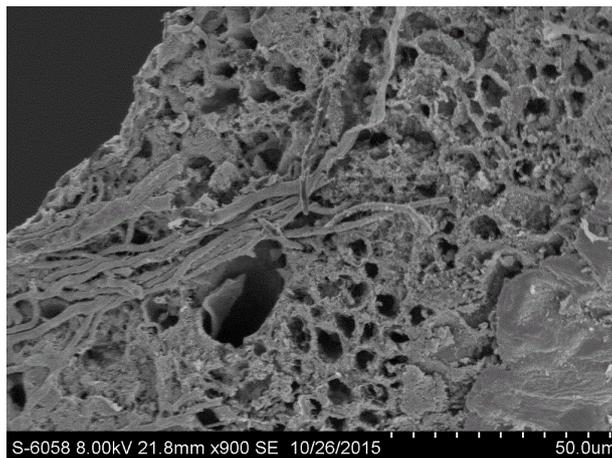


Figure 6. Cross section of *V. aestivalis* explant sample with fungal hyphae erupting from between vascular regions of the plant. Image taken at 900× magnification and 8.0 kV acceleration.

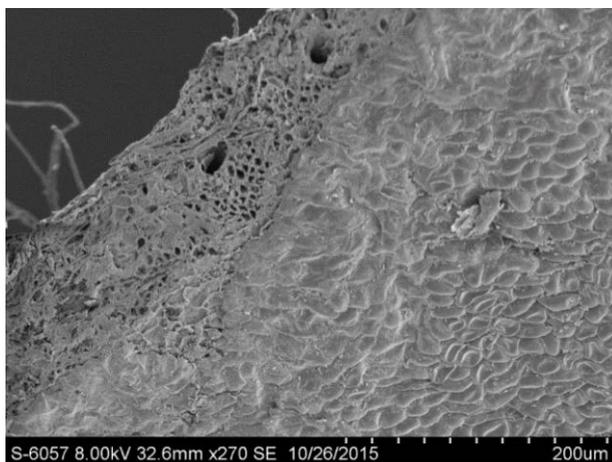


Figure 7. Cross section of *V. aestivalis* explant sample with fungal hyphae erupting from between vascular regions of the plant. Image taken at 270× magnification and 8.0 kV acceleration.

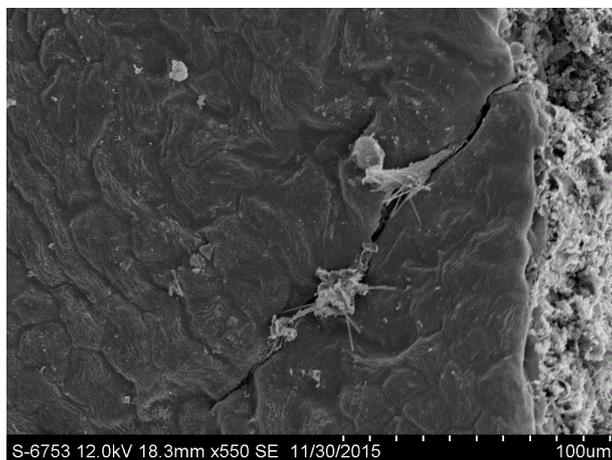


Figure 8. Surface of *V. aestivalis* explant sample with fungal hyphae erupting from between cells. Image taken at 550× magnification and 12.0 kV acceleration.

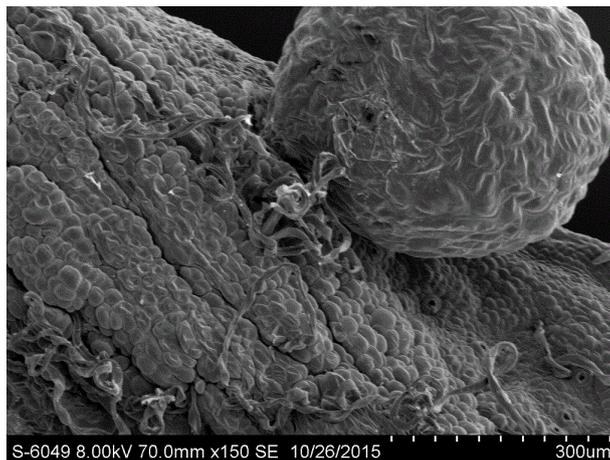


Figure 9. Surface of *V. aestivalis* explant sample with fungal hyphae erupting from between partially dedifferentiated cells. Image taken at 150× magnification and 8.0 kV acceleration.

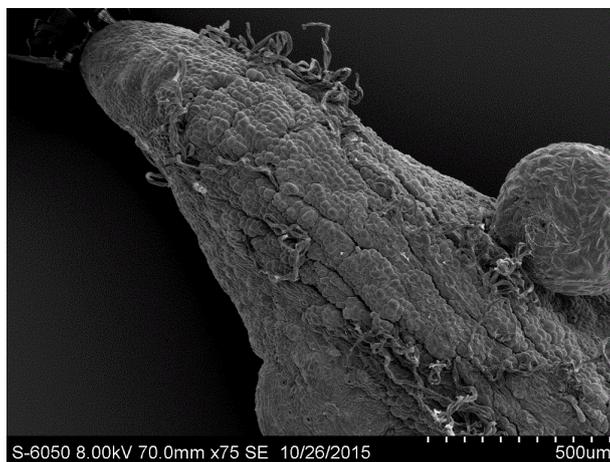


Figure 10. Surface of *V. aestivalis* explant sample with fungal hyphae erupting from between partially dedifferentiated cells. Image taken at 75× magnification and 8.0 kV acceleration.



Figure 11. Surface of *V. aestivalis* explant sample with fungal hyphae erupting from between partially dedifferentiated cells. Image taken at 110× magnification and 15.0 kV acceleration.

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 chamber trials, as sterilization steps were added, the rate of successfully initiating a contamination-free callus culture increased. In the non-selection 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 “Norton/Cynthiana”. 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 grown in a growth chamber, sterilizing with at least alcohol and bleach (and chlorine dioxide, if available) increases the success rate of callus production from “Norton/Cynthiana”.

Fungal hyphae were detected erupting from explants after surface sterilization (**Figures 7-10**). Imaging of the endophyte growth from within explant samples combined with the surface sterilization of said explants lends support to the idea that fungal contamination in “Norton/Cynthiana” cultures can result from insufficient sterilization of fungal endophytes, as well as, surface spores. Endophyte suppression through surface sterilization can prove difficult, as plant tissues are naturally resistant to deep tissue penetration by conventional surface sterilization efforts.

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 that are 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 [13], 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.

While SEM imaging indicates endophyte contamination can explain the high loss rate in tissue callus production of “Norton/Cynthiana” when surface sterilization is employed alone, the rate of survival and successful callus initiation indicates the efficacy of employing a selection pressure during the initial phase of callus initiation. 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 “Norton/Cynthiana”. However, the fungal endophyte associated with “Norton/Cynthiana” 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 a growth chamber simulating springtime conditions, sterilized in alcohol, bleach, and chlorine dioxide, and plated on a selection media 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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