Renewal Progress Report for CDFA Agreement Number #18-0397

Grape protoplast isolation and regeneration of plants for use in gene editing technology

Project Leader
David Tricoli
Plant Transformation Facility
University of California
Davis, CA 95616
dmtricoli@ucdavis.edu

Time Period Covered by this report: The results reported here are from work conducted from September 6, 2018 – February 20, 2019.

INTRODUCTION

The development of a system that achieves successful isolation of grape protoplasts, formation of callus from those protoplasts and ultimately the regeneration of protoplast-derived plants has significant relevance to the PD/GWSS Research Community and the wine-grape industry. It provides an excellent vehicle for deploying non-*Agrobacterium*-mediated non-integrating gene editing technology for fundamental research and product development. Even if the goal of regeneration of plants from protoplast is not achieved, efficient formation of protoplast-derived callus can be used for high throughput testing of potential gene editing guide RNAs. If regeneration of whole plants can be achieved, it will allow for the production of non-chimeric gene edited plants, which is critical for clonally propagated crops such as grape. Protoplast technology was actively researched in the 1980s and early 1990s, but the advent of transgenic technology resulted in this cell culture technique falling out of favor. Although there are published reports in the literature demonstrating successful isolation of protoplasts from grapes, production of callus from grape protoplasts has historically proven to be inefficient (Xu et al., 2007). In addition, to my knowledge, regeneration of grape plants from protoplast has not yet been achieved. We believe that utilizing embryogenic callus and rapidly dividing grape suspension cultures may provide advantages over other tissue sources. Encapsulating protoplasts in alginate beads and culturing them in conditioned medium or nurse cultures has enhanced the frequency of protoplast division in other crops. We believe this technique should also be applicable for culturing grape protoplasts. Encapsulation of protoplasts in alginate beads will also allow us to test many different media components by culturing beads in a 24 well plate format, which will allow us to test media addendums using a factorial design. Given that embryogenic callus and suspension cultures are highly efficient in regenerating embryos and plants, and given that the protoplasts will be produced directly from these tissues, we believe this material gives us the best possibility of regenerating embryos and plants from protoplast-derived callus. Over the past five month, we have develop a robust grape protoplast isolation and purification system which routinely produced high yields of protoplasts from embryogenic callus of the three grape genotypes we tested; Merlot, Thompson Seedless and 1103P. By encapsulating protoplasts in a calcium alginate matrix and culturing them in osmotically adjusted grape feeder cell suspensions, we can routinely stimulate callus development from isolated protoplast. We have discovered that the addition of antioxidants to the culture medium significantly improves callus formation from protoplasts. Over the following year, we will be testing various media formulations in an effort to regenerate plants from protoplast-derived callus.

OBJECTIVES

1. Develop protoplast isolation techniques for grape using actively dividing grape embryogenic cultures.
2. Culture grape protoplasts in calcium alginate beads and stimulate the formation of callus colonies.
3. Stimulate plant regeneration from protoplast-derived callus colonies.

Progress:

Objective 1. Develop protoplast isolation techniques for grape using actively dividing grape cultures

In 2018, we established new somatic embryogenic cultures for Merlot, 1103P and Thompson Seedless from anther filaments harvested from immature flowers collected from the Foundation Plant Service's vineyards **(Figure 1).**

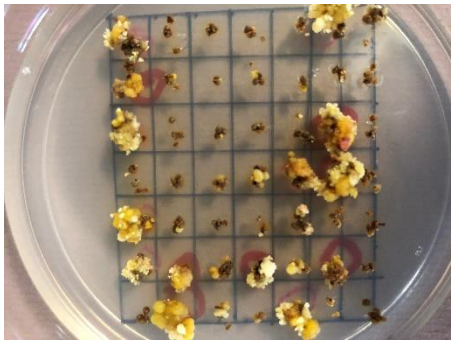

Figure 1. Somatic embryos of Merlot clone 3 generated from anther filaments and increased *in vitro*.

We have used these somatic embryo cultures to establish new embryogenic suspension cultures and embryogenic callus cultures. Somatic embryos from anther filaments were transferred from agar-solidified plates to liquid WPM medium (Lloyd and McCown, 1981) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 1 mM MES, 10.0 mg/l Picloram, 2.0 mg/l metatopolin, 2g/l activated charcoal, 100 mg/l ascorbic acid and 120 mg/l reduced glutathione (Pic/MTag) and grown in 125 ml shake flasks on a gyratory shaker at 100 rpms in the dark. (**Figure 2**).

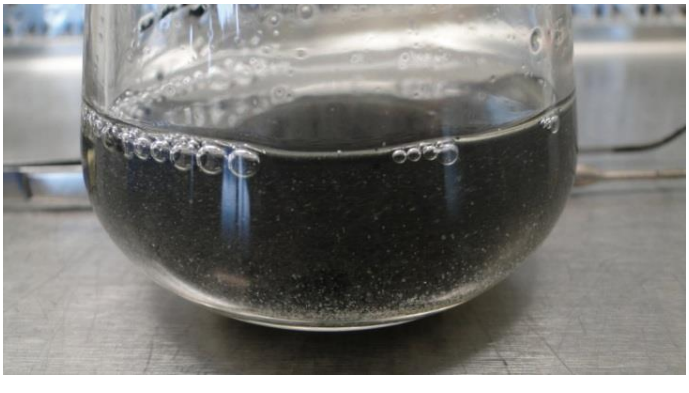

Figure 2. Fine suspension cultures of 1103P growing in WPM, 20 g/l sucrose, 1g/l casein, 1mM MES, 1,000 mg/l activated charcoal, 10 mg/l picloram, 2 mg/l meta-topolin, 100 mg/l ascorbic acid and 120 mg/l reduced glutathione (Pic/MTag)

We collected aliquots of these suspensions and plating them on agar solidified WPM medium (Lloyd and McCown, 1981) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 1 mM MES, 10.0 mg/l Picloram, 2.0 mg/l thidiazuron, 2g/l activated charcoal (PIC/TDZ) in order to generate embryogenic callus cultures which we are using for isolation of grape protoplasts **(Figure 3)**.

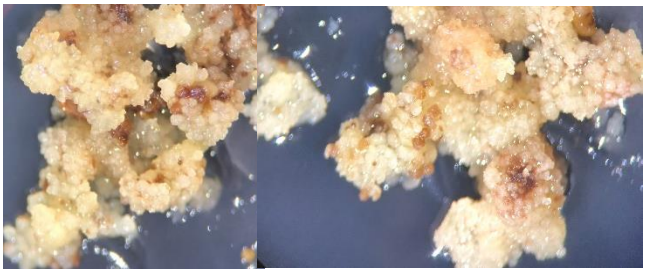

Figure 3 Embryogenic callus generated by plating a 200 ul aliquot of grape suspension culture onto agar solidified WPM medium (Lloyd and McCown, 1981) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 1mM MES, 10.0 mg/l Picloram, 2.0 mg/l thidiazuron, and 2g/l activated charcoal (PIC/TDZ).

Protoplast Isolation and purification

We collected aliquots of rapidly dividing embryogenic grape suspension cultures of 1103P and Thompson Seedless and centrifuged them at 2000 rpm for 8 minutes to harvest approximately 3 ml packed cell volume. We removed the supernatant, replaced it with 5 ml of protoplast isolation solution, and transferred the solution to a 60 x 15 mm petri dish. Alternatively, we harvested embryogenic callus of Merlot or Thompson Seedless from agar-solidified plates containing Pic/TDZ medium. We treated both sources of cells in an enzyme solution consisted of filter sterilized 0.5% Onozuka Cellulase R10, 0.25% pectinase, 0.25% macerozyme R10, 0.4 M mannitol, 5mM $CaCl_2$, 10 g/l BSA, and 5 mM MES. We subjected the cells to infiltration under house vacuum for three, 2-minute exposures and incubated the solution in the dark at 25 degrees centigrade on a platform shaker at 50 rpms. After approximately 16 hours incubation, we filtered the protoplast solution through a 40 um screen and collected the protoplasts by pelleting via centrifugation at 2000 rpm for 10 minutes. We washed the protoplasts twice in an osmotically adjusted wash solution containing 0.4 M mannitol, 2mM $CaCl_2$, 1g/l BSA and 1,191 mg/l HEPES. We initially tried to purify the protoplasts isolated from suspension cultures using a dextran gradient consisting of 4 ml of a 13% dextran solution, overlaid with 2 ml of a 9.1% dextran solution, overlaid with 1 ml of a 0.4 M wash solution. We have successfully used this dextran gradient to isolate lettuce and soybean mesophyll protoplast. However when this gradient was used for grape suspension cultures and centrifuged at 2000 rpms for 8 minutes, we found that the activated charcoal that is used in the suspension culture, layers at the same band as the protoplasts; the interface of the 9.1% dextran and the wash solution. We therefore modified the dextran gradient by adding a third dextran layer The new gradient consist of 4 ml of a 13% dextran solution, over overlaid with a 3 ml of a 9.1% dextran solution, overlaid with 2 ml of a 4.05% dextran solution, overlaid with 1 ml of wash solution. When centrifuged at 2000 rpms for 8 minutes, the protoplasts layer at the interface of the 4.05% dextran layer and the wash solution, effectively separating the protoplasts with minimal contamination from the activated charcoal. Protoplasts derived from embryogenic callus harvested from agar solidified Pic/TDZ medium could be purified using a dextran gradient consisting of 2 ml of a 13% dextran solution, overlaid with 1.5 ml of 0.4 M wash solution since no activated charcoal use in the preparation. The protoplast band was readily harvested with a sterile Pasteur pipette, and transferred to a 60 x 15 mm petri dish. Yields of protoplasts from 3 ml packed cell suspension volume or 500 mg fresh weight of embryogenic callus ranged from 2.5 to 8 $x10^6$ cells per ml **(Figure 4)**.

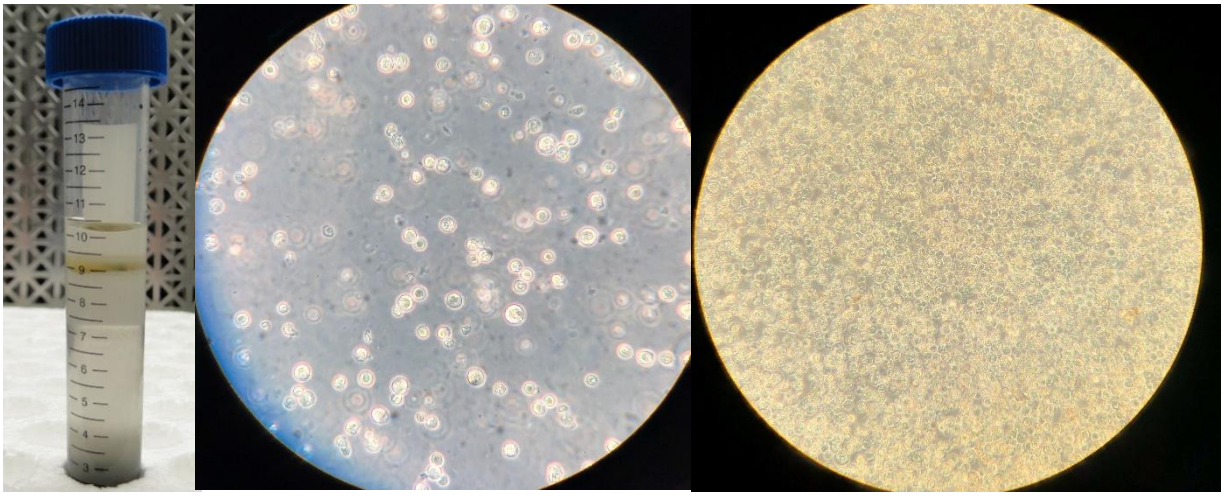

Figure 4. Dextran gradient separates grape protoplasts from debris (left). Harvested Merlot (middle) and Thompson Seedless (right) grape protoplast prior to encapsulation in calcium alginate beads.

Objective 2. Culture grape suspension protoplasts in calcium alginate beads and stimulate the formation of callus colonies.

Generating osmotically adjusted conditioned medium

The Plant Transformation Facility at UC Davis has developed a method for encapsulating protoplasts in calcium alginate beads and culturing them in an osmotically conditioned feeder suspension culture. The feeder suspension is use to stimulate the protoplasts to divide to form calli even at low protoplast culture density. We demonstrated this to be efficacious in soybean (Tricoli et al., 1986) and lettuce protoplasts (Tricoli unpublished). Protoplasts need to be cultured in high osmotic medium to prevent them from implosion or explosion prior to the reformation of their cell walls. The conditioned media we are testing are based on formulations used to stimulate somatic embryo development from isolated grape anther filaments. These include:

- Nitsch and Nitsch minimal organics medium (1969) supplemented with 60 g/l sucrose, 1.0 mg/l 2, 4-dichlorophenoxyacetic acid (2,4-D) and 2.0 mg/l benzylaminopurine (BAP) (PIV)
- MS minimal organics medium supplemented with 20 g/l sucrose 1.0 mg/l 2,4-D and 0.2 mg/l BAP (MSE)
- MS minimal organics medium supplemented with 30 g/l sucrose 1.0 mg/l 2,4-D and 1.0 mg/l BAP (MS1)
- WPM medium supplemented with 20 g/l sucrose 10 mg/l Picloram and 2.0 mg/l TDZ (Pic/TDZ)

We generated osmotically conditioned grape feeder suspensions of Thompson Seedless and 1103P by gradually increasing the osmotic potential of the suspension medium over time. During the bi-weekly subcultures of the suspension cultures, we removed one-half of the suspension and replaced it with grape suspension medium containing 72.87 g/L mannitol, 1191 mg/l HEPES and 1g/L BSA, pH 5.7 along with the appropriate plant growth regulators. During the subsequent bi-weekly subculture, we again removed one-half of the old suspension and replaced it with an equal volume of medium containing 72.87 g/L mannitol, 1191 mg/l HEPES and 1g/L BSA, pH 5.7. We repeated this process bi-week so the cells could gradually acclimated to the high osmotic medium over time. Once suspensions were actively growing on high osmotic medium, we harvested conditioned medium on a bi-weekly basis by centrifugation at 2000 rpm for 10 minutes. We collected the supernatant and stored it at 4 degrees centigrade or used it immediately to culture the bead-encapsulated protoplasts. Alternatively, we cultured encapsulated protoplasts in conditioned suspension cultures as opposed to conditioned medium lacking cells, but great care must be taken to ensure that suspension cells are completely removed prior to the alginate bead being dissolved. When using feeder suspension cultures, we always used a feeder suspension of a genotype different from the encapsulated protoplasts. Therefore, if we encapsulated merlot protoplasts, we used Thompson seedless or 1103P conditioned cells as the feed suspension.

Encapsulating the protoplasts

In order to generate the protoplast containing alginate beads, we adjusted the protoplast density to two time the desired final density with 0.4M mannitol/buffer solution. We mixed the protoplast solution with an equal volume of a 6.4% or 3.2% sodium alginate solution (adjusted to pH 5.7). We formed beads by drawing up the solution into a 12 ml sterile syringe and expelling the solution dropwise through a syringe needle into an osmotically adjusted 50 mM $CaCl_2$ solution. After 30 minutes in the $CaCl_2$ solution, we rinsed the beads one time in 0.4M mannitol/buffer wash solution **(Figure 5).** The size of the beads can be increased or decreased depending on the gauge of the needle. We used either a 30.5 or 23-gauge needle to make beads that were approximately 2 mm or 5 mm in diameter respectively. We have also modified the gel strength of the beads by using either 6.4% or 3.2 % sodium alginate. Varying both bead size and alginate concentration can effect diffusion of nutrients into the beads.

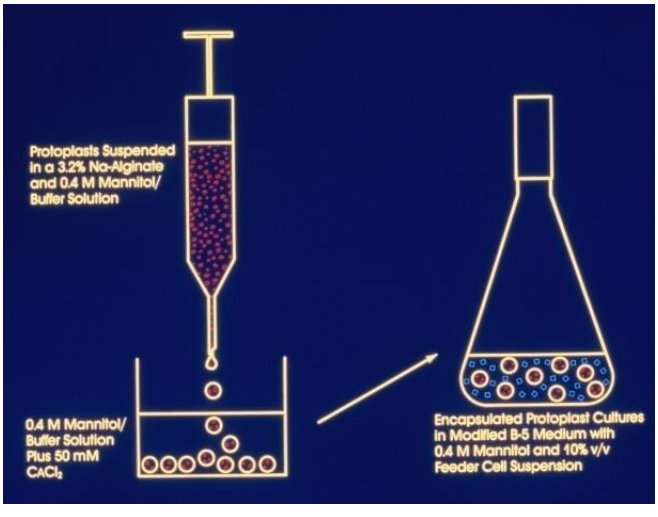

Figure 5. Diagram of the production of protoplasts encapsulated in alginate beads and cultured in conditioned medium.

In addition to allowing one to test various media formulation, embedding protoplasts in calcium alginate beads also insures that each protoplast-derived callus colony is from single cell descent. This will be important for gene editing experiments since if protoplasts are not fixed in a matrix; they will rapidly clump together making determining single cell descent impossible. Normally when cultured at low density, protoplasts fail to divide. However, culturing embedded protoplasts in conditioned medium or with feeder suspensions has been shown to stimulate protoplasts division in other species even at very low cell densities. Since the alginate matrix is permeable to nutrients, the conditioned medium serves as a nurse culture for the low-density cultured protoplasts. Previously, we have demonstrated that a single protoplast encapsulated in a 2-5 mm alginate bead could be stimulated to divide using this nurse culture system for both soybean and lettuce

We have successfully embedded grape protoplast of 1103P, Thompson Seedless and Merlot in calcium alginate beads and they have survived the embedding process. We initially cultured the embedded protoplasts in 24 well plates, which allowed us to test multiple hormone and media formulations for their ability to stimulate cell division using a factorial design. We placed one ml of medium osmotically adjusted with 0.4 M mannitol in each well along with 10-20 beads and incubated the plate in the dark on a platform shaker at 50 rpms. After 14 days, we added 1 ml of medium of the same formulation but lacking mannitol to each well, thereby reducing the osmotic of the medium in half. We monitored protoplasts for cell wall formation and division over a 4-6 week period. Using this system, we tested the osmotically adjusted media formulations of Gamborg OL, Miller RA, and K Ojima. 1968, Chee, R and R M Pool. 1987, Driver, J.A. and A.H. Kuniyuki, 1984, Lloyd and McCown, 1981, Murashige, T. and Skoog, F., 1962, Quoirin, M. and P Lepoivre 1977, Rugini, E. 1984, and Schenk, RU and AC Hildebrandt, 1972. We also tested a wide array of hormone combination using the 24 well format, which allows for a two or three-dimensional factorial design **(Figure 6).** Using this design, we were able to quickly test over 150 different combinations of hormones and 8 different salt formulations. To date, we observed the highest

amount of cell wall formation and first cell divisions on Murashige and Skoog and Lloyd and McCown medium. We also tested media known to stimulate embryogenic callus formation from anther filament tissue. We collected conditioned PIV, MES, MS1, and Pic TDZ cell suspension cultures that had been acclimated to growing under high osmotic as described above, pelleted the cells by centrifugation, transferred 1 ml of this medium to 24 well plates and cultured encapsulated protoplasts in the wells. We found that the best-conditioned medium for stimulating protoplast cell division was Pic/TDZ and this formulation was advances to studies involving cell suspension feeder cultures in 125 ml shake flasks.

The system has also allowed us to rapidly tested non-hormonal medium addendums including putrescene, spermidine, pluronic F68, resveratrol, citric acid, ascorbic acid, L-cysteine and reduced glutathione either alone or in various combinations. Although this 24 well format allows us to observe protoplast viability, cell wall formation and the first few cell divisions, the number of protoplasts that divide is low. In addition, although cells underwent a few divisions in 24 well plates, they failed to advance beyond the four to eight cell stage. Still, this 24 well format allowed us to determine which salt formulations, hormone combinations and non-hormone addendum to advance to feeder suspension studies.

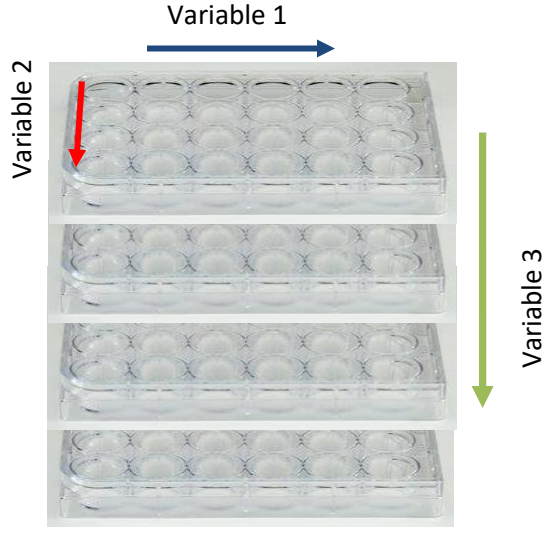

Figure 6. Culturing encapsulated protoplasts in 24 well plates allows one to test numerous salt formulation and hormone concentration in a single experiment.

Development of mini-calli from encapsulated protoplasts using feeder suspensions

Using feeder cell suspension, we have made significant advances in stimulating isolated protoplasts to divide and form callus. The use of a feeder suspension greatly improved cells division and callus colony formation from protoplasts when compared to conditioned medium alone. We re-suspended embedded protoplasts in conditioned osmotically adjusted grape cell suspension cultures in 125 ml shake flasks and incubated them at 100 rpm and 25 degrees centigrade. In this system, we utilize a grape feeder suspension culture that is of a different genotype than the genotype used to generate the protoplasts. For example, we used 1103P conditioned cell suspension cultures for embedded Thompson Seedless protoplasts. Viable protoplasts began dividing in 4-7 days. We added equal volume of grape suspension culture medium without mannitol to the flasks at day 14, thereby reducing the starting mannitol concentration to 0.2 M. After 14 additional days, we again added equal volume of grape suspension culture medium without mannitol to the flasks, thereby reducing the starting mannitol concentration to 0.1 M. By day 21 post encapsulating, small callus colonies were visible (**Figure 7**).

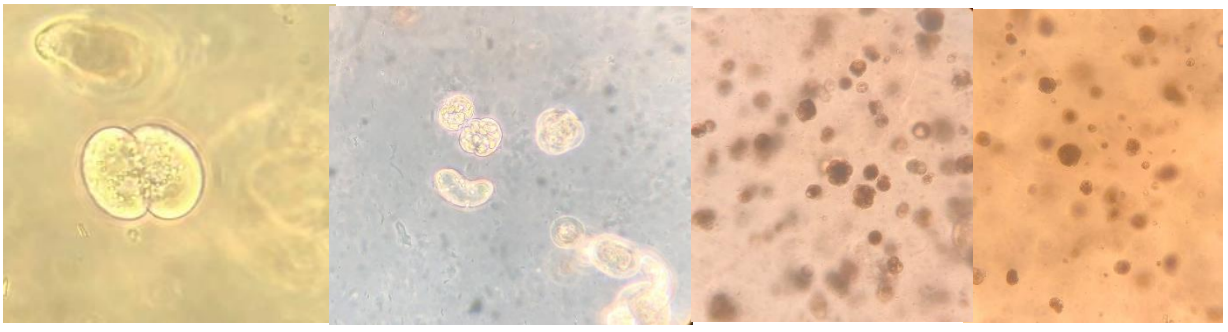

Figure 7. First cell division of a Thompson Seedless protoplast embedded within a calcium alginate bead (left) multi-cell stage (middle-left), Thompson seedless (middle-right) and Merlot (right) protoplasts forming mini callus colonies.

Callus colonies that develop in alginate beads often became discolored due to phenolic production. We were concerned that these compounds might be toxic to the growth and development of the protoplast-derived callus. We have develop and tested an antioxidant solution consisting of 100 mg/l ascorbic acid, 150 mg/l citric acid, 30 mg/l reduced glutathione and 100 mg/l L-cysteine (PTF AO). We tested the effect of this antioxidant addendum on protoplast viability and division on Thompson seedless and merlot protoplast culture. Protoplast were isolated and encapsulated in 1.6% calcium alginate beads. Calcium alginate beads from the same protoplast preparation were randomly transferred to shake flasks containing conditioned 0.4M Pic/TDZ 1103P feeder cell suspensions with or without the addition of 1x or 5x of the antioxidant solutions. After 14 days, the osmotic strength of the feeder suspension was reduced from 0.4 M to 0.2 M mannitol. At day 35, we randomly harvested five beads per treatment and counted the number of mini calli per bead. For both merlot and Thompson seedless protoplasts, a significantly higher percentage of callus colonies were observed developing in beads grown in the suspensions containing the antioxidant solution (**Table 1**). Merlot protoplasts tend to produce more phenolic than Thompson seedless protoplasts and the antioxidant addendum had a much more profound effect on the division of the merlot protoplasts than the Thompson seedless protoplasts. Without the addition of the antioxidant mixture, no mini calli were observed after 35 days in culture, whereas an average of 168 mini calli were produced per bead from protoplasts grown in feeder cultures with the antioxidant addendum (**Table 1**). We also observed increased Thompson seedless protoplast viability and increased callus development with increasing antioxidants concentration (**Table 2, Figure 8**) and we are currently exploring the effects of increased anti-oxidants on merlot protoplasts.

Table 1. Addition of antioxidant solution consisting of 100 mg/l ascorbic acid, 150 mg/l citric acid, 30 mg/l reduced glutathione and 100 mg/l L-cysteine enhances protoplast-derived callus formation.

Number of callus colonies per bead				
Bead	**TS wo/anti-oxidants**	**TS w/ anti-oxidants**	**Merlot w/o anti-oxidants**	**Merlot w/anti-oxidants**
1	**13**	**45**	**0**	**199**
2	**42**	**52**	**0**	**182**
3	**27**	**37**	**0**	**159**
4	**30**	**36**	**0**	**160**
5	**30**	**69**	**0**	**139**
Ave	**28.4**	**47.8**	**0**	**167.8**

Table 2. Increasing the concentration of the antioxidant formulation consisting of 100 mg/l ascorbic acid, 150 mg/l citric acid, 30 mg/l reduced glutathione and 100 mg/l L-cysteine from 1x to 5x enhances protoplast-derived callus formation in Thompson seedless protoplasts.

Bead	TS w/ 1x anti-oxidants	TS w/ 5x anti-oxidants
1	2	112
2	6	114
3	10	130
4	10	118
5	13	125
Ave		

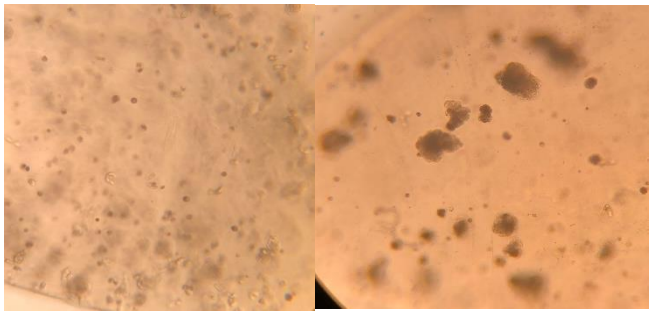

Figure 8. Callus formation from encapsulated Thompson seedless protoplasts cultured in grape feeder suspension medium with 1x antioxidant mixture (left) and 5x antioxidant mixture (right)

Callus colonies continued to develop within the calcium alginate beads and often grew large enough that they could be seen rupturing through the surface of the beads (**Figure 9**). Once protoplasts reached this stage, we dissolve the calcium alginate matrix to release the callus colonies.

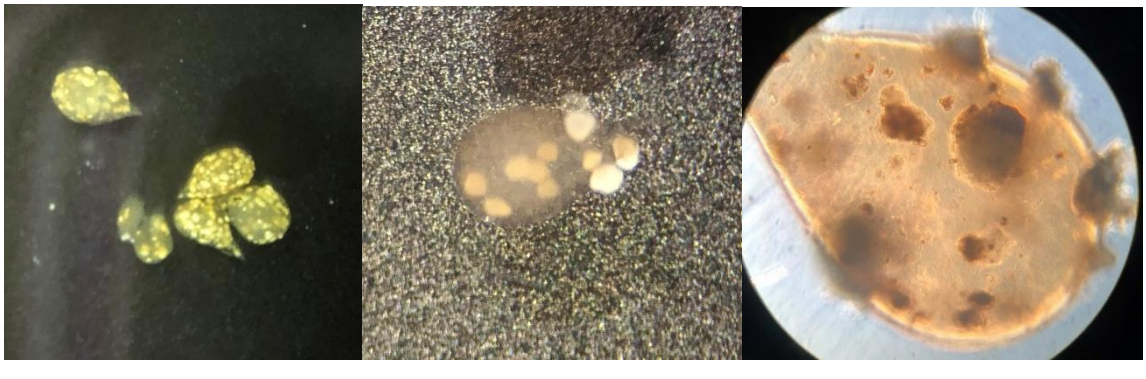

Figure 9. Mini colonies from Thompson seedless protoplasts encapsulated in calcium alginate beads and grown in conditioned cell suspension of 1103P (left). Callus colonies growing out of the alginate matrix (middle). Close up of an individual bead with developing callus colonies (right).

Dissolving the calcium alginate matrix and plating the protoplast-derived callus

Once protoplasts developed into callus colonies of approximately 16-32 cells within the alginate beads, we transferred the beads and conditioned feeder suspension into a 100 x 20 mm petri dish. Using forceps, we manually transferred individual beads to a 100 x 20 mm petri dish containing 40 ml of Lloyd and McCown

minimal organics medium supplemented with 30 g/l sucrose, 10 mg/l Picloram and 2.0 mg/l TDZ. This transfer/washing process is repeated 1-2 more times to eliminate any of the feeder suspension cells. We transferred washed beads into a 125 ml shake flask containing 20 ml of a 300 mM KH_2PO_4 solution. We pipetted the beads and solution up and down repeatedly through a 10 ml pipet up to ten time to break up the alginate matrix. We placed the flask on a gyratory shaker at 100 rpm overnight. After 16 to 24 hours, we again pipetted the suspension up and down through a 10 ml pipet, which competed the dissolution of the matrix, releasing the protoplast-derived callus colonies.

Objective 3. Stimulate plant regeneration from protoplast derived mini calli.

We centrifuged suspensions containing the dissolved protoplast-derived calli at 2000 rpms for 8 minutes and removed the potassium phosphate solution. We re-suspended the calli in Lloyd and McCown minimal organics medium supplemented with 30 g/l sucrose, 10 mg/l Picloram and 2.0 mg/l TDZ. We prepared agar-solidified plates containing various media formulations, plated 0.5 ml of an actively growing grape suspension medium of various formulations on top of the agar, and covered the plated suspension with an 85cm Whatman filter disk. We placed a 7 cm filter disc on top of the 85 cm filter and plated the solution containing the protoplast-derived colonies on top of the 7 cm disk creating a nurse over layer culture system. Over the next year, using this nurse over layer technique, we will test various media formulation in an attempt to create embryogenic callus colonies and eventually regenerate embryos and plants from these protoplast-derived callus colonies.

Publications produced and presentations made that relate to the funded project.

Tricoli D. M. 2018. Grape protoplast isolation and regeneration of plants for use in gene editing technology. Pierce's Disease Symposium Report pp 101-107.

Research relevance statement, indicating how this research will contribute towards finding solutions to Pierce's disease in California.

The development of a system that allows the isolation of grape protoplasts, formation of mini calli and the ultimate regeneration of protoplast-derived plants has significant relevance to the PD/GWSS Research Community and the wine-grape industry. It provides an excellent vehicle for deploying non-*Agrobacterium*-mediated gene editing technology for fundamental research and product development using CRISPR-Cas9 gene editing techniques. Even if the goal of regeneration of plants from protoplasts is not achieved, efficient protoplast isolation and formation of protoplast-derived mini calli can be used for high throughput testing of potential gene editing guide RNAs. Similar systems have been developed in other crops and allow for rapid testing of gene editing strategies. If regeneration of whole plants can be achieved, it will allow for the production of non-chimeric gene-edited plants, which is critical for clonally propagated crops such as grape.

Laypersons Summary

CRISPR-Cas9 is a gene editing technology that allow one to make precise changes in a plant's genome. There are a number of methods for delivering the CRISPR-Cas9 into the animal cells. However, unlike animal cells, plants cells are incased in cell walls that prevent easy introduction of DNA into the cell. This makes the utilization of CRISPR-Cas9 or other gene editing approached more difficult for plant cells. Protoplasts are plant cells, which have had their cell walls removed. These cells are very delicate and require careful manipulation of the solutions in which they are grown. If the pressure of the solution outside the protoplast is not adjusted to match the pressure of the conditions within the cell, the protoplast will implode or burst. However if protoplasts can be stably maintained in culture, they allow for gene editing delivery techniques that are used in animal cells to be employed for plant cells. The purpose of this work is to develop a robust method to generate protoplasts from grape tissue and stimulate the protoplasts to reform a cell wall and divide. Once the cells divide, we will test different growth factors to try to stimulate the small cell colonies to form into embryos and germinate into plants. These techniques will provide a valuable tool for deploying gene-editing techniques to produce non-chimeric gene edited plants.

Literature Cited:

Chee, R and RM Pool. 1987. Improved inorganic media constituents for in vitro shoot multiplication of Vitis. Sci. Hort. 32: 85.

Driver, J.A. and A.H. Kuniyuki. 1984. In vitro propagation of Paradox walnut rootstock. HortScience 19:507–509.

Gamborg OL, Miller RA, and K Ojima. 1968. Nutrient Requirements of suspension cultures of soybean root cells. Exp. Cell Research Vol. 50: 151-158.

Grosser, J. and Gmitter F. 2011. Protoplast fusion for production of tetraploids and triploids: applications for scion and rootstock breeding in citrus. Plant Cell Tissue Organ Culture 104:343–357.

Lloyd, G and BH McCown. 1981. Commercially-feasible micropropagation of Mountain Laurel, Kalmia latifolia, by shoot tip culture. Proc. Int. Plant Prop. Soc. 30:421-427.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nitsch, JP and C Nitsch. 1969. Haploid plants from pollen grains. Science 163: 85-87.

Quoirin, M. and P Lepoivre. 1977. Improved medium for in vitro culture of Prunus sp. Acta. Hort. 78:437-442.

Rugini, E. 1984. In vitro propagation of some olive (Olea europaea L.) cultivars with different root ability, and medium development using analytical data from developing shoots and embryos. Scientia Horticulturae 24: 123-134.

Schenk, RU and AC Hildebrandt. 1972. Medium and Techniques for Induction and Growth of Monocotyledonous and Dicotyledonous Plant Cell Cultures. Can. J. Bot. 50: 199-204.

Tricoli, D.M. Heins, M. B. Carnes M. G. 1986 Culture of Soybean mesophyll protoplasts in alginate beads. Plant Cell Reports 5:334-337.

Xu, X., Lu, J. Dalling, D., Jittayasothorn and Grosser, J.W. 2007. Isolation and Culture of Grape Protoplasts from Embryogenic Suspensions Cultures and Leaves of Vitus vinifera and Vitis rotuundifolia. Acta Hort. 738 pp 787-790.

K. Status of funds.

We anticipate that all funds allocated for fiscal year FY2018-2019 will be expended.

L. Summary and status of intellectual property associated with the project

No intellectual property has been submitted for this work