**Interim Progress Report for CDFA Agreement Number #18-0397**

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

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**Time Period Covered by this report:** The results reported here are from work conducted from March 1, 2018 – June 31, 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. 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. 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 have demonstrated that this technique is applicable for culturing grape protoplasts. Encapsulation of protoplasts in alginate beads allows us to test many different media components by culturing beads in a 24 well plate format, which allows us to test media addendums using a factorial design. 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 and polyamines to the culture medium significantly improves callus formation from protoplasts. We are now in the process of 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 suspension cultures.
2. Culture grape suspension protoplast in calcium alginate beads and stimulate the formation of mini calli.
3. Stimulate plant regeneration from protoplast derived mini calli.

**Progress:**

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

In 2019, we collected immature flowers from the Foundation Plant Service's vineyards for Chardonnay, 1103P and Thompson Seedless and plated anther filaments to generate new embryogenic cultures to serve as a new source material from which to islate protoplasts and to establish new conditioned feeder suspensions. We are particularly interested in generating callus from Chardonnay. Chardonnay has proven difficult to modify using agrobacterium-mediated transformation and would therefore benefit from protoplast-mediated gene editing. Furthermore, Chardonnay produces less phenolic compounds in culture which makes it a better candidate for protoplast culture than other genotypes such as merlot. We are starting to harvest callus for Chardonnay anther filaments and over the next few months we will be bulking it up for use in protoplast culture. New cultures of 1103 will be used to establish new embryogenic conditioned nurse suspension cultures **(Figure 1**). We will use the new Thompson seedless callus to generate somatic embryos cultures for use in continuing our studies in the development of the protoplast isolation, culture and plant regeneration

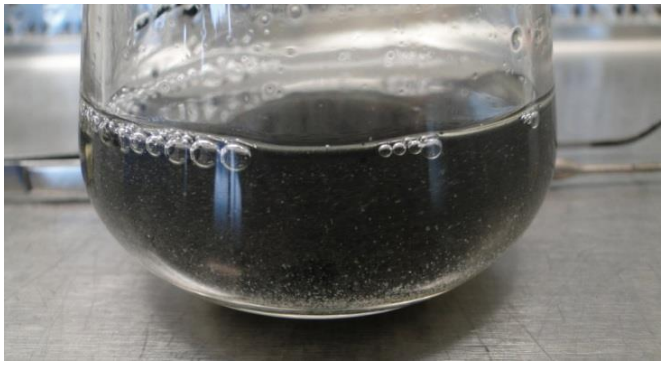

**Figure 1**. Fine suspension cultures of 1103P growing in WPM, 20 g/l sucrose, 1g/l casein, 1mM MES, 1,000 mg/l activated charcoal, 10 mg/l picloram, 2 mg/l meta-topolin, 100 mg/l ascorbic acid and 120 mg/l reduced glutathione (Pic/MTag)

We collected aliquots of 1103, merlot and Thompson seedless suspensions and plated 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 2)**.

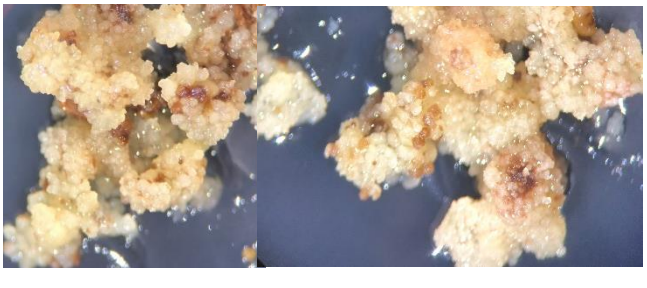

**Figure 2** Embryogenic callus generated by plating a 200 ul aliquot of grape suspension culture onto agar solidified WPM medium (Lloyd and McCown, 1981) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 1mM MES, 10.0 mg/l Picloram, 2.0 mg/l thidiazuron, and 2g/l activated charcoal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

**Protoplast Isolation and purification**

We harvest embryogenic callus of Merlot or Thompson Seedless from agar-solidified plates containing Pic/TDZ medium. We treated 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 purify protoplasts derived from embryogenic callus harvested from agar solidified Pic/TDZ medium using a dextran gradient consisting of 2 ml of a 13% dextran solution, overlaid with 1.5 ml of 0.4 M wash solution. We can readily harvest the protoplast band and transferred them to a 60 x 15 mm petri dish using a Pasteur pipette. Yields of protoplasts from 500 mg fresh weight of embryogenic callus ranged from 2.5 to 8 $x10^6$ cells per ml **(Figure 3)**.

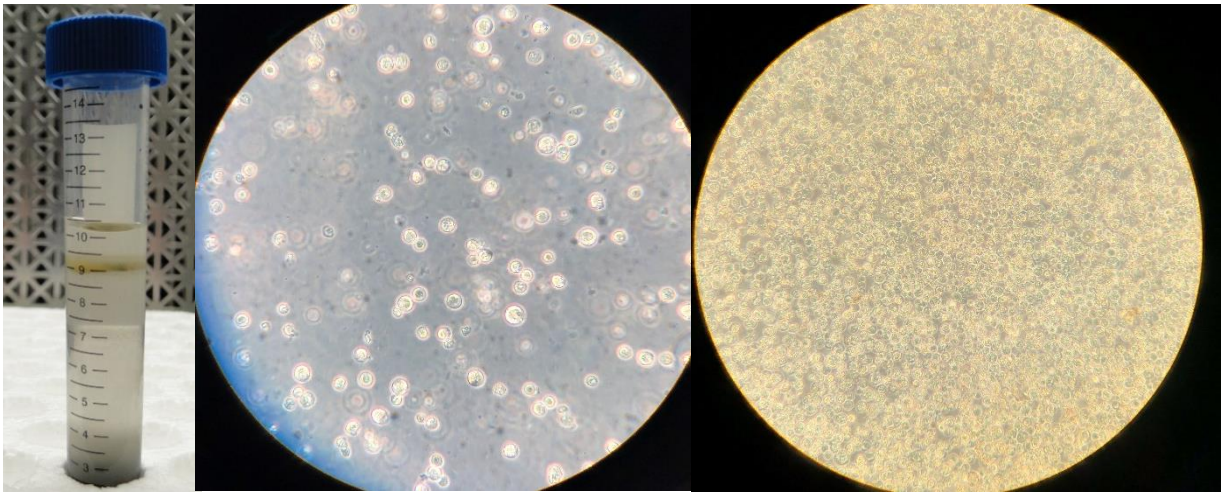

**Figure 3.** Dextran gradient separates grape protoplasts from debris (left). Harvested Merlot (middle) and Thompson Seedless (right) grape protoplast prior to encapsulation in calcium alginate beads.

**Objective 2. Culture grape suspension protoplast in calcium alginate beads and stimulate the formation of mini calli.**

**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 aviod 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.

**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 4).** 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.

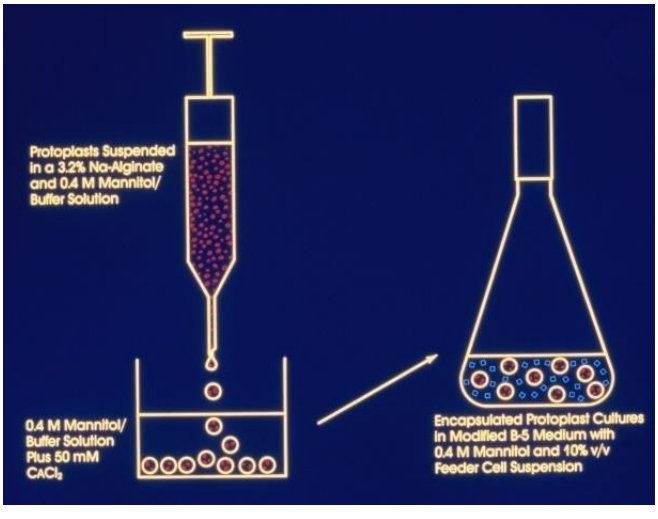


**Figure 4.** Diagram of the production of protoplasts encapsulated in alginate beads and cultured in conditioned medium.

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 et al., 1968, Chee, and Pool, 1987, Driver and. Kuniyuki, 1984, Lloyd and McCown, 1981, Murashige and Skoog, F., 1962, Quoirin, Lepoivre 1977, Rugini, 1984, and Schenk, and 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. 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.

**Development of mini-calli from encapsulated protoplasts using nurse 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 nurse 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 5**).

Recently, we have replaced the use of the shake flasks with 60 x 15 mm petri plates. This format allows for easy observation of the protoplasts using an inverted microscope and aids sampling of beads to evaluate the fate of the embedded protoplasts. Beads are cultured in 3.5 ml of Lloyd and McCown minimal organics medium supplemented with 30 g/l sucrose, 10 mg/l Picloram and 2.0 mg/l TDZ, 0.4 M mannitol, 1,191 mg/l HEPES, 50 mM $CaCl_2$, 5x antioxidant solution with 1.5 ml of conditioned 1103P cell suspension culture.

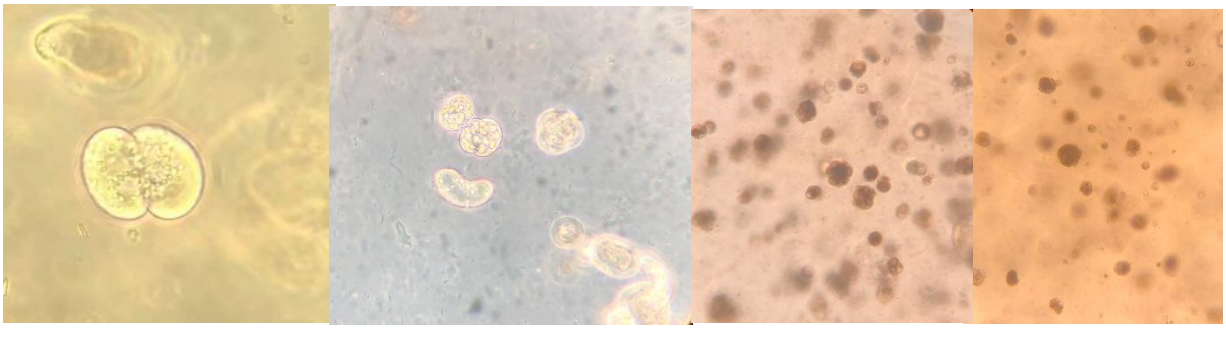

**Figure 5**. First cell division of a Thompson Seedless protoplast embedded within a calcium alginate bead (left) multi-cell stage (middle-left), Thompson seedless (middle-right) and Merlot (right) protoplasts forming mini callus colonies.

**Antioxidants**

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 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**).

**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/ 1x anti-oxidants** | **Merlot w/o anti-oxidants** | **Merlot w/ 1x 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 |
| **Average** | **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.**

| Number of callus colonies per bead | | |
|---|---|---|
| **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 |
| **Average** | **8.2** | **119.8** |

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 6**). Once protoplasts reached this stage, we dissolve the calcium alginate matrix to release the callus colonies.

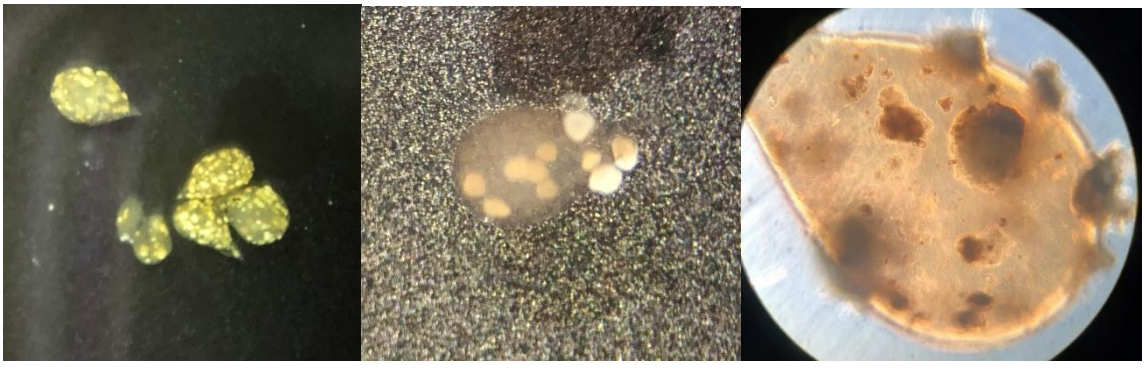

**Figure 6.** Mini colonies from Thompson seedless protoplasts encapsulated in calcium alginate beads and grown in conditioned cell suspension of 1103P (left). Callus colonies growing out of the alginate matrix (middle). Close up of an individual bead from experiment 18340 with developing callus colonies 6 weeks after encapsulation (right).

**Polyamines**

Polyamines are polycationic compounds, which effect many aspects of growth and stress response in plants including cell division, embryogenesis, organogenesis, floral, fruit and pollen development and senescence. The major polyamines in plants include Putrescine (PUT) spermidine (SPD), spermine (SPM), and cadaverine (CAD).

We are testing the ability of these polyamines to increase the plating efficiency of encapsulated protoplast as well as increase the rate of cell division. Initially we tested 1.0 mM putrescine in combination with 0.0 or 0.1mM spermidine since we have previously founds these levels to be beneficial in other plant species. We found the inclusion of 1.0 mM putrescine stimulated callus colony formation but the addition of 0.1 mM spermidine did not enhance the response. We also tested 1.0 mM putrescine in combination with 0.0, 0.1 and 1.0 mM spermine and 0.0 .01 or 1.0 mM spermidine. Interestingly, we have found that the addition of 1.0 mM spermine to the medium enhanced the number of protoplast that divided and the speed of protoplast derived callus formation irrespective of the spermidine concentration. Very large multicellular colonies developed within 21 days of protoplast isolation. We did not see any enhancement in growth with the addition of 0.1 mM spermine (**Table 3, Figure 7**).

**Table 3**. Callus colony formation 14 days after encapsulation of TS protoplast in calcium alginate beads and grown in conditioned 0.4M Pic/TDZ 1103P feeder cell suspensions containing 1mM putrescine, and varying levels of spermidine (SPD) and spermine (SPM).

| **# colonies /alginate bead Experiment 19129** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Bead** | | **1** | **2** | **3** | **4** | **5** | **Total** | **Average** |
| mM Polyamine | | | | | | | | |
| SPD | SPM | | | | | | | |
| 0.0 | 0.0 | 28 | 49 | 48 | 39 | 25 | 164 | 32.9 |
| 0.1 | 0.0 | 91 | 93 | 120 | 110 | 93 | 507 | 101.4 |
| 1.0 | 0.0 | 81 | 97 | 79 | 125 | 89 | 471 | 94.2 |
| 0.0 | 0.1 | 96 | 81 | 79 | 59 | 84 | 399 | 79.8 |
| 0.1 | 0.1 | 135 | 119 | 79 | 130 | 85 | 548 | 109.6 |
| 1.0 | 0.1 | 147 | 107 | 103 | 137 | 90 | 584 | 116.8 |
| 0.0 | 1.0 | 275 | 126 | 238 | 169 | 221 | 1029 | 205.8 |
| 0.1 | 1.0 | 219 | 168 | 154 | 84 | 188 | 813 | 162.6 |
| 1.0 | 1.0 | 212 | 262 | 196 | 220 | 162 | 1052 | 210.4 |

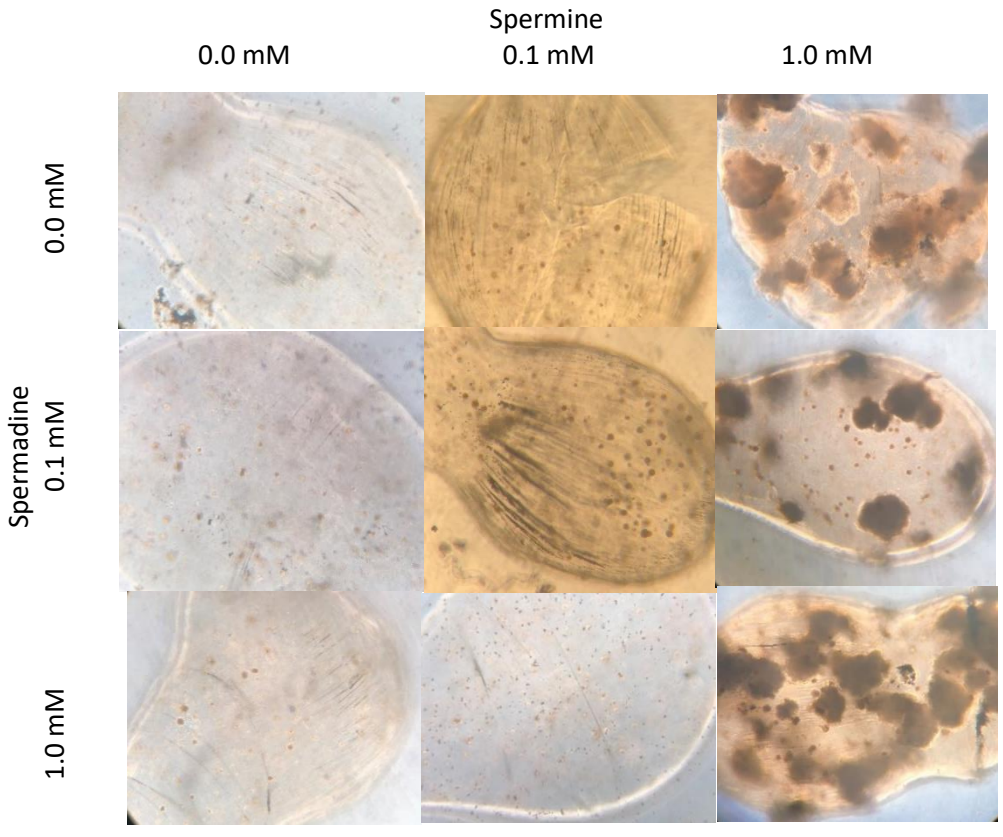


**Figure 7.** Enhanced growth of TS mini calli when grown in the presence of 0.0, 0.1 or 1.0 mM spermidine in combination with 0.0. 0.1 or 1.0 mM spermine 21 days post encapsulation. All treatments also contained 1.0 mM putrescine and 5x antioxidant solution.

We also tested cadaverine at 0.0, 0.1 or 1.0 mM in combination with 1.0 mM putresciene, 0.1 mM spermidine, and 0.0, 0.1 or 1.0 mM spermine. Cadaverine does not seem to enhance cell division or cells growth from grape protoplasts. However, we again saw a significant enhancement in callus colony growth with treatments that contained 1.0 mM spermine after only 21 days of culture regardless of the level of CAD tested **(Table 4, Figure 8).**

**Table 4**. Callus colony formation 14 days after encapsulation of TS protoplast in calcium alginate beads and grown in conditioned 0.4M Pic/TDZ 1103P feeder cell suspensions containing 1.0 mM putrescine, 0.1 mM spermidine (SPD) and varying levels of spermine (SPM) and Cadaverine (CAD).

| **colonies /alginate bead Experiment 19136** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Bead** | | **1** | **2** | **3** | **4** | **5** | **Total** | **Average** |
| mM Polyamine | | | | | | | | |
| SPM | CAD | | | | | | | |
| 0.0 | 0.0 | 99 | 103 | 116 | 67 | 73 | 458 | 91.6 |
| 0.1 | 0.0 | 68 | 79 | 64 | 65 | 86 | 362 | 72.4 |
| 1.0 | 0.0 | 133 | 121 | 115 | 133 | 98 | 600 | 120 |
| 0.0 | 0.1 | 100 | 115 | 92 | 104 | 95 | 506 | 101.2 |
| 0.1 | 0.1 | 90 | 85 | 71 | 66 | 79 | 391 | 78.2 |
| 1.0 | 0.1 | 154 | 155 | 106 | 105 | 101 | 621 | 124.2 |
| 0.0 | 1.0 | 104 | 82 | 87 | 92 | 73 | 438 | 87.6 |
| 0.1 | 1.0 | 66 | 125 | 88 | 60 | 79 | 424 | 84.8 |
| 1.0 | 1.0 | 141 | 114 | 132 | 11 | 127 | 633 | 126.6 |

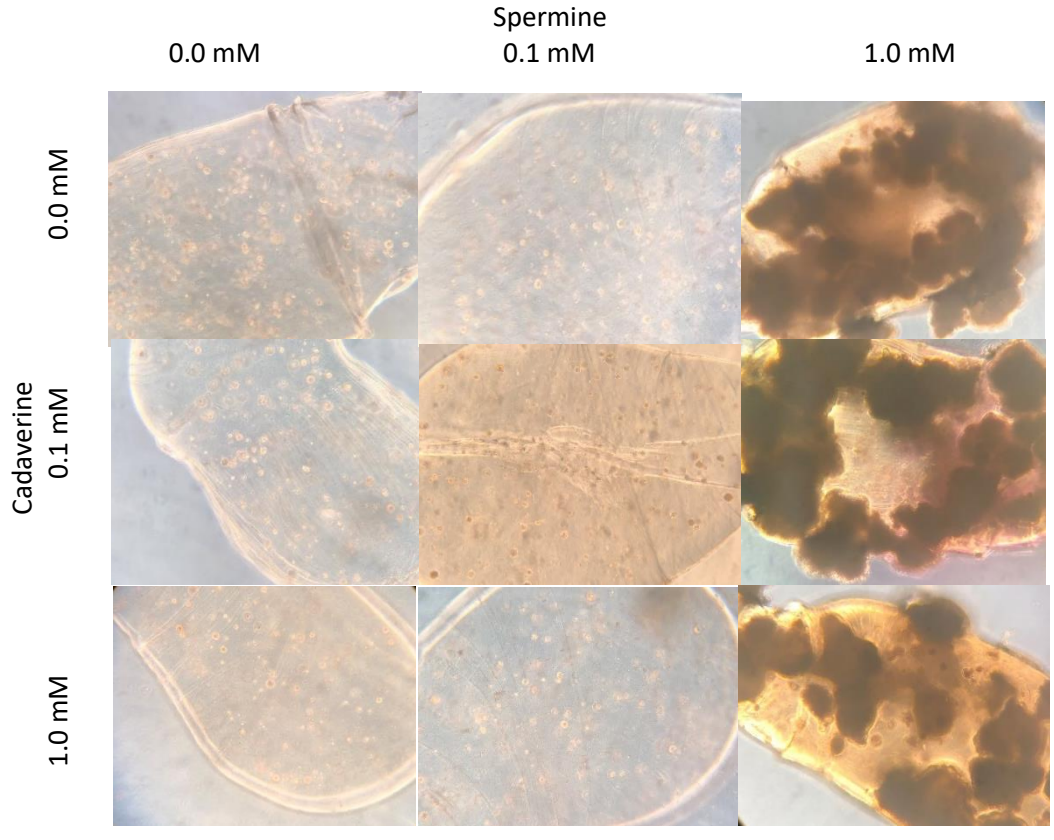


**Figure 8.** Enhanced growth of TS mini calli when grown in the presence of 0.0, 0.1 or 1.0 mM Cadaverine in combination with 0.0. 0.1 or 1.0 mM spermine 14 days post encapsulation.. All treatments also contained 1.0 mM putrescine. 0.1 mM spermidine and 5x antioxidant solution.

**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 2 additional 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. 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.

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

We prepared agar-solidified plates containing Pic/MT, Pic/TDZ and BN-Sorb and 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 85mm Whatman filter disk. We placed a 70 mm filter disc on top of the 85 cm filter and plated the solution containing the protoplast-derived colonies released from the calcium alginate matrix on top of the 7 cm disk creating a nurse over layer culture system. We have transferred intact calcium alginate beads containing

protoplast-derived mini callus colonies to the top of the 70.0 mm filter paper and incubated them at 23 degrees centigrade in the dark

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.

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**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**

I have submitted a Record of Invention to the University of California Innovation Access Group describing the production of callus colonies from grape protoplasts.