Renewal Progress Report for CDFA Agreement Number 11-0416-SA

Development of a Grape Tissue Culture and Transformations Platform for the California Grape Research Community

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Introduction

Tissue culture of grape plants remains an inefficient process for many genotypes. The procedure is labor intensive, limited to specific genotypes, and requires a significant amount of time to establish embryogenic cell cultures and convert cell cultures into whole plants. The efficiency of establishing embryogenic callus cultures and regenerating plants from callus remains very low for many important grape genotypes and is not at the level required to allow for the predictable, cost effective and timely recovery of tissue culture plants needed to successfully offer grape tissue culture and transformation through a self-sustaining service-based facility. These challenges include the successful establishment and multiplication of embryogenic cultures, prevention of tissue necrosis caused by oxidation, conversion of embryos into true-to-type plants, transformation of embryogenic callus, and the regeneration of non-chimeric transgenic plants from embryogenic cells. The goal of this agreement is to leverage the expertise of the National Research Laboratory of Chile, (INIA), and the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis (UCDPTF) to significantly increase the efficiency of tissue culture and transformation technology in grape genotypes important to their respective countries. The proposed collaboration combines pre-existing expertise and technical know-how to expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the PD/GWSS research community. Results of this collaboration will accelerate the delivery of PD/GWSS research results in genotypes that are relevant to the research community. This report outlines the progress that has been made to date toward achieving that goal and highlights improvements in growth of high quality embryogenic callus as well as the production of transgenic 1103,101-14 and Chardonnay embryos.

List of objectives.

1. To establish an international collaboration between leading laboratories in the US and Chile that share a common goal of accelerating the development of efficient tissue culture and transformation protocols for grape varieties of importance to the viticulture industries in their respective countries.
2. To develop a self-sustaining service facility that will provide grape tissue culture and transformation services for at least one rootstock and one wine grape genotype in support of the PD/GWSS Research Community

Description of activities conducted to accomplish each objective, and summary of accomplishments and results for each objective.

1. Adapt tissue culture and transformation methodologies developed by the Chilean partner, for grape genotypes of importance to California including 1103, 101-14, Cabernet and Chardonnay.

Progress:

A. Production of embryogenic callus from anthers of 11-03, Chardonnay and Cabernet Sauvignon.

We harvested anthers from grape genotypes 11-03, Chardonnay and Cabernet Sauvignon in the spring of 2011 and 2012 and have plated them onto two different callus induction media; either Murashige and Skoog minimal organics medium supplemented with 60 g/l sucrose, 1.0 mg/l 2, 4-D and 2.0 mg/l BAP (PIV) or Murashige and Skoog minimal organics medium supplemented with 20 g/l sucrose, 1.0 mg/l NOA and 0.2 mg/l BAP (NB medium). Although the percentage of anthers that develop embryogenic callus is low, we have been able to establish robust embryogenic callus for all three genotypes from filaments (figure 1 and Table 1).

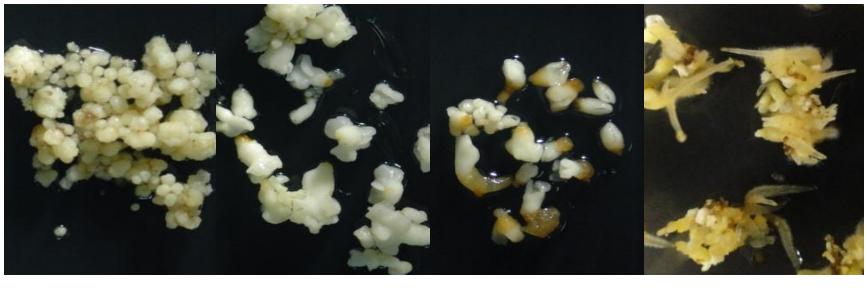

Figure 1. From left to right, somatic embryo cultures of 101-14 from cultured meristems, somatic embryo cultures 11-03, Chardonnay and Cabernet Sauvignon from cultured anther.

Table 1. Embryogenic callus production from anthers harvested in 2011 and 2012 plated on PIV or NB medium

Year	**Genotype**	**PIV #/(%)**	**NB #/(%)**
2011	Cabernet	3/400 (0.8%)	NT
	Chardonnay	4/400 (1%)	NT
	1103	2/150 (1.3%)	NT
2012	Cabernet	0/200 (0%)	0/280 (0%)
	Chardonnay	9/184 (4.9%)	2/56 (3.6%)
	1103	0/75 (0%)	1/196 (0.5%)

B. Production of embryogenic callus from leaf explants

C.

The National Research Laboratory of Chile (INIA) and the UC Davis Plant Transformation Facility (UCDPTF) are exploring an alternative method to generating embryogenic callus utilizing leaf pieces from *in vitro* grown plants. Unlike generating callus from anthers which have a short window of availability in the spring of each year, leaf tissue from *in vitro* plantlets are available year round. In addition, unlike meristem explants which are time consuming and difficult to excise, leaf explants are easy to isolate and can be secured from known pathogen-free tissue culture plantlets. We received disease free cultures of

Chardonnay and 101-14 and Cabernet Sauvignon from Foundation Plant Services (FBS), and have established *in vitro* shoot cultures from field plantings of 11-03 that came from disease indexed cultures. These shoot cultures will be maintained in culture and used as a source of tissue for experiments designed to establish embryogenic callus culture from young leaf explants. We are continuously harvesting very young unopened terminal leaves and plated them on Nitsch and Nitsch medium with 20 g/l sucrose and 1.0 mg/liter 2, 4-D and 0.1 mg/l BAP as per INIA's formulation and methodology.

We have produced callus from leaf explants of genotypes Cabernet, Chardonnay, 1103, 101-14 and Thompson Seedless (TS). TS leaf callus has begun forming embryos (figure 2). Although TS is not one of the targeted genotype of this grant, these results will provide a valuable guide for us in the identification of the appropriate callus morphology for other genotypes. We are transferring samples of the putative pro-embryogenic callus from all genotypes to X6 medium as per INIA's protocol to determine if somatic embryos can be generated.

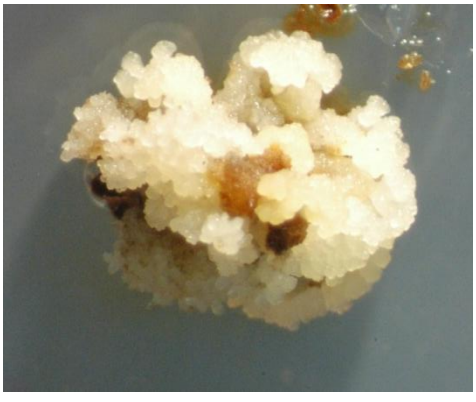

Figure 2. Embryogenic callus formation from leaf explant of Thompson Seedless plated on INIA's NB2 medium formulation

D. Increasing embryogenic callus tissue.

We have made significant progress on improving the production of embryogenic grape callus across a range of genotypes including 1103, 101-14, Cabernet and Chardonnay using a modification of INIA's method of cycling the callus between agar-solidified medium and liquid media in shake flasks. We are now maintaining stock cell suspension cultures as per INIA, but we are using a modification of UCDPTF's Pic/TDZ medium formulation consisting of WPM medium supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 10.0 mg/l Picloram, 2.0 mg/l meta-topolin, 2g/l activated charcoal, 100 mg/l ascorbic acid and 30 mg/l reduced glutathione (Pic/MTag) instead of INIA's RM medium. Embryogenic grape callus grown on agar-solidified medium is transferred to 20 mls of Pic/MTag in 125 ml shake flasks and grown on a gyratory shaker at 100 rpms in the dark. After the suspensions are established, 10 ml of the suspension is withdrawn each week from the flask and replaced with 10 mls of fresh medium. One to two mls of the removed suspension is transferred to agar solidified Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA 400 mg/l (BN). Embryogenic callus plated on BN medium produces high quality embryogenic callus at the appropriate stage for use in transformation in approximately 4-8 weeks. If the callus is not required for transformation it can be discarded rather than sub-cultured, since new agar-solidified plated are established each week as part of the subculture process for the cell suspension cultures. Previously we maintained stock callus exclusively on agar-solidified plates which were subcultures every 3-4 weeks. Sub-culturing these plates was labor intensive since often the callus becomes oxidized when maintained on plates for long time intervals requiring non-oxidized callus to be carefully separated from oxidized callus. By using this new procedure of maintaining cells in suspension and plating 2 mls for the cell suspension at each weekly subculture, one can easily generates high quality callus for use in transformations while eliminating the need to maintain and subculture large numbers of stock callus plates. It appears that once high quality suspensions are created this process can be repeated indefinitely allowing for a constant and reliable supply of embryogenic callus for use in transformation experiments. The process does not require cycling material between agar and liquid medium as per INIA protocol, but maintains cells in low density cell suspension by continually sub-

culturing them on a weekly basis which allows for the continual production of non-phenolic producing embryogenic callus (figure 3 and 4). We have noted however that it is important to plate the cell suspension at a low density. If plated at too high a density the callus oxidized and turns brown. We are exploring various plating technique to determine the best methods for producing the highest quality callus.

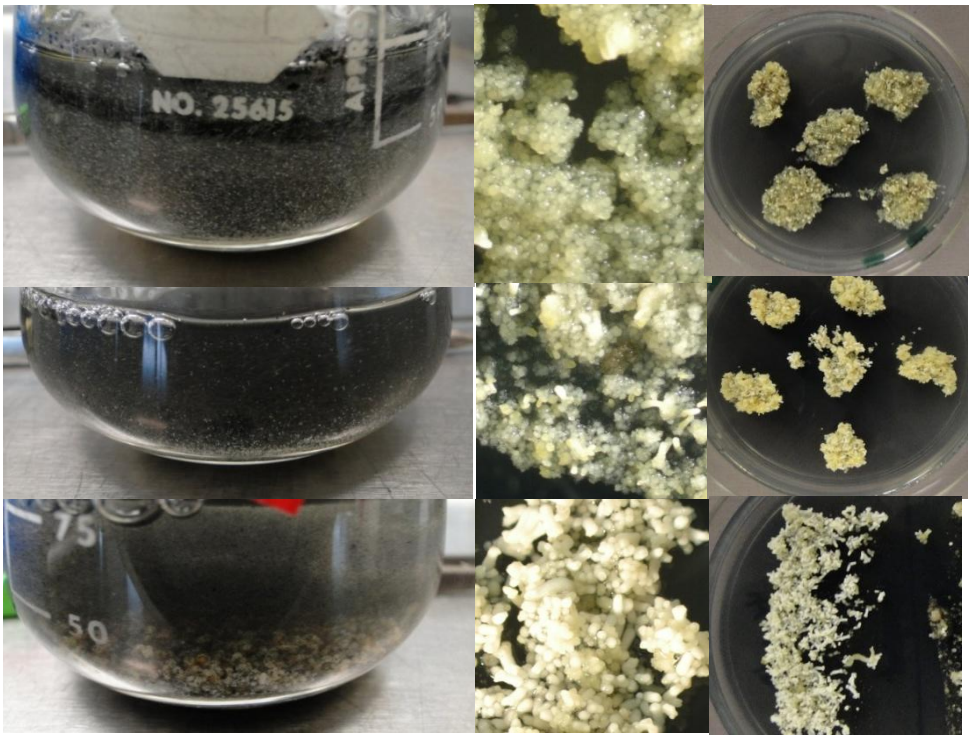

Figure 3. Production of high quality embryogenic callus from cell suspensions. Chardonnay (top), 1103 (middle) and 101-14 (bottom) in cell suspension cultures on WPM medium supplemented with 1g/l casein, 1M MES, 1000 mg/l activated charcoal,10 mg/l picloram, 2 mg/l meta-topolin (left), close up of cell suspension cultures (middle) and embryogenic callus developing 4 weeks after plating 1-2 ml of suspensions on WPM medium supplemented with 1g/l casein, 1M MES, mg/l activated charcoal,0.5 mg/l BAP and 0.1 mg/l NAA.

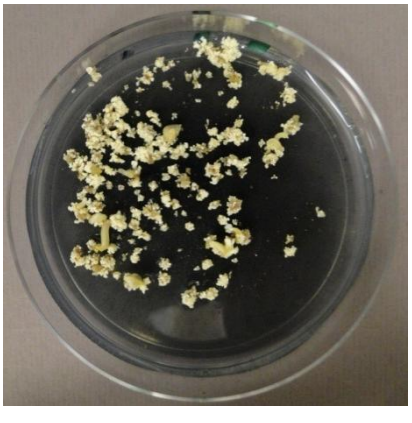

Figure 4. Cabernet stock callus after plating 2 ml of a cell suspension cultures grown on WPM + 1g/l casein + 1M MES + 1000 mg/l activated charcoal + 10 mg/l picloram + 2 mg/l meta-topolin onto WPM + 20 g/liter sucrose, 50 g/liter sorbitol, 1g/l casein + 1M MES + 500 mg/l activated charcoal + 0.5 mg/l BAP + 0.1 mg/l NAA and 14 g/liter agar

At INIA they are testing embryogenic callus growth rates of additional grape cultivars in suspension cultures on INIA's RM medium and UCDPTF's PT medium. Cultivars under investigation at INIA include

Freedom, 110-R, Salt Creek and Moscatel de Alejandria. Images of the cell suspensions growing on the two media are shown in Figure 5.

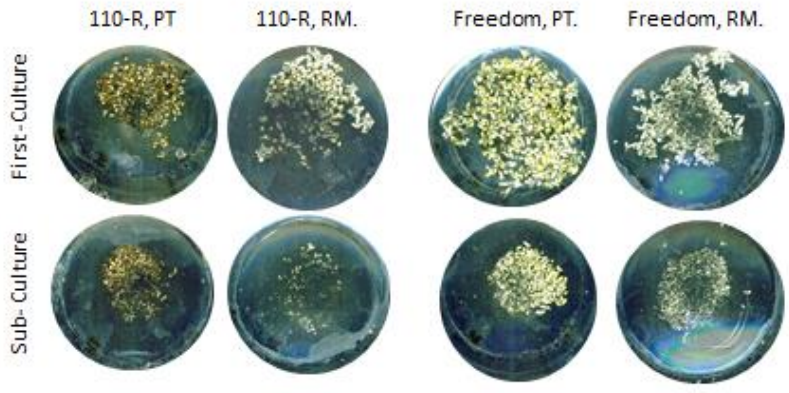

Figure 5. Results from Dr. Tapia at INRA comparing cell suspension growth of Freedom, 110-R, S on INIA's RM medium compared to UCD's Pic/TDZ (PT) liquid medium. Suspension on the top row are initial cultures whereas cultures on the bottom row have been sieved and subcultured.

We have previously shown that grape embryogenic callus can be maintained for very long intervals without subculturing by growing them in temporary immersion bioreactors. Viable embryogenic cultures can still be harvested after six months in culture without fresh medium exchanges (figure 6). These previous studies were conducted in the light and the callus became oxidized and turn brown in the bioreactors. This did not appear to inhibit callus viability when transferred to fresh agar-solidified medium. However we began repeating the bioreactor studies on 101-14, 1103 and Chardonnay while maintaining the vessels in the dark. Growth rates and callus quality will be monitored. This method could prove to be a very cost effective method for maintaining a steady supply of stock embryogenic callus for tissue culture and transfortmation use. Unlike cell suspension cultures which need to be fed each week, this technique requirs no manipulation for at least 3-4 months.

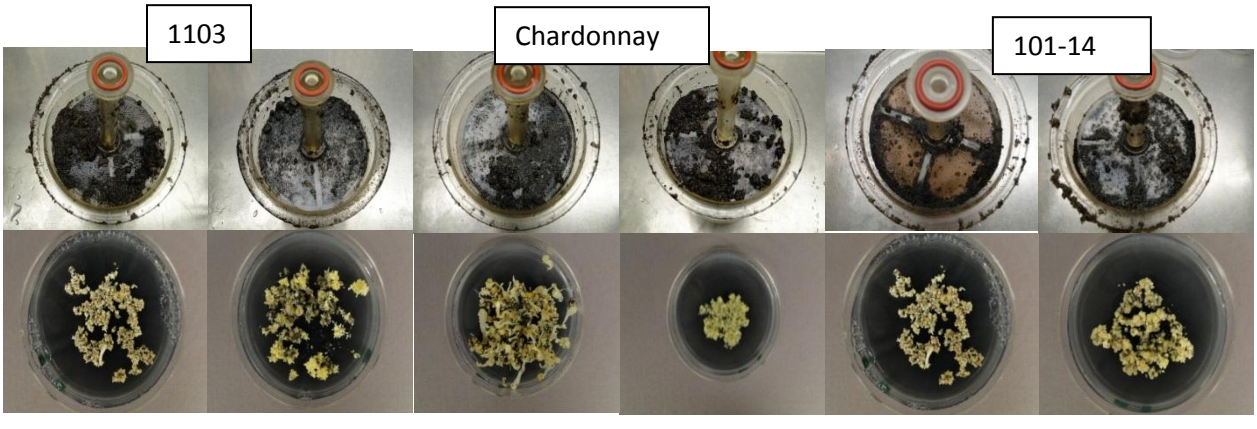

Figure 6. Callus growth of 1103, Chardonnay and 101-14 in temporary immersion system (TIS) on Pic/TDZ (top left of each panel) or Pic/MT (top right of each panel) and subsequent callus development after transfer to agar-solidified BN medium (lower left and lower right of each panel).

D. Enhance the efficiency of whole plant regeneration from embryogenic callus of grape cultures.

We have begun investigating variation of our current regeneration medium by testing various concentrations of BAP in combination with NAA on both 1103 and Chardonnay to improve the efficiency

of plant recovery from embryos. Our standard regeneration medium consists of WPM + 1g/l casein + 1M MES + 500 mg/l activated charcoal + 0.5 mg/l BAP + 0.1 mg/l NAA and was developed for use with Thompson Seedless grapes. Preliminary results indicate that this medium is appropriate for regeneration of Chardonnay, but in not optimal for 1103. Genotype 1103 benefits significantly from a reduction in the BAP concentrations. (See Table 2 and figure 7). Studies are now under way to evaluate lower levels of both BAP and NAA on regeneration efficiency in 1103. Decreasing the time required to regenerate whole plants from callus could significant improve the efficiency of our grape tissue culture and transformation systems.

Table 2. Regeneration response of Chardonnay and 1103 on WPM + 1g/l casein + 1M MES + 500 mg/l activated charcoal + 0.5 mg/l BAP + 0.1 mg/l NAA with various concentrations of BAP and NAA (0 = no regeneration, 5 = efficient regeneration).

Germination Ratings for Chardonnay

	NAA mg/l			
	0.1	0.5	1.0	5.0
BA mg/l				
0.1	5	4	3	2
0.5	5	3	3	2
1	5	1	3	2
5	5	3	2	1

Germination Ratings for 1130

	NAA mg/l			
	0.1	0.5	1.0	5.0
BA mg/l				
0.1	3	3	2	1
0.5	3	2	2	1
1	2	2	2	1
5	1	1	1	1

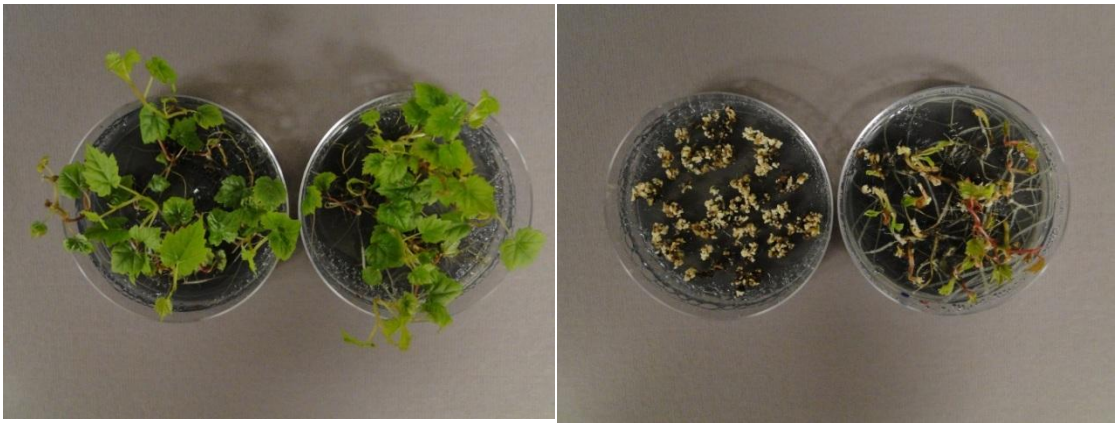

Figure 7. Regeneration of non-transgenic Chardonnay grape (left) and 1103 (right) on WPM + 1g/l casein + 1M MES + 500 mg/l activated charcoal + 0.5 mg/l BAP + 0.1 mg/l NAA (left side of each photo) vs. WPM + 1g/l casein + 1M MES + 500 mg/l activated charcoal + 0.1 mg/l BAP + 0.1 mg/l NAA (right side of each photo)

Next steps for Objective 1:

- Continue to explore development of embryogenic callus from leaf tissue
- Continue to improve maintenance of embryogenic callus in suspension and bioreactors
- Continue to Improve regeneration efficiency for 101-14 and 1103

Objective 2. **Develop a cost effective grape tissue culture and transformation platform for at least one priority California wine grape, and one California grape rootstock which will provide PD/GWSS Research Community with a predictable supply of experimental plant material while reducing labor and maximizing tissue culture and transformation efficiency.**

Progress:

The generation of transgenic callus and regeneration of plants from that callus is a long term process often requiring 6 or more months for transgenic embryogenic callus to form and up to 6 to 8 additional months for transgenic embryogenic callus to convert into whole plants. We believe that these problems may be related to the extended length of time that callus remains in a non-differentiated state on high hormone containing medium such as our Pic/TDZ medium formulation. Therefore we are evaluating media formulations to determine if we can speed up the process of transgenic embryogenic callus production and whole plant regeneration. We have shown that when callus maintained on Pic/MT is transformed, the development of embryogenic callus occurs more rapidly than from callus maintained on Pic/TDZ or Pic/BA (figure 8).

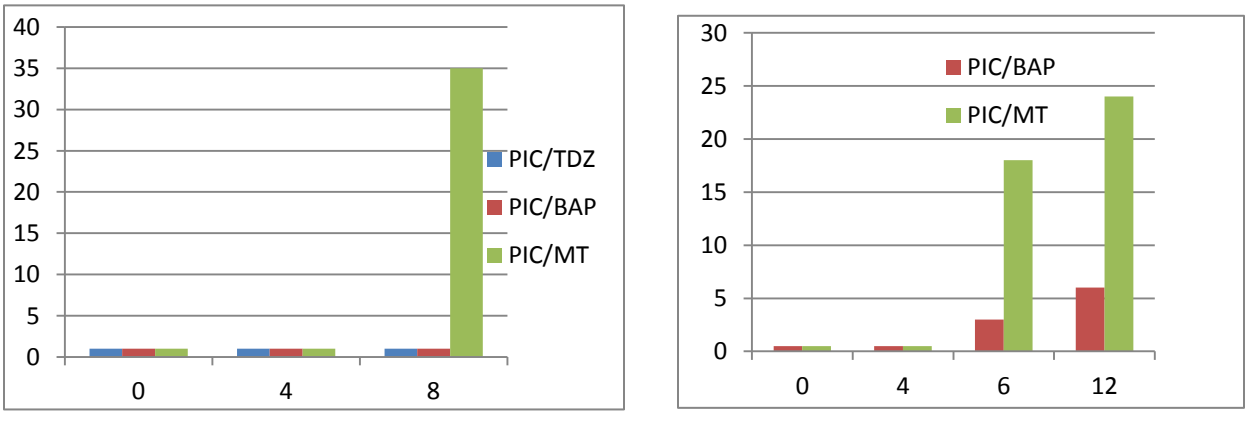

Figure 8. The percentage of transgenic Thompson Seedless callus colonies produced, eight-twelve weeks after co-cultivation and cultured on induction medium composed of WPM supplemented with 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin 10 mg/l picloram and 2.0 mg/l TDZ, BAP or MT.

To further evaluate the impact of the maintenance medium on transgenic callus production, we initiated four transformation experiments with TS comparing the speed at which transgenic callus was produced after maintaining callus on Pic/BAP verse Pic/ MT. Transgenic embryogenic callus consistently developed faster from inoculated callus that was previously maintained on Pic/MT than callus maintained on Pic/BA (Table 3).

Table 3. Transformation efficiency of Thompson Seedless across four parallel experiments 6 and 10 weeks after co-cultivation with *Agrobacterium* and selected on induction medium composed of WPM supplemented with 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin 10 mg/l picloram and 2.0 mg/l BAP verses 10 mg/l picloram and 2.0 mg MT.

Callus selection medium	Experiment #	# (%) transgenic colonies	
		6 weeks	10 weeks
10 mg/l Picloram / 2.0 mg/l BAP	111018	1/25 (4%)	1/25 (4%)
	111020	1/25 (4%)	2/25 (8%)
	111022	0/25 (0%)	0/25 (0%)
	111024	1/25 (4%)	4/25 (16%)
10 mg/l Picloram / 2.0 mg/l MT	111017	5/25 (20%)	8/25 (32%)
	111019	2/25 (8%)	2/25 (8%)
	111021	8/25 (32%)	10/25 (16%)
	111023	3/25 (12%)	4/25 (16%)

This period we also investigated how the developmental stage of the stock callus influences the speed at which transgenic embryos are produced. We transferred embryogenic Thompson Seedless callus to WPM + 1g/l casein + 1M MES + 500 mg/l activated charcoal + 0.5 mg/l BAP + 0.1 mg/l NAA (BN) or Pic/MT medium. Note that callus maintained on BN medium consists of more developed heart and early torpedo staged embryos whereas callus maintained on Pic/MT consisted of pro-embryogenic masses. After three consecutive four week subcultures on BN or Pic/MT medium, calli were inoculated with the *Agrobacterium* strain EHA105 carrying the kanamycin plant selectable marker gene and the scorable marker gene, dsRed, co-cultivated for 2-3 days and then transferred to selection medium composed of WPM supplemented with 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin plus 0.5 mg/l BAP and 0.1 mg/l NAA. Callus was cultured in the dark and sub-cultured to fresh medium of the same formulation every 3 to 4 weeks and monitored for the presence of transformed embryogenic cells. After nine week, transgenic callus colonies appeared on nearly 50% of the callus colonies maintained on BN medium prior to inoculation, while only 6 percent of the inoculated callus maintained on Pic/MT medium prior to inoculation produced transgenic colonies. Furthermore transgenic embryos developed much faster on callus from callus maintained on BN compared to callus maintained on Pic/MT treatment (figure 9), Since callus maintained on BN medium is more advanced in development, we will need to track the dsred expression of embryos forming on BN medium to verify that they are not chimerics.

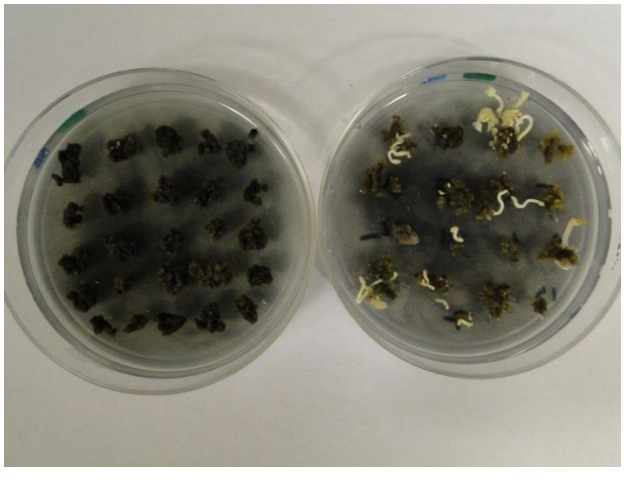

Figure 9. Transformation response of inoculated Thompson Seedless callus maintained on Pic/MT (left) verses BN (right) 9 weeks after co-cultivation and selected on BN with 200 mg/l kanamycin.

We have initiated transformation experiments with 101-14 and 1103 callus using the *Agrobacterium* strain EHA105 carrying the kanamycin plant selectable marker gene and the scorable marker gene, dsRed, We have produced our first dsred expressing transgenic embryos for 101-14,1103 and Chardonnay (figure 10 and 11). We now need to increase the efficiency by employing advances mad with Thompson Seedless and determine if transgenic embryos can be efficiently converted into plants.

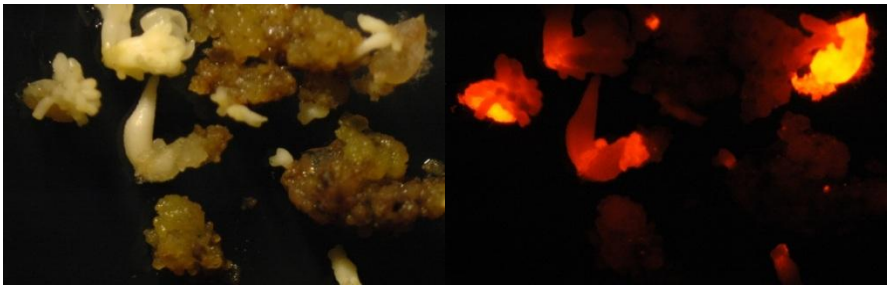

Figure 10. 101-14 embryogenic callus transformed with the dsred scorable marker gene under white light (left) vs. fluorescence (right). Transgenic and non-transgenic sectors are clearly visible based on dsred expression.

Figure 11. Dsred expressing grape embryos of 1103 (left), transgenic embryo of 101-14 (middle) and Chardonnay (right) after selection on 200 mg/l kanamycin.

Next Steps:

- Determine if the improvements developed for increasing the speed of the production of transgenic embryogenic callus and the regeneration of transgenic plants for Thompson Seedless can be applied directly to transformation of 1103,101-14 Chardonnay and Cabernet.
- Determine the incidence of chimeric plant using the dsred marker gene

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

Tricoli D. M. 2012. Development of a Grape Tissue Culture and Transformation Platform for the California. Pierce's Disease Research Progress Reports, Grape Research Community pp. 225-232

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

This strategic and mutually beneficial partnership leverages the expertise of the National Research Laboratory of Chile, (INIA), and the Ralph M. Parsons Foundation Plant Transformation Facility (PTF) at UC Davis and will accelerate the delivery of PD/GWSS research results. The proposed collaboration combines pre-existing expertise and technical know-how that will expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the PD/GWSS research community. The development of a highly efficient service-based tissue culture and transformation platform for grape varieties of relevance to the PD/GWSS Research Community and the wine grape industry will have major benefits for the grape research community, by accelerating the gene function/validation process of identifying PD/GWSS practical control strategies. To ensure broad utility of this technology platform within the research community, once established, we propose offering the

enabling technologies through UC's existing core service facility on a time efficient and cost-effective fee-for service basis.

J. Layperson summary of project accomplishments.

This proposal is aimed at establishing an international collaboration between leading laboratories in US and Chile to reduce the time and cost of tissue culture and transformation for grape varieties of importance to the viticulture industries in their respective countries. The collaboration leverages pre-existing expertise and technical know-how to expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the PD/GWSS research community.

The two labs have exchanged their latest media formulations and protocols for increasing embryogenic callus and both labs are comparing the effectiveness of each other's techniques using germplasm important to their particular country. The availability of a reliable supply of highly embryogenic grape callus is crucial for the cost effective service-based grape transformation system. Eduardo Tapia Rodríguez from the National Research Laboratory of Chile (INIA) is currently comparing and contrasting the growth rate and quality of cell suspension of Chilean genotypes including 110R, Freedom, Muscatel and Salt Creek when maintained on INIA's medium verses UCDPTF's medium. By modifying INIA's cell suspension strategy and media components for increasing embryogenic cultures, UCDPTF has been able to produce a very reliable supply of highly embryogenic callus for 1103, 101-14, Chardonnay and Cabernet. Aliquots of the suspensions can be plated onto agar solidified medium weekly, providing a steady supply of high quality embryogenic callus for use in tissue culture and transformation experiments for genotypes important to California's PD/GWSS Research Community. UCDPTF has also demonstrated that embryogenic callus can be increased in temporary immersion bioreactors for a period of over 3 months without any input of labor. This technique could significantly reduce the amount of labor currently required to maintain cell suspension or callus cultures and serves as a safe germplasm bank of embryogenic callus.

We have also made improvements in medium formulations which allow for faster recovery of transgenic embryogenic callus and plants and have started applying these new protocols to the recovery of tissue cultures plants of 1103, 101-14, Chardonnay and Cabernet. While the medium we currently employ to convert callus into plants works very well for Chardonnay, we found that it is not effective for regeneration of plants from 1103 callus. By testing various combinations of auxins and cytokinins, we have made significant improvements in the medium formulation for regeneration of 1103 callus into whole plants. Most significantly this period, we have produced our first transgenic dsred expressing embryos of 1103, 101-14 and Chardonnay and we are working on regenerating whole plants from these embryos. Now that we have demonstrated that we can transform these genotypes it will allow us to test various transformation parameters to increase the transformation efficiency and reduce the time required to generate transgenic lines.

K. Status of funds.

We anticipate that all funds allocated for fiscal year FY2012-2013 will be expended by June 30, 2013.

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

This collaboration will continue to work closely with PIPRA to address access to intellectual property and know-how for this work in order to make new services widely accessible to the research community. Individual protocols are already being shared between the two groups. Any protocol improvements developed through this collaborative grant will be shared between INIA and UC Davis and will be integrated into an effective grape tissue culture and transformation recharge based service. PIPRA will serve as an interface with INIA to develop strategies to access the products of this research collaboration. The two labs have already benefited from the exchange of information on media formulations and techniques which can be applied to Chilean grape varieties including Salcrik, Freedom and Harmony and California genotypes 11-03, 101-14, Cabernet Sauvignon and Chardonnay which are the targets of the US effort.