

A. Renewal Progress Report for CDFA Agreement Number 201120901

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

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D. Time period covered by the report: January 13, 2012 – March 5, 2012

E. 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 and regenerating plants for many important grape genotypes remains very low and are 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, with concrete benefits already realized by both groups from the creation of this collaboration.

F. List of objectives:

- I. 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.

II. 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

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

Objective I 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.

1. **Adapt tissue culture and transformation methodologies developed by the Chilean partner, for grape genotypes of importance to California including; 11-03, 101-14, Cabernet Sauvignon, and Chardonnay.**

Progress:

- A. Production of embryogenic callus of 11-03, 101-14, Chardonnay and Cabernet Sauvignon.

In anticipation of the project, in May 2011 we collected anthers of Cabernet Sauvignon, Chardonnay, 101-14 and 11-03 and we are in the process of establishing embryogenic callus for all four genotypes. We currently have embryos developing on anther filaments of 11-03, Cabernet Sauvignon and Chardonnay.

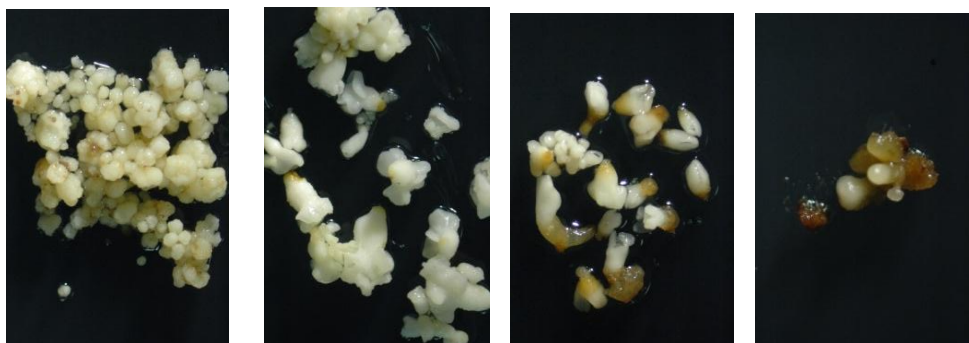


Figure 1. From left to right, somatic embryo cultures of 101-14 from cultured meristems, somatic embryo cultures 11-03, Chardonnay and Cabernet Sauvignon from cultured anther.

Next Steps:

- We will harvest significantly more anthers in spring 2012 from 11-03, 101-14, Chardonnay and Cabernet Sauvignon to ensure we generate ample material for our work.
- B. Production of embryogenic callus from leaf explants

Currently embryogenic callus cultures in grape can be accomplished by culturing anthers harvested in the spring of each season or by meristem culture. After 4-9 months in culture, a small percentage of the explanted tissue (less than 5%) will produce embryogenic callus. Although establishing embryogenic callus from anther filaments is effective for most grape genotypes, it has a number of disadvantages, one of which is that the tissue is collected from field grown material which could be infected by various pathogens. In addition, anthers are available for harvest for only a limited number of weeks each year. The National Research Laboratory of Chile (INIA) and UC Davis' Plant Transformation Facility (UCDPTF) are exploring an alternative method to generating embryogenic callus which utilizes 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 very difficult to excise, leaf explants are easy to isolate and can be secured from known pathogen-free tissue culture plantlets. We have secured disease-free *in vitro* plantlets produced from meristem culture of Cabernet Sauvignon, Chardonnay and 101-14 from UC Davis' Foundation Plant Services (FBS). Utilizing this material, we have isolated leaf explants to attempt to established embryogenic callus from young leaf tissue using the protocol INIA's uses for their Chilean genotypes.

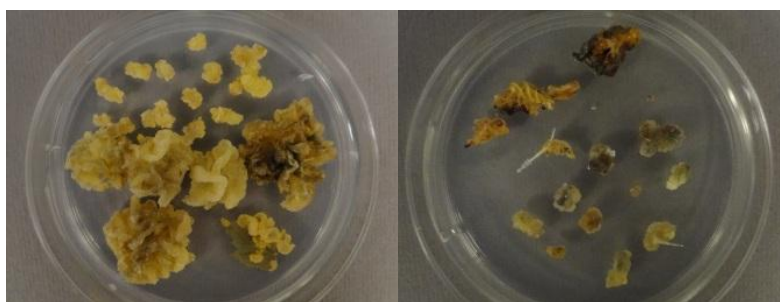


Figure 2. Leaf explants from disease-free *in vitro* shoots of Chardonnay (left) and 101-14 (right). Callus has been produced and we are monitoring for the formation of embryos.

Next Steps:

- We received disease free cultures of Chardonnay and 101-14 last fall and have recently been provided cultures of Cabernet Sauvignon thanks to the efforts of FBS. Unfortunately FBS no longer has *in vitro* cultures of 11-03. However, they do have recently established field plantings of 11-03 that came from disease indexed cultures and we are coordinating with them to take shoot tips/meristems from this material in the spring 2012 and will establish *in vitro* plantlets to be used to generate embryogenic callus from leaf explants.

C. Increasing embryogenic callus tissue

Our existing grape transformation system has several inefficient steps which make generation of transgenic plants time consuming and expensive. One factor is the time required to generate significant amounts of embryogenic callus. Regardless of the explant source used (anthers, leaves or meristems), the embryogenic callus takes many months to form and once developed grows very slowly, requiring many months in tissue culture before significant quantities of the callus are produced. Normally, one full year is required to establish and increase the callus in order to obtain sufficient quantities for use in research experiments. Therefore most experiments initiated in 2011, use callus from material isolated in 2009 or 2010. INIA has developed a method of rapidly increasing embryogenic callus by cycling the callus between solid and liquid media.

This technique allows for rapid increase in callus fresh weight while minimizing oxidation and the development of detrimental phenolic compounds in the callus.

Eduardo Tapia Rodríguez from INIA visited UC Davis' Plant transformation Facility (UCDPTF) and instructed us on INIA's method for increasing embryogenic callus by cycling tissue between solid and a liquid media. Eduardo first evaluated whether our genotypes (101-14 and Thompson Seedless TS-10) could be grown on INIA's standard agar solidified callus medium formulation composed of Nitsch and Nitsch medium supplemented with 20 g/l sucrose, 200 mg/l glutamine, 100 mg/l inositol, 0.225 mg/L BAP, and 1.1 mg/L 2,4-D (NB2). His studies demonstrated that NB2 medium could be used to effectively maintain and increase 101-14 and Thompson Seedless callus, with monthly sub-cultures. The callus produced in NB2 was subsequently used for growth in INIA's liquid rootstock callus induction medium consisting of ¼ strength Murashige and Skoog salts supplemented with 400 mg/liter glutamine, 60 g/liter sucrose, 1.0 mg/liter 2, 4-D, 1.0 mg/liter kinetin, and 250mg/liter activated charcoal (RM). If callus exhibited signs of oxidation during the liquid phase, the callus was collected and sieved through a 520 micro screen and the callus that was retained on the screen was transferred back to agar solidified NB2 medium and cultured in the dark (figure 3).



Figure 3. Callus tissue of 101-14 after growing on liquid PT medium and sieving through a 520 micro screen. This fraction is returned to NB2 medium in the dark for further increase.

Eduardo also compared growth rates of 101-14 and TS-10 in INIA's liquid RM to growth rates in UCDPTF's liquid callus induction medium consisting of Lloyd and McCown minimal organics medium, supplemented with 20 g/liter sucrose, 1g/liter casein, 1 mM MES, 10 mg/liter picloram, 2 mg/liter tridiazuron (TDZ) and 600 mg/liter activated charcoal (PT) in the liquid phase of INIA's solid to liquid cycling methodology. Increase in fresh weights during the liquid phase was recorded over a 2 week period. The statistical kinetic growth comparison in Tukey HSD test, confirmed the advantage of using UCDPTF's PT medium during the liquid phase of the process with significantly greater increases in callus fresh weight in PT medium verses RM medium for both 101-14 and TS-10 (figure 4 and 5).

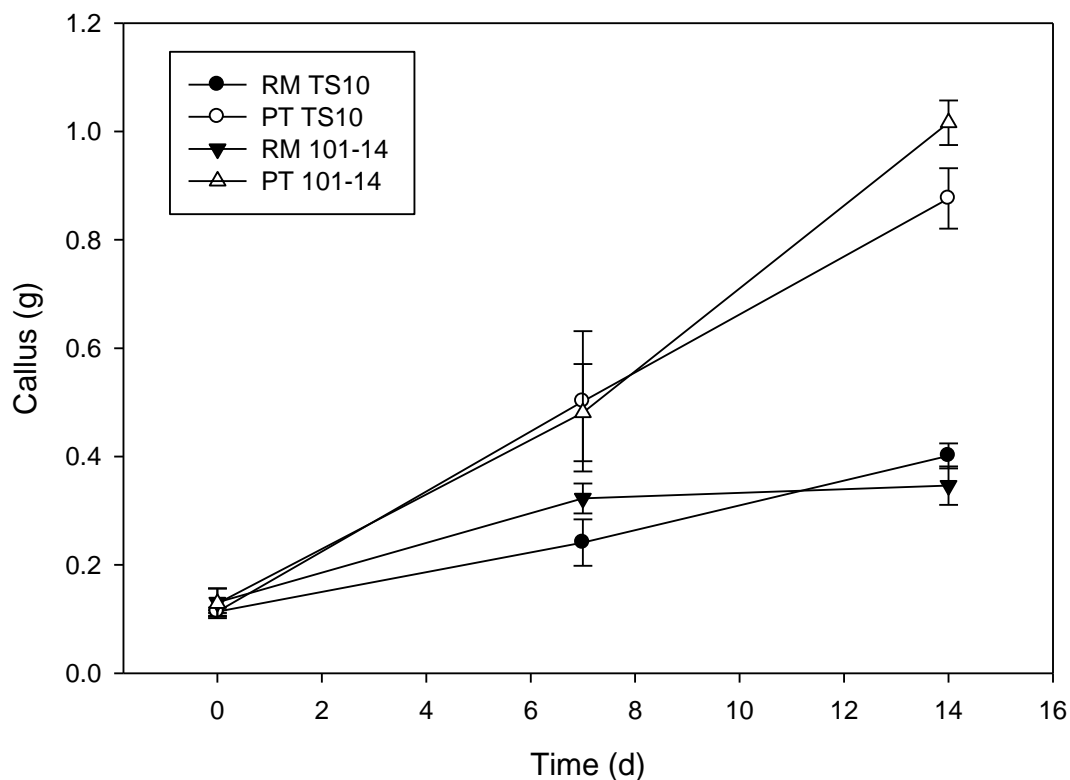


Figure 4. Comparison of the kinetic growth rates of Thompson Seedless (TS-10) and 101-14 embryogenic callus on INIA's liquid medium formulation (RM) versus PTF's liquid medium (PT) after 2 weeks in culture. (RM) Rootstock Media, Prieto's Lab, (PT) Tricoli's Lab.

μ (d⁻¹) comparison

	Count	Mean
RM 101-14	3	0.070299
RM TS10	3	0.0901098
PT 101-14	3	0.13913
PT TS103	3	0.145717

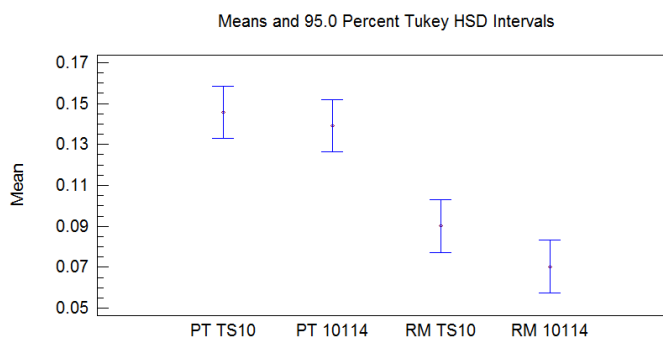


Figure 5. The statistical growth kinetics comparison in Tukey HSD test, showing the mean increase in fresh weight (g/liter) and confirming the improvement in biomass production using PT versus RM medium during the liquid phase of INIA's methodology.

Next Steps:

- UCDPTF will test INIA's methodologies on 11-03 and Cabernet Sauvignon.
- Humberto's lab is repeating the comparison of INIA's liquid RM medium formulation verse UCDPTF's liquid PT medium formulation on their Chilean grape varieties including Salcrik, Freedom and Harmony, to determine if PT medium can be used to enhance biomass production in their genotypes.
- We need to explore methods to reduce the amount of labor associated with INIA's liquid/solid medium methodology for increasing the embryogenic callus. The protocol requires frequent handling of the liquid suspensions and transition from liquid to solid state. These procedures, although effective in increasing callus, are time consuming and will put pricing pressure on a service-based transformations system for grape. We also hope to find methods of preventing or reducing oxidation of embryogenic callus tissue in the liquid or solid phase.

2. Increase the efficiency and reduce the time required for *in vitro* regeneration of grape plants from embryogenic callus by adapting INIA's grape bioreactor technology and UC Davis Plant Transformation Facility's temporary immersion system (TIS) for use in grape tissue culture and transformation.

Progress:

In addition to the use of INIA's liquid/ solid cycling system, we want to explore UCDPTF's temporary immersion system (TIS) for use in rapidly increasing embryogenic callus. We have already begun testing INIA's liquid shake flasks methodology on 101-14 which INIA has developed for table grapes (see above). Once sufficient amounts of callus are available, we will begin comparing INIA's shake flask system to UCDPTF's TIS technology for increasing callus of 101-14, 11-03, Cabernet Sauvignon, Chardonnay.

Next Steps:

- We will compare the efficiencies of increasing embryogenic callus in shake flasks using INIA's technology compared to increasing embryogenic callus in UCDPTF's Temporary Immersion System.

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

Progress:

In our hands, regeneration of plants from embryos is currently a significant bottleneck for efficient grape tissue culture and transformation. Therefore, we have begun investigating various medium addendums in combination with testing various developmental stages of embryogenic tissue used for transformation/regeneration in an attempt to decrease the time required to produce transgenic callus and regenerated whole plants after *Agrobacterium-mediated* transformation. Embryogenic grape callus can be maintained for long periods of time as pro-embryonic masses (PEMS) by culturing tissue on medium with high levels of plant hormones. However maintaining cultures under these conditions for long periods of time inhibits the ability of embryos formed from PEMS to convert to whole plants. Alternatively, callus cultures maintained on low hormone or hormone-free medium remain more developed and can be more readily converted to whole plants, but eventually all the callus coverts into germinating embryos and therefore material cannot be maintained indefinitely on these media formulations. In addition, when used for transformation,

highly developed embryos can result in the production of a high percentage of chimeric transgenic plants.

Our current media formulation consists of Lloyd and McCown's WPM supplemented with 10mg/liter Picloram and 2.0 mg/liter tridiazuron (TDZ). This combination of hormones has the advantage of allowing us to maintain embryogenic callus as pro-embryonic masses for an indefinite period of time. However both picloram and TDZ are powerful plant growth regulators and the concentrations used in our medium are quite high compared to other systems. For example in embryogenic cultures of onion, picloram is used at only 1-2 mg/liter. In addition, the cytokinin TDZ, once absorbed into the tissue, is known to break down slowly and plants grown on TDZ often exhibit inhibition in rooting due to residual cytokinin effects. Stock callus grown on picloram/TDZ and inoculated with *Agrobacterium* will produce transgenic callus on selection, but on average, resistant callus does not develop for 4-6 months. In addition growth of embryogenic callus on high levels of picloram and TDZ appears to inhibit conversion of embryos into whole plants. For Thompson Seedless, the grape genotype most amenable to transformation, conversion of embryos into whole plants requires repeated monthly subcultures over a period of 6-8 months before whole plants can be obtained. The efficiency of conversion of transgenic embryos into whole plants remains a major stumbling block with the percentage of embryos converting to whole plants in the range of ten percent or less. Therefore, we are testing the viability of replacing TDZ with the cytokinin meta-topolin; (MT) a highly active aromatic cytokinin from poplar leaves *populus x canadensis moench*, cv. Robusta. We will determine if this formulation can maintain callus as pro-embryogenic masses while allowing more rapid plant regeneration. We are also examining the feasibility of maintaining stock tissue as globular, heart and small torpedo embryos as opposed to more suppressed PEM.

Increasing the osmoticum of the medium has proven to be beneficial for the regeneration of embryos to plants for other plants species in particular rice and cucumber. We are exploring the use of the sugar alcohol sorbitol to increase the osmoticum of our grape regeneration medium in an attempt to improve the speed and efficiency of conversion of embryos into plants.

Next Steps:

- Evaluate the feasibility of maintaining embryogenic callus in a less suppressed state.
- Begin transformation studies on 11-03, 101-14, Chardonnay and Cabernet Sauvignon on various developmental stages of callus using the scorable marker gene dsRed to allow us to monitor for chimeric plants.
- Compare transformation efficiencies using UCDPTF's new media formulations and embryo development stages vs. INIA's transformation protocol in an attempt to reduce the time required to generate transgenic plants.

Objective II. 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:

Improvements made in objective I above, will be incorporated into a grape tissue culture and transformation platform through the University of California Ralph M. Parsons Foundation Plant Transformation Facility and will provide grape tissue culture and transformation service to grape researchers at a self-sustaining, cost effective recharge rate.

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

None

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 the 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.

Eduardo Tapia Rodríguez from the National Research Laboratory of Chile (INIA) visited UC Davis' Plant Transformation Facility (UCDPTF) to instruct us on INIA's methods for generating embryogenic callus from *in vitro* leaf tissue and for increasing embryogenic callus by cycling tissue between solid and a liquid medium. Eduardo has compared and contrasted the two facility's agar-solidified media formulations (INIA's NB2 medium verses UCDPTF's PT medium) using INIA's methodology for increasing embryogenic callus on agar-solidified medium. Eduardo found that for California genotypes, 101-14 and TS-10, callus culture could be grown effectively on INIA's agar-solidified medium when sub-culture at monthly intervals and exhibited less oxidation than on UCDPTF's standard agar-solidified maintenance medium. In contrast, when the callus produced on solid medium was used to generate more biomass in liquid shake cultures using INIA's agar/liquid cycling system, UCDPTF's liquid medium formulation exhibited improved growth rate compared to INIA's standard liquid medium, producing significantly more biomass based on statistical kinetic growth comparisons. Eduardo has returned to Chile and is testing UCDPTF's liquid medium formulation on Chilean genotypes including Salcrik, Freedom and Harmony, while we are testing INIA's methodologies on 11-03, Cabernet Sauvignon, Chardonnay. This is a concrete example of how both labs have already benefited from the collaboration. UCDPTF has obtained expertise from INIA on increasing embryogenic callus by cycling tissue between solid and a liquid medium using INIA's NB2 medium, while INIA has gain access to UCDPTF's callus multiplication medium (PT), which was shown to enhance biomass production in liquid shake cultures compared to the INIA's standard liquid medium.

K. Status of funds.

We anticipate that all funds allocated for fiscal year FY2011-2012 will be expended by June 30, 2012.

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.