A. Interim Progress Report for CDFA Agreement Number11-0416-SA

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

C. Principal investigators:

- David M. Tricoli, Plant Transformation Facility, UC Davis
  College of Agricultural and Environmental Sciences, University of California, One Shields Avenue, Davis, CA 95616
  Phone: (530) 752-3766 | dmtricoli@ucdavis.edu.

Cooperators:

- Cecilia Chi-Ham, Director of Biotechnology Resources, PIPRA
  University of California, One Shields Avenue, PRB Mail Stop 5, Davis, CA 95616-8631
  Tel: 530 754 6717 | clchiham@ucdavis.edu
- Humberto Prieto, The Plant Research Station, National Research Laboratory of Chile (INIA)
  Chile | humberto.prieto.encalada@gmail.com

D. Time period covered by the report: March 5, 2012- June 30, 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.

We harvested additional anthers from grape genotypes 11-03, 101-14, Chardonnay and Cabernet sauvignon in the spring of 2012 and have plated them onto two different callus induction media (PIV and NB medium) in order to generate additional callus to ensure that we have ample material for our research. We have established embryogenic callus cultures of varying quality for each of the four genotypes (figure 1).

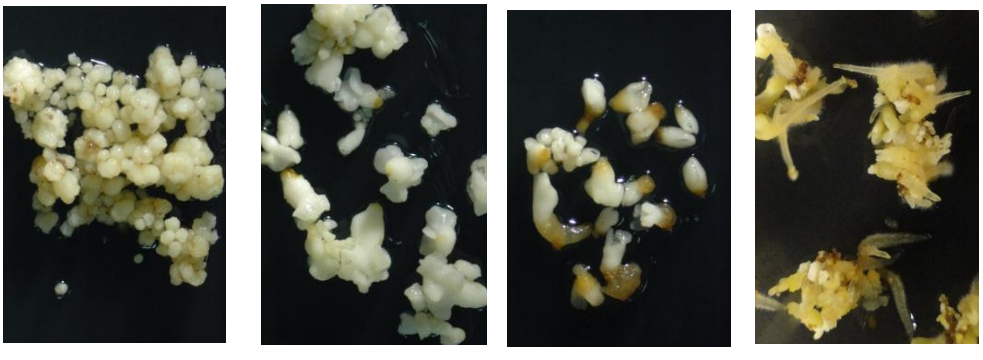


Figure 1. From left to right, somatic embryo cultures of 101-14 from cultured meristems, somatic embryo cultures 11-03, Chardonnay and Cabernet sauvignon from cultured anther.

As can be seen in figure 2 below, each of the four genotypes responds quite differently to our standard media regime. Our standard media formulation established for Thompson Seedless (TS) consists of Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 10 mg/l picloram, 2 mg/l thidiazuron (TDZ), and 8g/l TC Agar (PT medium). On this medium TS callus can be grown indefinitely as compact pro-embryogenic masses (PEM). However callus maintained in this suppressed state is slow to regenerate into whole plants and can result in plants with an abnormal phenotype (figure 3). We have recently found that if we maintain callus on Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, and 0.1 mg/liter NAA 400 mg/l (BN), plants can be regenerated in significantly less time. However most genotypes cannot be maintained on BN medium indefinitely, since eventually all the callus regenerates into whole plants. This is especially evident for Cabernet Sauvignon, Chardonnay and 1103 (figure 2 bottom panels). The duration of time that the callus can be maintained on BN callus prior to regenerating plants varies from genotype to genotype. Chardonnay, Cabernet Sauvignon and 1103 germinate very quickly on this medium formulation and can only be maintained for very short periods of time (1-2 months) before the entire callus regenerates into plantlets. Germinating embryos cannot be used as source tissue for transformation. To overcome this problem we have begun cycling

callus back and forth between PT and BN media on a bi-monthly timeframe in an attempt to maintain callus at the appropriate developmental stage.

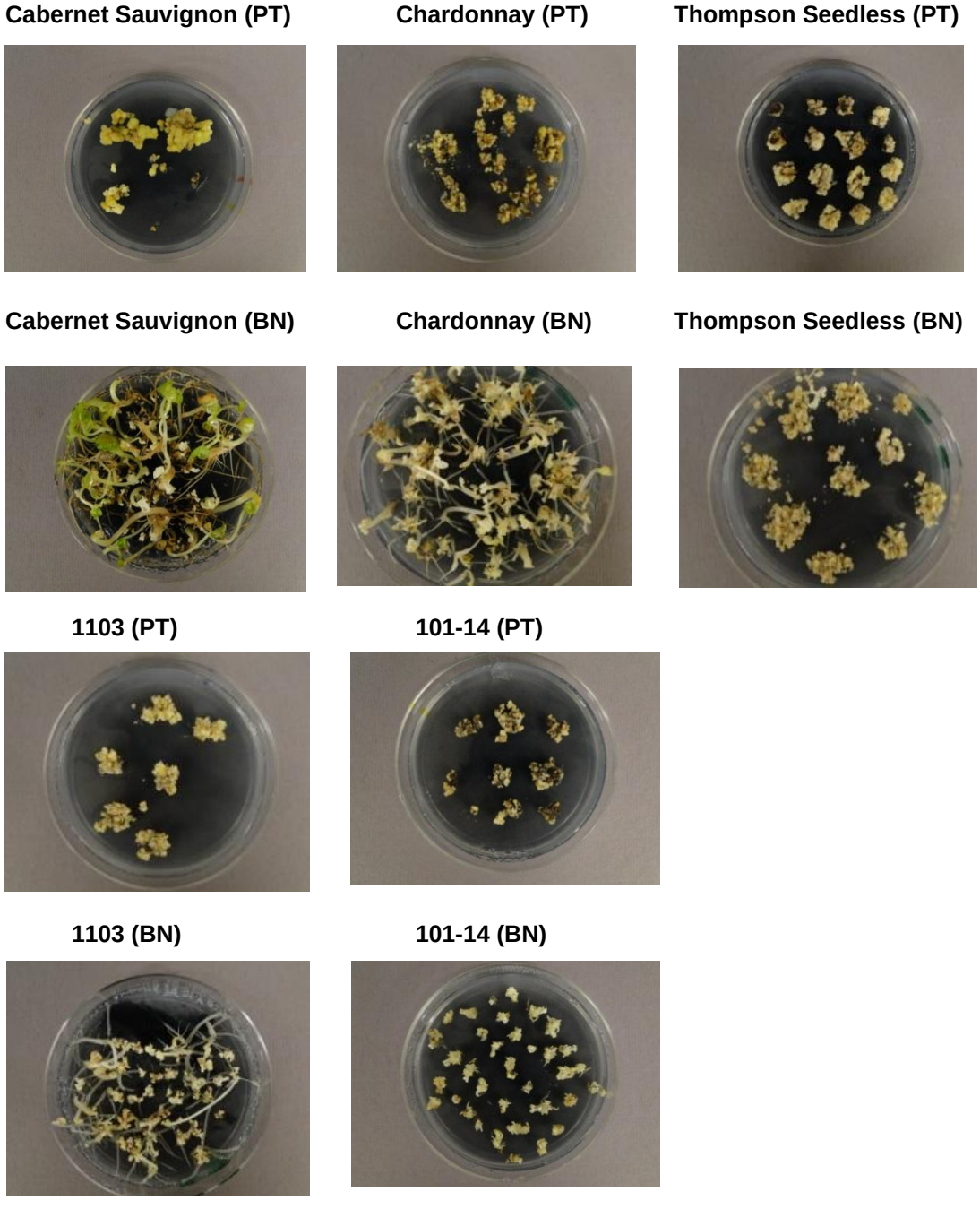


Figure 2. Grape embryogenic callus cultures of Cabernet sauvignon, Chardonnay, Thompson Seedless, 1103 and 101-14 maintained on Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 10 mg/l picloram, 2 mg/l thidiazuron (TDZ), and 8g/l TC Agar (PT) medium (top panels) or maintained on 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) (bottom panels).

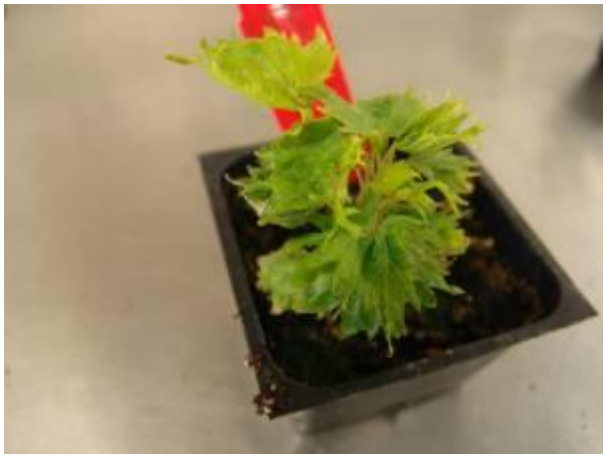


Figure 3. Abnormal phenotype observed in some plants regenerated from callus maintained on Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 10 mg/l picloram, 2 mg/l thidiazuron (TDZ), and 8g/l TC Agar (PT medium.

Next Steps:

- Determine culture conditions which allow long term maintenance of embryogenic callus at the appropriate stage of development for Chardonnay, 101-14, 1103 and Cabernet sauvignon for use in transformation experiments.

B. Production of embryogenic callus from leaf explants

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 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 last fall and have recently been provided cultures of Cabernet sauvignon thanks to the efforts of Foundation Plant Services (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. We secured shoot tips from this material this spring and established *in vitro* plantlets to be used to generate embryogenic callus from leaf explants (figure 4)

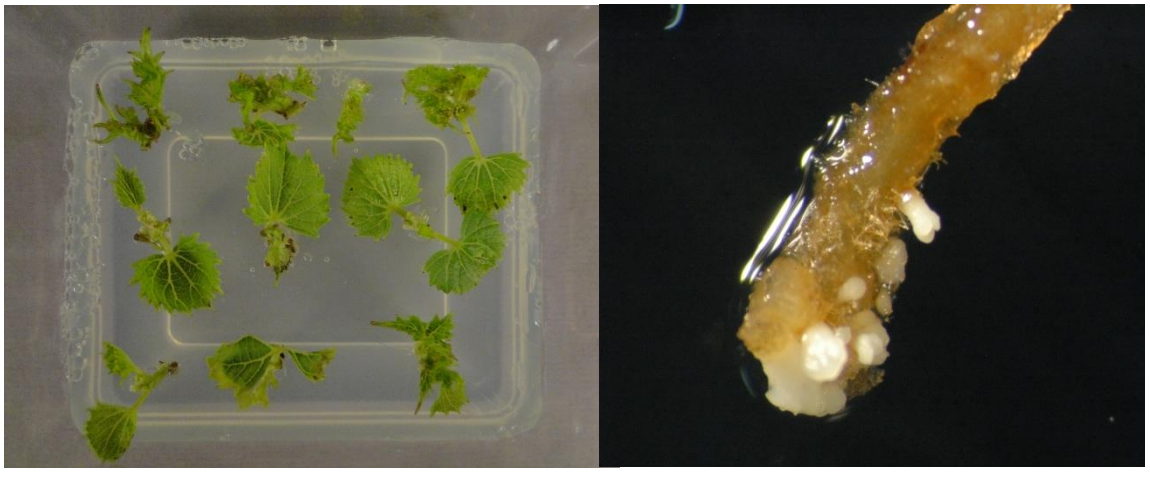


Figure 4. Shoot tip cultures of 1103 established *in vitro* for use in generating embryogenic callus from leaf tissue (left) and somatic embryo developing off the epidermis of root tissue of Cabernet sauvignon (right)

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. However, to date we have not been successful in producing embryogenic callus from leaf explants. After consulting with Humberto's lab, it appears that we need to collect younger stage leaves and increase the osmotic potential of the medium for the protocol to work properly. Recently we have noted the development of secondary somatic embryos on root tissue of Cabernet sauvignon. We may also be able to exploit this phenomenon for the production of embryogenic callus.

Next Steps:

- Working with our Chilean partners, trouble shoot the generation of embryogenic callus from leaf explants of Chardonnay, Cabernet sauvignon, 101-14 and 11-03
- Evaluate utility of *in vitro* root explants as a replacement of leaf tissue for generating somatic embryos

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 agar-solidified medium and liquid media in shake flasks. 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. He 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. 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 weigh in PT medium verses RM medium for both 101-14 and TS-10 (see March 2012 interim report).

Next Steps:

- Based on Eduardo's results using liquid PT medium, UCDPTF will test INIA's shake flask methodologies on 11-03, Chardonnay and Cabernet Sauvignon using UCDPTF's PT liquid medium or modifications thereof.
- 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.
- Explore methods to reduce the amount of labor associated with INIA's liquid/solid medium methodology for increasing the embryogenic callus. Due to phenolic oxidation of the suspension cultures, the protocol requires frequent handling of the cultures. As the tissue begins to oxidize in

suspension, it needs to be transferred from liquid to solid culture. These procedures, although effective in increasing callus, are time consuming and will put pricing pressure on a service-based transformations system for grape.

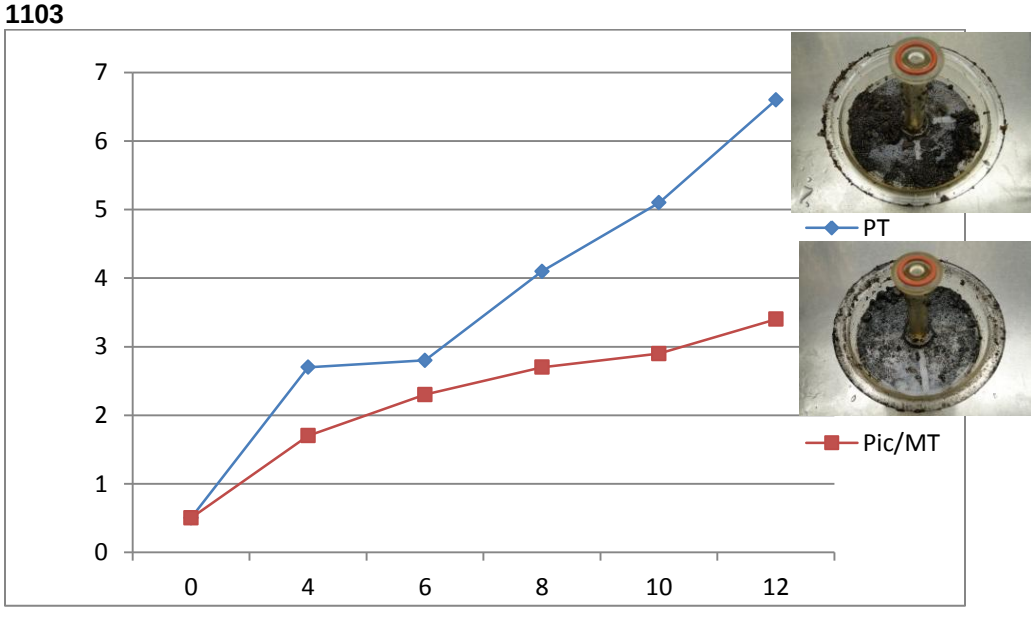
- Find methods of preventing or reducing oxidation of embryogenic callus tissue in the liquid or solid phase.

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 evaluating INIA's liquid shake flasks methodology on grape genotypes 101-14, 1103, Chardonnay and Cabernet sauvignon, we are exploring UCDPTF's temporary immersion system (TIS) for use in rapidly increasing embryogenic callus. We have inoculated RITA bioreactors with 0.5 g fresh weight of Chardonnay, 101-14 or 1103 embryogenic callus. The RITA vessels contain Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 10 mg/l picloram, 2 mg/l thidiazuron, (**PT medium**) or WPM supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal 10.0 Picloram, 2mg/l meta-topolin (**Pic/MT medium**) or 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 medium**). Fresh weight measurements and observations were taken at 2 week intervals. Results are presented in figures 5 and 6. Callus increase moderately on PT and Pic/MT media with more rapid growth seen on PT medium than on Pic/MT medium. However, the quality of the callus on both media formulations was compromised by excessive accumulation of phenolic compounds in the tissue. It is not known if this phenomenon is detrimental to further growth, so to test this, we have transferred samples of callus from the bioreactors to agar solidified BN medium to determine if white non-phenolic callus will develop. When callus was transferred to RITA vessels containing BN medium, 1103 and Chardonnay quickly regenerated into whole plants making it unfeasible to maintain the callus on this medium. On the other hand, 101-14 cultures remained as embryos which enlarge in size but did not germinate (figure 6).

1103



Chardonnay

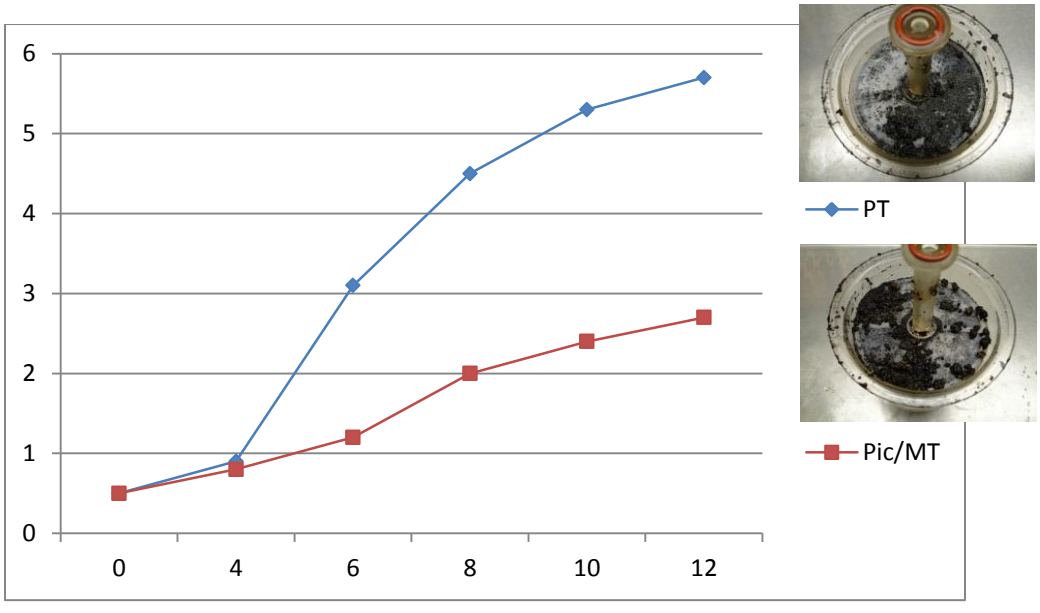
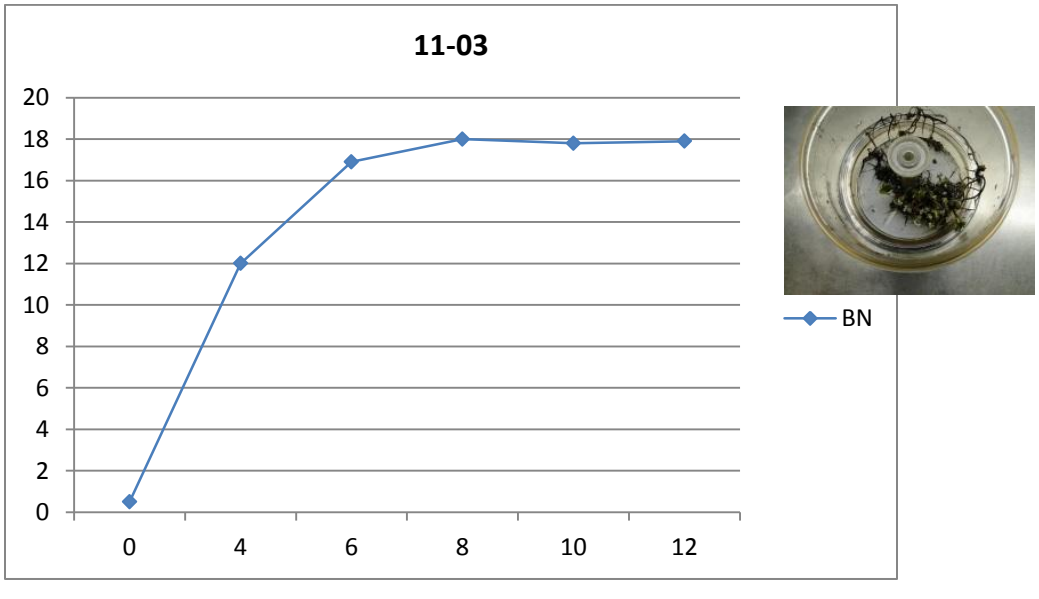


Figure 5 Growth rate increase (g fresh weight) of 11-03, and Chardonnay in a temporary immersion system grown in Woody Plant Media WPM supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 10 mg/l picloram, 2 mg/l thidiazuron (TDZ), (PT medium) or WPM supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal 10.0 Picloram , 2mg/l MT (Pic/MT)



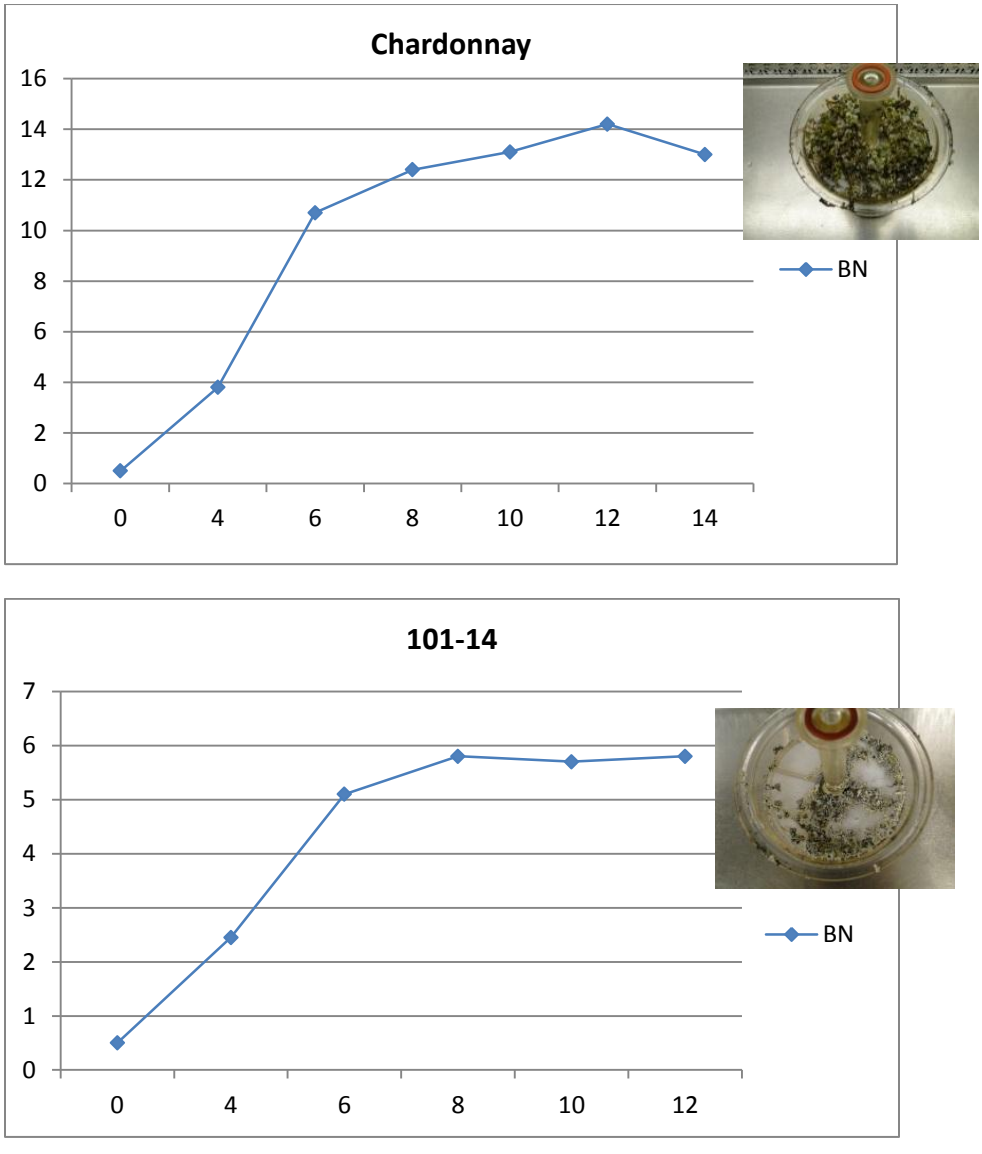


Figure 6. Growth rate increase (g fresh weight) of 11-03, Chardonnay and 101-14 in a temporary immersion system grown in (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA 400 mg/l (BN)

Next Steps:

- Determine if callus produced in the Rita vessels can be converted into non-phenolic callus by transfer to agar solidified BN medium.

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 often taking 6-9 months for transgenic embryogenic callus to convert into whole plants and some of these plants display distorted and variegated leaf morphology (figure 3). 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 PT medium formulation. Therefore, we have begun investigating various medium addendums in combination with testing various developmental stages of embryogenic tissue used for transformation and regeneration in an attempt to decrease the time required to produce transgenic callus and regenerated whole plants after *Agrobacterium-mediated* transformation. Our current media formulation consists of Lloyd and McCown's WPM supplemented with 10mg/liter Picloram and 2.0 mg/liter tridiazuron (TDZ). We have begun 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. Preliminary results indicate that embryogenic callus can be maintained as pro-embryogenic masses when MT is substituted for TDZ. We need to determine if callus maintained on medium containing MT regenerates whole plants faster than callus maintained on medium containing TDZ and does not negatively impact transformation efficiencies.

Next Steps:

- Develop media formulations that will allow maintenance of embryogenic callus of 1103, 101-14 and Chardonnay in a less suppressed developmental stage and which can rapidly regenerate into non-chimeric 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:

We have initiated preliminary transformation studies on callus of 11-03, 101-14 and Chardonnay using a construct containing the scorable marker gene dsRed. This protein allows us to non-destructively track transformation over the course of the transformation process and access the presence or absence of chimeric plants (figure 7)

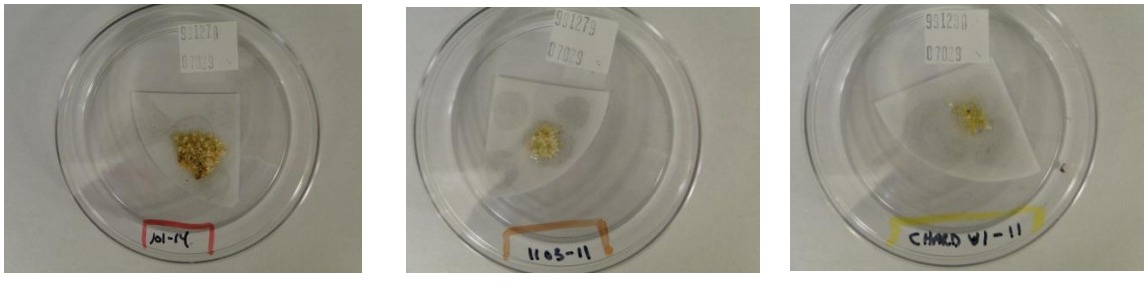


Figure 7. Co-culture of embryogenic callus of 101-14, 1103 and Chardonnay with the scorable marker gene dsRed

Next Steps:

- Continue transformation studies on 11-03, 101-14, Chardonnay and Cabernet Sauvignon on various developmental stages of callus using the scorable marker gene dsRed.

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.

We are exploring Eduardo Tapia Rodríguez from the National Research Laboratory of Chile methods for increasing embryogenic callus by cycling tissue between solid and a liquid medium. While visiting the UCDPTF, he found that when Thompson Seedless and 101-14 callus produced on solid medium was used to generate more biomass in liquid shake cultures using INIA's agar/liquid cycling system, UCDPTF's PT liquid medium formulation exhibited improved growth rate compared to INIA's standard liquid medium, producing significantly more biomass based on statistical kinetic growth comparisons. We are expanding these studies by investigating modifications of the UCDPTF PT liquid medium in an attempt to further improve growth quality of the cell suspensions in order to eliminate the need to cycle callus between the agar and liquid phase using grape genotypes; 1103, 101-14, Chardonnay and Cabernet Sauvignon. We have also begun experiments exploring increasing grape callus using UCDPTF's temporary immersion system.

K. Status of funds.

We anticipate that all funds allocated for fiscal year FY2012-2013 will be expended by the end of the fiscal year.

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.