

- **TITLE OF REPORT:** Interim Report for CDFA Contract Number 12-0442
- **TITLE OF PROJECT: FIELD EVALUATIONS OF GRAFTED GRAPE LINES EXPRESSING POLYGALACTURONASE INHIBITING PROTEINS (PGIPS)**

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- **TIME PERIOD COVERED BY THE REPORT:** The results reported here are from work conducted from 1 March 2014 to 31 July 2014.

- **INTRODUCTION:**

The project was designed to establish two field sites with typical vineyard practices that would allow grape lines to be evaluated in order to assess whether polygalacturonase inhibiting proteins (PGIPs) restrict *Xylella fastidiosa* (*Xf*) spread and Pierce's Disease (PD) and whether expression of pPGIP impacted the performance and attributes of the vines.

The PI, co-PI and others had shown that the expansion of *X. fastidiosa* from the infection site throughout the vine, creates a systemic infection that causes PD and vine death (Krivanek and Walker, 2005; Labavitch 2006, 2007; Lin, 2005; Lindow, 2006, 2007a, b; Rost and Matthews, 2007). The grapevine water-conducting xylem elements are separated by pit membranes, pectin-rich cell wall "filters" whose meshwork is too small to permit movement of *Xf* (Labavitch et al., 2004, 2006, 2009a). *Xf* produces cell wall-degrading enzymes to digest the pit membrane polysaccharides (Labavitch et al., 2009b), opening xylem connections and permitting spread of the bacteria.

The *Xf* genome contains a polygalacturonase (*XfPG*) and several  $\beta$ -1,4-endo-glucanase (EGase) genes, whose predicted enzyme products could participate in the digestion of pectin and xyloglucan polymers in pit membranes and, thereby, facilitate PD development by the movement of *Xf* within vines. Labavitch et al. (2006, 2007, 2009a; Perez-Donoso et al., 2010) reported that introduction of PG and EGase into uninfected grapevines caused pit membrane breakage. Roper et al. (2006, 2007) developed an *XfPG*-deficient *X. fastidiosa* strain and showed it was unable to cause PD symptoms, so *XfPG* is a PD virulence factor.

The over-all research aim is to use plant PGIPs to limit *Xf* spread in grapevines. PGIPs are produced by plants, including in flowers and edible fruits, and are selective inhibitors of pathogen and pest PGs (Powell et al., 2000; Shackel et al., 2005; Stotz et al., 1993, 1994). Transformed grapevines expressing the pPGIP-encoding gene from pears have reduced susceptibility to *Xf* and pPGIP is transported across the graft junction from pPGIP expressing grape and tomato rootstocks into wild-type scions (Agüero et al., 2005, Haroldsen et al., 2012).

Grafting pPGIP-producing rootstocks to scions, which do not contain an introduced pPGIP gene, is an opportunity to provide a beneficial plant fruit protein (i.e., pPGIP) without introducing a pPGIP gene into the scion itself. This project has been designed to scale up the grafted and own-rooted pPGIP expressing grapevines, plant them in field settings, and evaluate their agronomic performance and their resistance to PD in settings comparable to commercial fields.

- **OBJECTIVES:**

1. Scale up the number of grafted and own-rooted pPGIP expressing lines.

2. Plant and maintain grafted and own-rooted lines in two locations with different PD pressure.
3. Evaluate relevant agronomic traits of vines in two locations.
4. Determine PD incidence in pPGIP expressing grafted and own-rooted lines. Test for *X. fastidiosa* presence and, if present, determine the extent of infection.

- **DESCRIPTION OF ACTIVITES:**

*Objective 1: Generate enough grafted and own-rooted grapevines for the field trial*

**Progress:** The pPGIP expressing Chardonnay and Thompson Seedless (TS) grapevines originally generated by Agüero et al. (2005) have been maintained at the UC Davis Greenhouses. Vegetative cuttings of non-lignified stem sections from transgenic and control plants of both cultivars were rooted in an aeroponic cloning manifold (EZ-Clone Inc., Sacramento, CA). These plants are referred to as “own-rooted plants.” Rooted cuttings were transferred to soil and maintained in the greenhouse before being transferred to the field sites. A sufficient number of grafted and “transgrafted” plants were generated for the field trials and were made by green grafting rootstock stem sections with budding scion tissue. Transgrafted plants had rootstocks from the pPGIP expressing lines and scions that do not express pPGIP. The number of plants of each genotype and grafting protocol for the field sites in Solano and Riverside Counties are shown in Table 1. DNA was prepared from the vines used as source tissue for grafting and the genotypes were confirmed by PCR.

**Results:** Sufficient plants of both the Chardonnay and TS varieties have been self-grafted, transgrafted or propagated by rooting to complete the Solano and Riverside County plots. The genotypes of the plants have been verified. All of the vines needed for the trail were transplanted to the two sites by mid-summer 2013. All activities for this objective have been completed.

**Table 1.** Numbers of grapevines planted in Solano and Riverside Counties. Dashed fill represents pPGIP expressing rootstocks and/or scions; black fill is null-transformants, no pPGIP, controls; white fill is non-transformed controls. In Solano County, own-rooted vines were mechanically inoculated in the summers of 2011-2013; grafted vines were inoculated in July, 2013. Vines planted in Riverside County were assessed in response to “natural” infections.

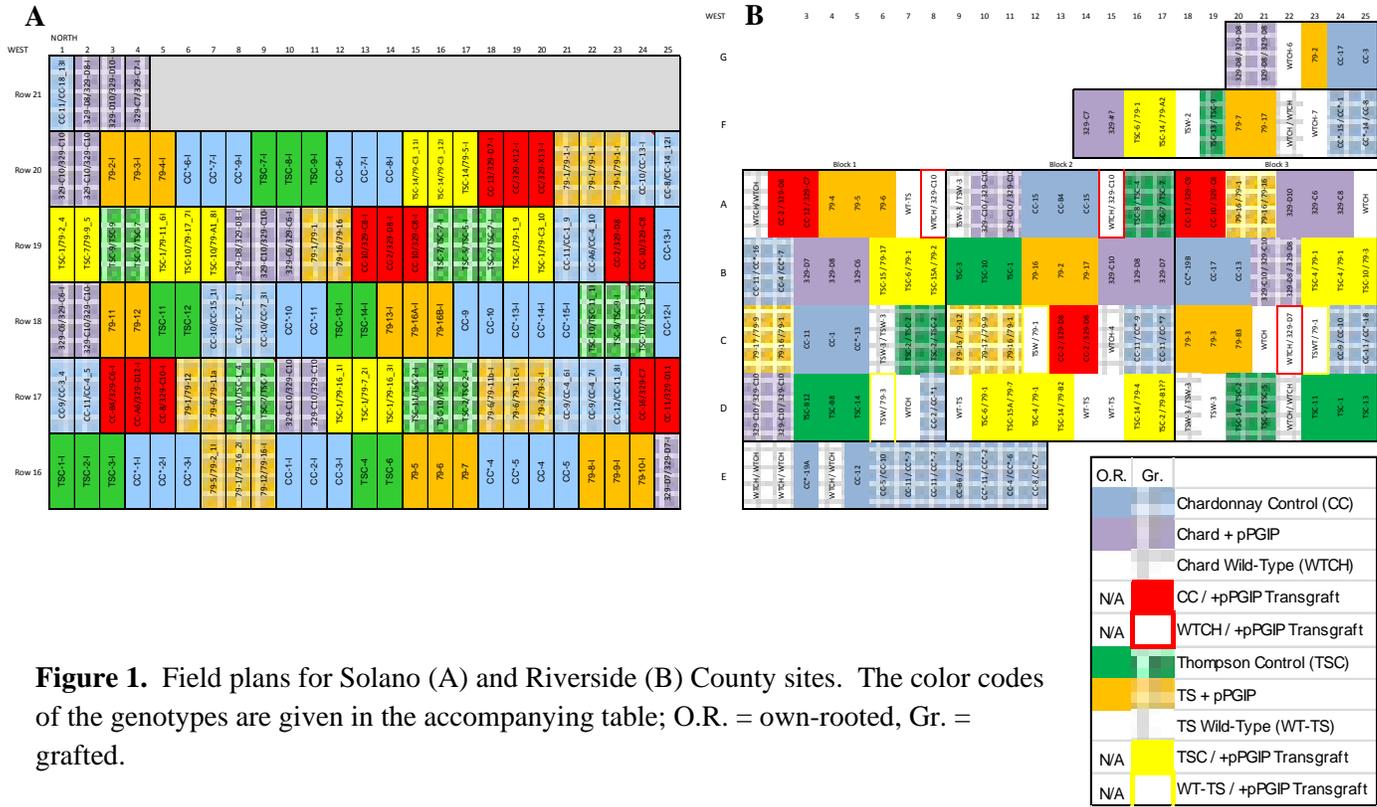
**Table 1.**

<b>SOLANO</b>		Chardonnay					Thompson Seedless				
	Grafting Strategy (Scion/root)										
	Hatch – pPGIP expressing										
Own-Rooted (#)	Inoculated	9	-	9	-	-	16	-	9	-	-
	Non-Inoculated	5	-	4	-	-	7	-	5	-	-
Grafted (#)	Inoculated	3	8	9	-	-	15	10	9	-	-
	Non-Inoculated	1	3	4	-	-	7	5	4	-	-
<b>RIVERSIDE</b>											
Own-Rooted (#)	Natural Infections	12	-	11	6	-	10	-	12	6	-
Grafted (#)	Natural Infections	8	5	8	6	3	15	15	7	3	3

*Objective 2: Establish field trial sites*

**Progress:** Field trial sites in Solano and Riverside Counties have been established to assess the PD resistance and general agronomic viability of own-rooted and grafted pPGIP expressing grapevines. The field plans of the Powell trial plots in Solano and Riverside Counties are shown in Figure 1. The location of the plots for this trial is within fields shared by other projects testing PD resistance of other

transgenic grapevines. The vines satisfying our initial PCR analysis were hand-planted in a randomized block design with blocks of two or three individuals with the same treatment (Table 1). Two plants have died (row 21 plant 2 and row 18 plant 13) have been damaged and are no longer viable. The underlying grape variety of each vine is being confirmed with assistance from Mark Greenspan.



The vines have been pruned both to maximize potential cane number for inoculations and to establish vigorous positions for future growth. With the permit amendment granted by the BRS-USDA in March 2012, flowers and fruiting clusters have been allowed to persist. All own-rooted Chardonnay vines were cordon trained and spur pruned and the majority of the Thompson Seedless vines were cane pruned in an attempt to maintain proper vine balance and ensure fruit development. The Solano field site was observed weekly during the 2013 growing season and has been observed in March, May and June of 2014. The vines in Riverside County planted in early June, 2013 and grew in the 2013 season. Observations of the vines in Riverside were made by members of the Powell team twice during the 2013 growing season and 4 April 2014.

**Results:** As of 3 June, 2013, both the Riverside and Solano County sites have been established with all the planned plantings for this project. All activities for this objective have been completed. *Objective 3: Evaluate relevant agronomic traits of vines in two locations.*

**Progress:** Both the Solano and Riverside vineyards continue to be maintained by appropriately pruning and training the vines. Eleven uninoculated grafted plants did not survive the arid 2013 summer in Solano County – these were replaced in the Fall of 2013. Otherwise, growth of the vines at both locations has been vigorous (Figure 2). Eleven own-rooted vines from Chardonnay control to TS control were reclassified based on cluster morphologies; this identification is being confirmed in the Summer of 2014. The previously observed leaf morphologies were supportive of this reclassification, but fruiting habit and form permitted definitive identification. Data for the agronomic and phenotypic observations in 2013 has been included in previous reports. Prior to the annual pruning on 20 March, 2014 in Solano

County, visual assessments of bud growth on inoculated canes and throughout the plants were made twice on 14 and 19 March on all vines in the Solano County site. The vines were re-pruned 28 and 29 July, 2014

In 2013, detailed analysis of plant performance and phenotypes was done on berries collected from uninoculated and inoculated plants of each own-rooted genotype at the Solano. The grafted plants in the plot were too juvenile to bear fruit in 2013 and had been inoculated until three weeks earlier; they were not sampled. The pH and °Brix of samples of crushed and free-run filtered juice were measured and reported previously. Soluble solids ranged from 21.7-24.4 °BRIX and pH values were 3.56-4.00 and showed no significant differences due to the pPGIP. A smaller subsample was repeated on 4 September 2013 with similar results. Some inoculated own-rooted vines did not bear fruit; grafted plants, with one exception, were fruitless in 2013 due to their immaturity. Total cluster numbers per plant and cluster weight, length, and peduncle length were counted. The data collected in 2013 (Figure 5) will be critically analyzed in 2014 to select appropriate measurements for meaningful comparisons between genotypes, treatments and plots.

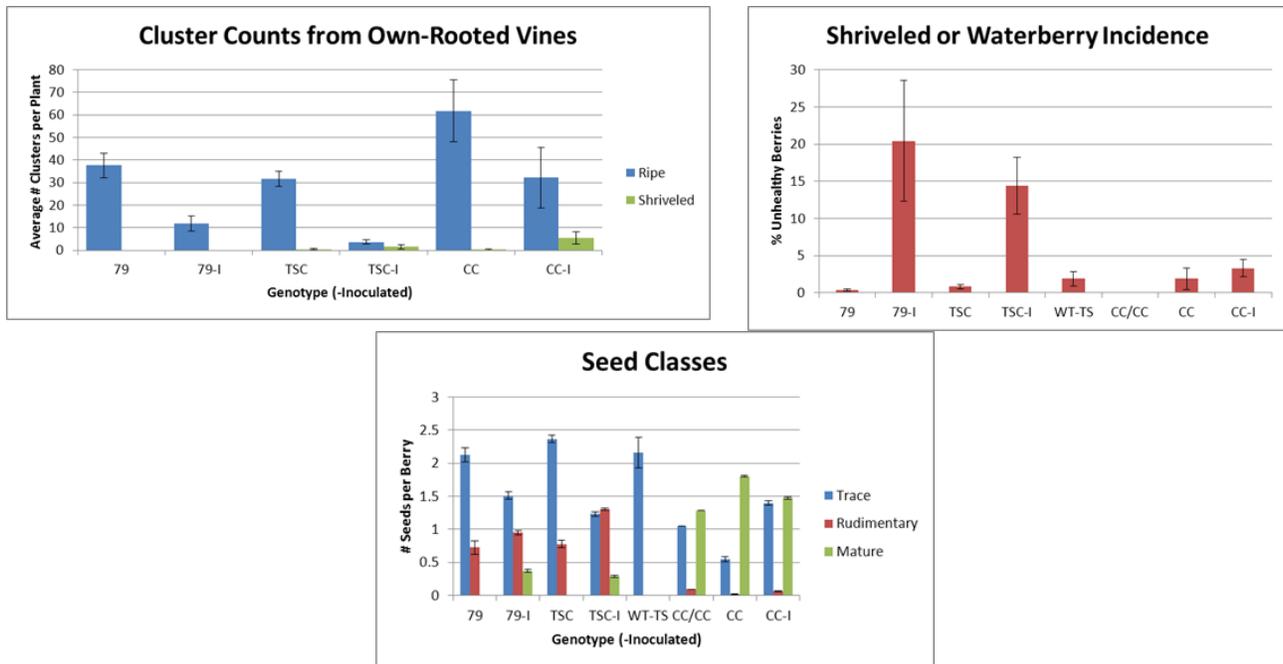
**Results:** Based on observations in 2013, the own-rooted TS plants expressing pPGIP had a slightly larger average yield than control vines. Expression of pPGIP in the Thompson Seedless variety did not significantly affect the berry cluster morphology or the juice characteristics. In 2014, initial bud growth data has just been collected and is currently being analyzed.



**Figure 2.** Representative inoculated Thompson Seedless plants in Solano County: top row, pPGIP expressing vine; bottom row, control vine. Pictures taken (from left to right) May 3, June 26, Sept. 24, and Sept. 11, 2013.

*Objective 4: Determine PD incidence in pPGIP expressing grafted and own-rooted lines.*

**Progress:** Two-thirds of the own-rooted vines at the Solano County site were first mechanically inoculated with approximately  $2 \times 10^4$  *X. fastidiosa* Temecula cells on 21 July 2011. On the inoculated vines, no visual evidence of PD infection was observed in the 2011 growing season or early in 2012 after bud break. The same 34 own-rooted vines were re-inoculated on 29 May 2012 with a mixture of *X. fastidiosa* Temecula and Stag's Leap strains (3:2, v:v). 3-4 canes of young, green tissue per vine were mechanically inoculated with approximately  $1.5 \times 10^7$  cells. Inoculations were repeated 6 August 2013 on own rooted and small transgrafted plants in our plots (see below). All grafted plants in our plots (but not own rooted vines) were inoculated again 28 May 2014. The inoculations were performed simultaneously with the other field site collaborators in 2011- 2014. The bacterial suspensions were provided by D. Gilchrist.



**Figure 3.** Number of clusters per plant (A), Percentage of shriveled unhealthy berries per cluster (B) and seed morphologies within clusters (C) of own-rooted TS expressing pPGIP (“79”), TS control (TSC) and Chardonnay (CC) from uninfected and infected (-I) plants.

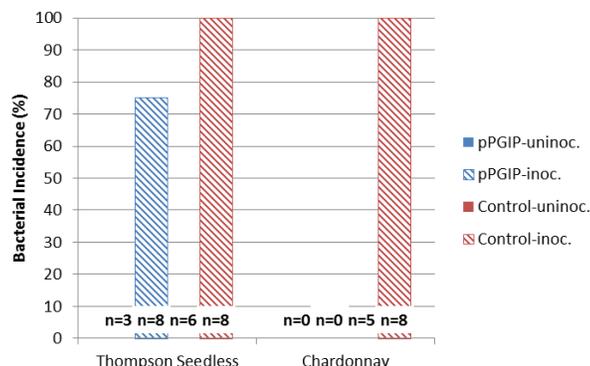
Once the remaining grafted and transgrafted vines at the Solano County site had been established after transplantation in June 2013, the complete trial of pPGIP vines in our plot was mechanically inoculated in the third year, on 8 August 2013. The own-rooted vines previously infected and the selected newly planted grafted vines were inoculated with a mixture of the *X. fastidiosa* Temecula and Stag’s Leap strains (3:2, v:v) prepared in our laboratory from glycerol stocks provided by the Kirkpatrick and Gilchrist labs. Inoculations were performed as in previous years, except only one site was inoculated per grafted vine because the vines were quite small (they had only been transplanted in June, 2013); larger, own-rooted vines were inoculated at 2 to 3 sites per plant. The inoculum cell density was estimated by optical density and confirmed by serial plating to be  $1.2 \times 10^6$  cells per inoculation site.

PD symptoms were first observed on the twice-inoculated vines in Solano County on April 24, 2013. The most frequent symptoms were inhibition of bud break along inoculated shoots and excessive growth from the base of plants, potentially indicating a disruption in the vasculature or more severe die-back of cordons and mature canes. Outside viticulturists and pathologists confirmed that these vines had PD. Their opinions were sought because traditional PD symptoms were mostly absent during the previous two growing seasons. Since the initial observation, each vine was photographed and initially scored for the presence of similar stunting or “blind” phenotypes (see previous reports). Initial observations of the thrice-inoculated own-rooted and the once-inoculated grafted and transgrafted vines in March 2014, indicates that, in general, bud growth is retarded on inoculated vines, especially on non-pPGIP-expressing or non-transgrafted vines.

PCR was used to detect *Xf*DNA sequences in leaves and petioles from inoculated and un-inoculated vines in 2013 (Figure 4). *Xf*DNA sequences were only detected in inoculated, and not in uninoculated, plant leaves. All DNA preparations were checked to see that PCR amplification of grape DNA sequences was possible.

An initial collection of canes was contemporaneous with those collected from other plots and represents an initial determination at 1 month following inoculations. On 28 July 2014, a single cane inoculated in May 2014 was excised from each plant and brought back to the lab to be processed for extraction of DNA. Cane material from 4-8cm from the point of inoculation was frozen at  $-80^{\circ}\text{C}$  for grinding once additional samples are collected later in the season. The amount of *Xf* DNA will be

determined by qPCR. The number of leaves with leaf scorch symptoms was made for each of the excised canes as well as all other canes inoculated in 2014 on 29 July 2014. The first proximal leaf and up to 12 distal leaves were counted on each inoculated cane and the number of leaves with scorching symptoms among the total number of leaves was determined.



**Figure 4.** Results of PCR detection of *Xf* DNA sequences in inoculated vines in Solano.

**Results:** In both 2013 and 2014, a higher percentage of bud positions were able to grow out on mechanically inoculated pPGIP expressing vines than on control vines. Preliminary analysis of 2014 observations suggests that both Thompson Seedless and Chardonnay vines had the pPGIP-dependent differences in susceptibility. In 2013, it was noted that inoculations with *Xf* promote shoot growth from the base of the vines. In the assessments in 2013, inoculated pPGIP expressing TS vines had 40% fewer clusters with aborted or abnormal berries than infected controls. However, 1 of 5 uninoculated pPGIP expressing TS vines had abnormal berry clusters and the un-inoculated controls had none. Three times as many mechanically inoculated pPGIP expressing TS vines had leaves with signs of marginal necrosis than infected control vines. In 2014, we expect to collect similar data with the Chardonnay genotype and the transgrafted plants.

Later in the season, inoculations with *Xf* resulted in a noticeable decline in clusters for all genotypes. However, this decline was 20% less in TS plants expressing pPGIP. Shriveled clusters were only observed in control genotypes and did not always correspond to infection with *Xf*. An increase in the percentage of unhealthy berries per cluster was measured in inoculated plants, but no significant difference in clusters from plants with or without pPGIP was observed. Berries from inoculated plants contained slightly more developed seeds than those from uninoculated plants; TS plants expressing pPGIP had fewer rudimentary seeds. Other observations reflect that uninoculated plants produce longer clusters with more berries.

*Xf* DNA sequences were detected by PCR in the inoculated samples. No *Xf* DNA sequences were detected in un-inoculated controls. Preliminary visual assessments indicate that expression of pPGIP reduces PD symptoms (bud outgrowth, abnormal berry clusters and leaf scorching along inoculated canes).

## CONCLUSIONS:

All of the own-rooted, transgrafted and grafted plants necessary for the studies in Solano and Riverside Counties for this project have been planted at the sites. The genotypes of the grafted plants were confirmed. An initial attempt to infect the vines in Solano County in 2011 was made but no symptoms were observed. A second attempt in 2012 resulted in detectable *Xf* DNA in infected vines in November, 2012 and visual symptoms of PD in April, 2013. The performance of the own-rooted Chardonnay and Thompson Seedless vines in the field thus far has been appropriate for commercial settings. Symptoms of PD infection were visible on the inoculated vines beginning in the Spring of 2013 following the introduction of *Xf* in 2012. The initial evaluations of the performance and productivity suggest that pPGIP expression in a table grape variety (Thompson seedless) or a wine grape (Chardonnay) improves resistance of vines to PD but does not otherwise affect growth.

- **PUBLICATIONS AND PRESENTATIONS PRODUCED:** Results as of mid-December 2013 were presented orally at the Annual Pierce's Disease Symposium in Sacramento by Ann Powell.
- **RESEARCH RELEVANCE STATEMENT:**

Work in this project evaluates the performance of grafted grapevine lines that produce a protein that is a candidate for control of Pierce's Disease (PD). The vines have been established in vineyards in a manner that approximates typical commercial settings in regions of Solano and Riverside Counties with low and high PD disease pressure, respectively. The CDFA PD and Glassy-winged Sharpshooter Board's Research Scientific Advisory Panel had established a priority to evaluate the potential commercial use of the strategy to deliver PGIPs from grafted rootstocks to control PD in the scion, fruit-bearing portions of grapevines. Established transformed 'Thompson Seedless' and 'Chardonnay' grapevines expressing a PGIP from pear fruit (pPGIP) showed reduced PD incidence when inoculated with *X. fastidiosa* (Agüero *et al.*, 2005). The pPGIP that was produced in the transformed rootstock was identified in samples of xylem exudate that were collected from grafted, but not transformed scions (Agüero *et al.*, 2005). Therefore, cuttings from these grapevines were grafted with non-pPGIP producing scions to make comparisons of the effectiveness and outcomes between vines producing pPGIP in grafted rootstocks, those producing pPGIP throughout the vine, and vines with no pPGIP.

- **LAY PERSON SUMMARY:**

Two vineyard plots containing own-rooted and transgrafted (rootstocks expressing pPGIP grafted to fruit producing scions with no genetic modifications that, thus, do not themselves produce pPGIP) combinations of Chardonnay and Thompson Seedless grapevines were established and the identities of the genotypes were established by June 2013. Mechanical inoculations with *X. fastidiosa* bacteria were done in 2011, 2012, 2013 and 2014 in Solano County and natural infections were allowed to occur in Riverside County. Data describing the agronomic and disease traits of the vines have been collected. Since this trial evaluates grape varieties grown for fresh fruit and for wine production in California, we are testing varieties important to most California grape growers. The initial evaluations of the performance and productivity suggest that pPGIP expression in a table grape variety (Thompson seedless) or a wine grape (Chardonnay) improves resistance of vines to PD but does not otherwise affect growth.

- **STATUS OF FUNDS:**

	<b>Budget (through 2014)</b>	Feb 1, 2013 – June 30, 2014  (actual)	<b>Budget (July 1, 2014 – June 30, 2015)</b>	<b>Budget (July 1, 2015 – June 30, 2016)</b>
Personnel				
Professional, 8% Ann Powell, Feb 13 – Oct 13, 16% Nov 13 – June 14, 8% Jul 14 – Jun 16	<b>19,510</b>	17,250	<b>7,900</b>	<b>8,137</b>
Lab Asst. I, 1 month (monthly base \$2,368)	<b>2,368</b>	4,355	<b>0</b>	<b>0</b>
Student Asst., \$10/hr	<b>1,500</b>	4,290	<b>1,800</b>	<b>1,800</b>
Employee Benefits	<b>7,559</b>	8,307	<b>3,041</b>	<b>3,310</b>

<b>SUBTOTAL</b> (Personnel + Benefits)	<b>30,937</b>	34,202	<b>12,741</b>	<b>13,247</b>
Supplies and Expenses	<b>9,907</b>	6,893	<b>2,000</b>	<b>2,000</b>
Equipment				
Travel	<b>2,000</b>	1,490	<b>1,000</b>	<b>0</b>
Computer Time				
Other				
Indirect Costs*	<b>0</b>	0	<b>0</b>	<b>0</b>
<b>SUBTOTAL</b> (Supplies, Expenses, Equipment, etc.)	<b>11,907</b>	8,383	<b>3,000</b>	<b>2,000</b>
<b>TOTAL</b>	<b>42,844</b>	42,585	<b>15,741</b>	<b>15,247</b>

- **SUMMARY AND STATUS OF INTELLECTUAL PROPERTY ASSOCIATED WITH THE PROJECT:** None is relevant.

- **LITERATURE CITED:**

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