"Interim Progress Report for CDFA Agreement Number 12-0445-SA"
Project Title: CAP and PGIP transgenic grapevines field trial.

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Introduction

'Thompson Seedless' (TS, *Vitis vinifera*) grapevines were transformed with a gene that encodes a chimeric anti-microbial therapeutic protein with a recognition domain from a neutrophil elastase (NE) that specifically binds to the *Xylella fastidiosa*(*Xf*) outer-membrane protein MopB and a lytic domain, Cecropin B (CB) that clears *Xf* the causative agent for Pierce's Disease (PD) in grapevines (Dandekar et al., 2012). We have also similarly transformed TS grapevines with a gene encoding polygalacturonase inhibitory protein (PGIP) that results in the expression of a PGIP that inhibits the action of a *Xylella* encoded polygalacturonase (PG), a virulence factor expressed by *Xf*, to interfere with long distance movement of *Xf* that provides resistance to Pierce's Disease in grapevine (Aguero et al. 2005). Transgenic grapevines expressing NE-CB and different PGIP constructs were first tested under greenhouse conditions and lines several lines that showed resistance to PD as compared to controls were identified by mechanically inoculating with *Xf* (Dandekar et al. 2012).

Selected transgenic grapevine plants expressing either NE-CB or PGIP, own-rooted or grafted with untransformed Thompson Seedless (TS), were planted in 2010-11 and since then, plants are being tested for PD resistance under field conditions at two locations.

At the Riverside County, a site with natural PD pressure, plants have been naturally infected, from 2011 to 2013 PD symptoms were observed, grapevines vigor was scored and *Xf* was detected in Xylem sap, petiole and stem extracts by ELISA and plating. Unexpectedly, during the 2014 spring season we observed that all of our 224 Thompson seedless vines at Riverside site were in decline; most of them had no new growth. Those that had growth did not follow any particular genotypic pattern with transgenic and controls appearing to be equally affected, in all cases the root zone was limited to the first six inches of soil and was heavily infected with Root Knot nematodes. Four soil samples were taken and all four were positive for Root Knot. Roots taken had four knots per inch whereas normally it would be one knot/gall

per four inches. Nematodes population data in soil and root samples, already submitted for analysis, will be available in the next progress report. Since the Riverside site was previously planted with Chardonnay for about 15 years and the vineyard was removed and replanted without fumigation. Our initial conclusion is that the field location had selected for an aggressive population of Root Knot nematode during the previous grapevine planting that then attacked our vines once they were planted in that same location, resulting in a lethal infection.

At the Solano County field, half of the non-grafted transgenic grapevine lines were manually inoculated as described by Almeida et al., (2003) for the first time in July 2011, for a second time on May 2012. Half of the grafted transgenic grapevines lines in Solano County field were manually inoculated on May 2012. The remaining un-inoculated, non-grafted and grafted grapevines that remained un-inoculated in the previously two inoculations (2011 and 2012) were manually inoculated on June 17, 2013, completing the manual inoculation of all grapevines at this location. At Solano County site PD symptoms have also been detected, grapevine vigor and cane survival have been scored and *Xf* has been detected in petiole and stem extracts using ELISA. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four current year canes per grafted transgenic and control plants at the Solano field were mechanically inoculated with *Xf*. On July 22, 2014 one cane per plant was harvested for *Xf* quantification. During the summer of 2014 two more set of canes will be harvest at two different sampling dates and one more in the spring of 2015. Quantification of *Xf* will be measured by qPCR. Severity or absence of PD symptoms will be recorded at each sampling date on all inoculated canes.

List of objectives

The goals of this project are to finish the field test of 4 NE-CB and 4 PGIP transgenic grapevine clones, to evaluate their horticultural characteristics and their resistance to PD. Transgenic grapevines have been tested in two field locations as non-grafted plants and as transgenic rootstocks grafted with wild type grapevine scion. One field location has PD pressure and plants were naturally infected with *Xf* and in the location with no PD pressure, grapevines were mechanically inoculated with *Xf* .

Objective 1. Validate the efficacy of *in planta*-expressed chimeric NE-CB and PGIP with different signal peptides to inhibit and clear *Xf* infection in xylem tissue and to pass through the graft union under field conditions.

Activity 1. Propagation, field planting, and grafting of HNE-CecB and PGIP transgenic grapevines.
Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.
Activity 3. Evaluate PD resistance of HNE-CecB and PGIP transgenic grapevines after inoculation with *Xf*.

Description of activities conducted to accomplish each objective

Activity 1. Propagation, field planting, and grafting of HNE-CecB and PGIP transgenic grapevines.
Four independent transgenic events expressing NE-CB (40-41, 40-89, 40-92 and 41-151) and four expressing different PGIP constructs (31-25, 45-77, 52-08, TS50) were planted in two experimental fields. Two hundred and ten transgenic or untransformed vines, own-rooted or grafted with untransformed TS scions, were planted in Riverside County on 5/18/10 and the remaining 10 were planted on 3/6/11, completing the planting at this location **(Table 1)**. We also planted 110 transgenic and untransformed vines on their own roots on 8/2/2010 and 110 vines grafted with untransformed TS scions on 6/27/11 in Solano County, completing the planting at this second location (**Table 1**). NE-CB- and PGIP-expressing transgenic grapevine lines in Solano County have also been genotyped, confirming the presence of the inserted transgene in all lines.

Table 1. Transgenic and control grapevines planted at Riverside and Solano fields			
Non-grafted		**Grafted**	
Event ID (Vector)	**# Planted**	**Event ID (Vector)**	**# Planted**
HNE-CecB lines			
40-41 (pDU04.6105)	12	40-41G (pDU04.6105)	12
40-89 (pDU04.6105)	12	40-89G (pDU04.6105)	12
40-92 (pDU04.6105)	12	40-92G (pDU04.6105)	12
41-151 (pDU04.6105)	12	41-151G (pDU04.6105)	12
PGIP Lines			
31-25 (pDU05.1002)	12	31-25G (pDU05.1002)	12
45-77 (pDU06-0201)	12	45-77G (pDU06-0201)	12
52-08 (pDU05.1910)	12	52-08G (pDU05.1910)	12
TS50 (pDU94.0928)	12	TS50G (pDU94.0928)	12
Control line			
TS	16	TS-G	12

Activity 2. Evaluate preservation of varietal characteristics in transgenic grapevines grown as whole plants or used as rootstocks.

To verify that horticultural and varietal characteristics of the parental genotype TS were unchanged, NE-CB and PGIP expressing transgenic grapevine lines in Solano and Riverside Counties were evaluated phenotypically in September 2011 and November 2011, respectively. This examination was accomplished using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV, 1983). The descriptors used were 1) aperture of young shoot tip/opening of young shoot tip, 2) density of prostrate hairs between main veins on 4th leaf lower side of blade, 3) number of consecutive shoot tendrils, 4) color of upper side of blade on 4th young leaf, 5) shape of mature leaf blades, 6) number of lobes on mature leaf, 7) area of anthocyanin coloration on main veins on upper side of mature leaf blades, 8) shape of teeth on mature leaves, 9) degree of opening of mature leaves/overlapping of petiole sinuses, 10) mature leaf petiole sinus bases limited by veins, 11) density of prostrate hairs between main veins on lower side of mature leaf blades, and 12) density of erect hairs on main veins on lower sides of mature leaf blades. Riverside and Solano Counties, NE-CB and PGIP-expressing transgenic grapevines lines were also phenotypically evaluated in the fall of 2012 and 2013. Up to date no difference between transgenic grapevines and parenteral genotype TS were observed.

Activity 3. Evaluate PD resistance of NE-CB and PGIP transgenic grapevines after inoculation with *Xf*.

At the Riverside Site, grafted and non-grafted transgenic grapevine lines naturally infected in the field were scored for Pierce's disease symptoms for the last time on May 2013. Stem samples harvested on May 2013 from Riverside grapevines were tested for number of *Xf* cells by ELISA, the standard curve was created using *Xf* from liquid culture. Pierce's disease symptoms and ELISA cell count results confirmed *Xf* infection in Riverside County field. Unexpectedly, during the 2014 spring season we observed that all of our 224 Thompson seedless vines planted at Riverside site were in decline; most of them had no new growth. Those that had growth did not follow any particular genotypic pattern with transgenic and controls appearing to be equally affected. We dug up the soil about 18 inches from the trunk and observed in all cases the root zone was limited to the first 6 inches of soil and was heavily infected with root knot nematode. Four soil samples were taken and all four were positive for Root Knot. Roots taken had four knots per inch whereas normally it would be one knot/gall per four inches. We did consult with Andrew Walker, Howard Ferris and Michael Mckenry, which is how we came up with the Root-Knot diagnosis. Nematodes population data in soil and root samples, already submitted for analysis,

will be available in the next progress report. Since the Riverside site was previously planted with Chardonnay for about 15 years and the vineyard was removed and replanted without fumigation. Our initial conclusion is that the field location had selected for an aggressive population of Root Knot nematode during the previous grapevine planting that then attacked our vines once they were planted in that same location, resulting in a lethal infection. Heavy feeding of nematodes in the first year of a vine likely resulted in root leakage in the first year leading to a field that would continue to be highly susceptible to poor cultural practices and or environmental stresses for the remainder of the life of the vineyard.

At the Solano County field, half of the non-grafted transgenic grapevine lines were manually inoculated as described by Almeida et al., (2003) for the first time in July 2011, for a second time on May 2012. Half of the grafted transgenic grapevines lines were manually inoculated on May 2012. Solano non-grafted and grafted grapevines that were not inoculated in the previously two inoculations (2011 and 2012) were manually inoculated on June 17, 2013, completing the manual inoculation of all grapevines at this location. The manually inoculated runners of grafted and non-grafted transgenic grapevine were scored for Pierce's disease symptoms on May 2014. Cane survival was scored using a 0-1 scale, where 0 = alive runner and 1 = dead runner (Fig. 1).

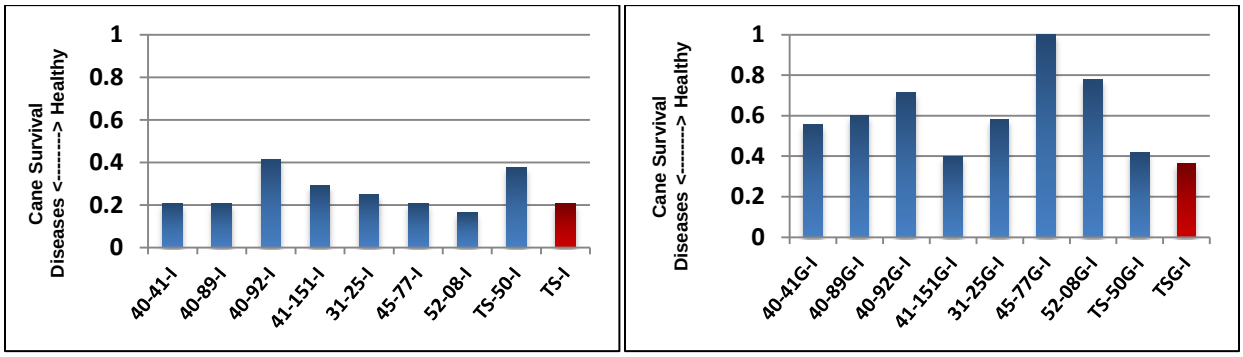

Figure 1: Solano non-grafted lines inoculated on 2011/2011 (left) and grafted lines inoculated on 2012 (right) cane survival. Non-grafted and grafted lines inoculated on 2013 have a cane survival score of 1. Spring of 2014.

Solano grapevines vigor was scored using a 0-4 scale, where 0 = healthy no PD symptoms, 1 = a few leaves on a few shoots that are symptomatic on cane(s), 2 = Many symptomatic leaves on multiple canes (in a mature bilateral cordon trained vine), 3 = dieback/death of canes/codons, and 4 = death of whole vine (Fig. 2 and 3)

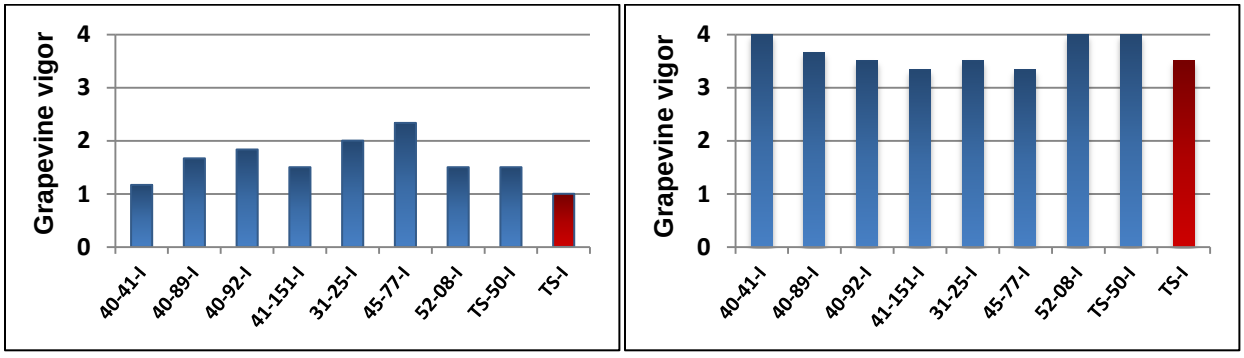

Figure 2: Solano non-grafted lines inoculated on 2011/2012 (left) and non-grafted lines inoculated on 2013 (right) grapevine vigor. Spring of 2014.

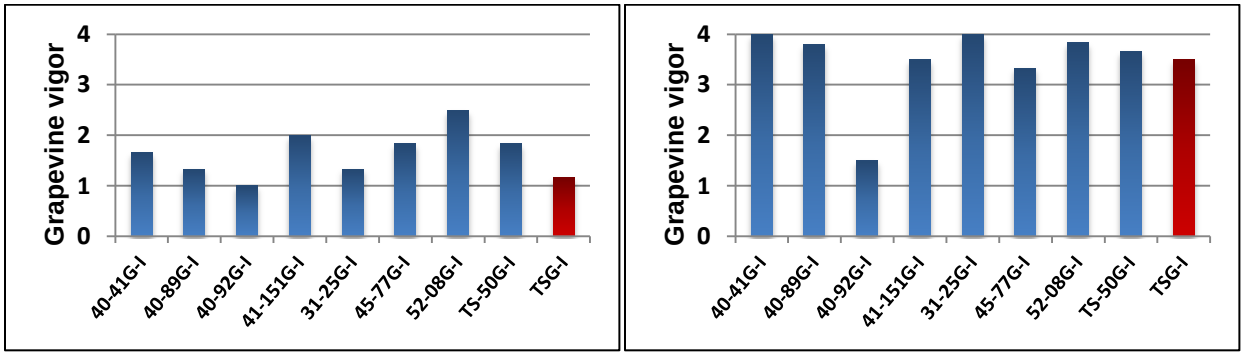

Figure 3: Solano grafted lines inoculated on 2012 (left) and grafted lines inoculated on 2013 (right) grapevine vigor. Spring of 2014.

Stem samples from runners in the Solano plot inoculated in 2011, 2012 and 2013 were harvested in Spring and Fall 2013 and number of *Xf* cells were determined using ELISA, the standard curve was created using *Xf* cells obtained from liquid culture. Pierce's disease symptoms and ELISA cell count results confirmed *Xf* infection in Solano County field (Fig. 4 and 5).

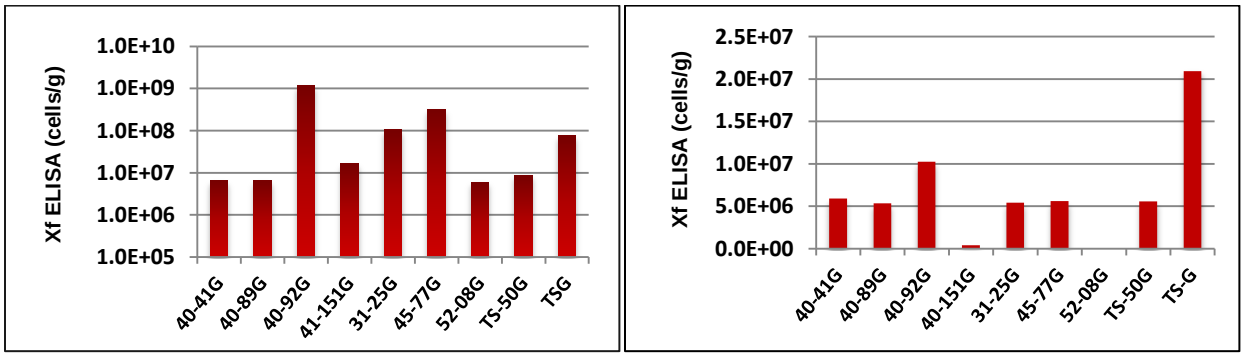

Figure 4: *Xylella fastidiosa* ELISA detection on Solano stem samples from grafted vines inoculated on 2012 (left) andgrafted lines inoculated on 2013 (right) and collected on Fall of 2013..

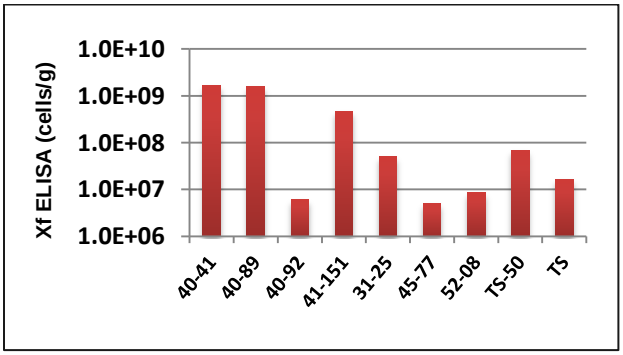

Fig 5. *Xylella fastidiosa* ELISA detection of Solano stem samples from non-grafted vines collected on Fall of 2013

On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four current year canes per grafted transgenic and control plants at the Solano field were mechanically inoculated with *Xf*. On July 22, 2014 one cane per plant was harvested for *Xf* quantification. During the summer of 2014 two set of canes will be harvest at two

different sampling dates and one more in the spring of 2015. Quantification of *Xf* will be measured by qPCR. Severity or absence of PD symptoms will be recorded at each sampling date on all inoculated canes.

Objective 2. Assume permit holder status for existing USDA-APHIS field permit 12-340-102r and maintain regulatory oversight and compliance with permit reporting requirements.

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Activity 4. Participate with PIPRA during transition and assume permit holder status.

The permit holder Prof. Alan Bennett of the current APHIS permit #12-340-102r was changed to Prof. Abhaya Dandeker in January 2014. The permit was extended by APHIS and the new end date is April 1, 2016.

Activity 5: Maintain regulatory oversight of both field locations and compliance with reporting requirements.

During the transition period that initiated on Oct.1 2013, personnel from the Dandekar's Laboratory worked closely with PIPRA personnel to obtain all of the documentation and records necessary to maintain the regulatory oversight of the field trial. This process was completed in Jan 2014 with the transfer of full responsibility coinciding with the transfer of the permit holder. We have also been working closely with UC Davis EH&S and to modify our existing BUA to include this permit a process that includes the institutional biosafety committee into the chain of custody for the regulatory oversight compliance management. The responsibility for regulatory compliance rests with the new permit holder but the university has to be brought into the loop during the transition period to maintain their oversight of campus BUAs. The issues that require regulatory oversight are listed in the permit and timely reporting and inspections have been conducted to maintain compliance with USDA APHIS.

Activity 6. Maintain active regulatory compliance inspections.

Regulatory compliance has been enforced by working closely with the two field managers and their crew, obtaining information related to regulatory compliance from the individual investigators and carrying out periodic inspection of field sites to maintain/enforce regulatory compliance. Two personnel from the Dandekar lab are entrusted with the tasks of documentation, training and inspection to ensure regulatory compliance.

<u>Summary of accomplishments and results for each objective</u>

We have successfully established two field trials to validate two greenhouse-tested strategies to control the movement and clearance of *Xylella fastidiosa* (*Xf*), a xylem-limited, Gram-negative bacterium that is the causative agent of Pierce's Disease (PD). A key virulence feature of *Xf* resides in its ability to digest pectin-rich pit pore membranes that interconnect the host plant's xylem elements, enhancing long distance movement and vector transmission. The first strategy evaluated the ability of a xylem-targeted polygalacturonase-inhibiting protein (PGIP) from pear to counter virulence associated with *Xf* PG activity. Our second strategy enhances clearance of bacteria from *Xf*-infected xylem tissues using a chimeric antimicrobial protein, NE-CB. The expectation is that expressing these proteins will prevent *Xf* movement and reduce its inoculum size, curbing the spread of PD in California vineyards.

Transgenic grapevine plants expressing either PGIP or NE-CB along with untransformed controls have been successfully planted in two locations. In Riverside County, planting was completed with 220 vines in the ground: 210 planted on 05/18/2010 with the remaining 10 planted on 03/06/2011. In Solano County, where planting was also completed with all 220 vines in the ground, 110 were planted on 08/02/2010 and the remaining 110 on 6/27/2011. These transgenic grapevines have been evaluated as plants on their own roots and as rootstocks grafted with untransformed Thompson Seedless (TS) scions. NE-CB- and PGIP-expressing transgenic grapevine lines in Riverside and Solano County have been

evaluated phenotypically using the first 12 descriptors from the "Primary descriptor priority list" proposed by the International Organization of Vine and Wine (OIV). No phenotypical/horticultural differences were observed between transgenic and untransformed TS vines. NE-CB- and PGIP-expressing transgenic grapevine lines in Solano County have also been genotyped, confirming the presence of the inserted transgene in all lines.

At the Riverside County site, the plants have been naturally infected by wild populations of GWSS and *Xf* presence in petioles extracts was confirmed by ELISA, and plate cell count in fall 2011. *Xf* presence was also confirmed in Riverside xylem sap samples collected in spring 2012, in petiole and stem extracts collected in fall 2012 and in stem extracts collected in the spring 2013. PD symptoms were earlier assessed using a standardized score based on percentage of leaf area scorching; in 2013 we used a 0-4 scale to evaluate grapevine vigor. Unexpectedly, during the 2014 spring season we observed that all of our 224 Thompson seedless vines at Riverside site were in decline; most of them had no new growth. Those that had growth did not follow any particular genotypic pattern with transgenic and controls appearing to be equally affected, in all cases the root zone was limited to the first six inches of soil and was heavily infected with root knot nematode. Four soil samples were taken and all four were positive for Root Knot. Roots taken had four knots per inch whereas normally it would be one knot/gall per four inches. Nematodes population data in soil and root samples, already submitted for analysis, will be available in the next progress report. Since the Riverside site was previously planted with Chardonnay for about 15 years and the vineyard was removed and replanted without fumigation. Our initial conclusion is that the field location had selected for an aggressive population of Root Knot nematode during the previous grapevine planting that then attacked our vines once they were planted in that same location, resulting in a lethal infection.

At the Solano County site, half of non-grafted vines were mechanically inoculated with the *Xf* type strain (Temecula 1) in 2011 and re-inoculated in spring 2012 to validate resistance to PD under field conditions, *Xf* presence was confirmed by ELISA in fall 2011, but no *Xf* growth in plate or PD symptoms were detected. Half of Solano County grafted plants were for the first time mechanically inoculated with *Xf* in 2012 and the other half in 2013. Leaf scorching the characteristic symptom of PD was observed in Solano Country for the first time in fall 2012 and *Xf* presence was confirmed by ELISA in petiole extracts collected in the same season and in stem samples from own-rooted and grafted lines collected on the spring and fall of 2013. Solano non-grafted and grafted grapevines that were not inoculated previously were manually inoculated on June 2013, completing the manual inoculation of all grapevines at Solano site. PD symptoms were earlier assessed using a standardized score based on percentage of leaf area scorching; now we are using a 0-1 scale to evaluate cane survival and a 0-4 scale to evaluate grapevine vigor. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four current year canes per grafted transgenic and control plants at the Solano field were mechanically inoculated with *Xf*. On July 22, 2014 one cane per plant was harvested for *Xf* quantification. During the summer of 2014 two more set of canes will be harvest at two different sampling dates and one more in the spring of 2015. Quantification of *Xf* will be measured by qPCR. Severity or absence of PD symptoms will be recorded at each sampling date on all inoculated canes.

The permit holder of the current APHIS permit #12-340-102r was transferred from Prof A. Bennett to Prof. A. Dandekar in January 2014. The permit was extended by APHIS and the new end date is April 1, 2016. During the transition period personnel from the Dandekar Laboratory worked closely with PIPRA personnel to obtain all of the documentation and records necessary to maintain the regulatory oversight of the field trial. The issues that require regulatory oversight are listed in the permit and timely reporting and inspections have been conducted to maintain compliance with USDA-APHIS.

For field trials, please include information on the status of the field trial, including planting and sampling activities, the condition of the plants, and any factors impacting the progress of the field trial. Also, please include photos of the field planting.

Riverside and Solano fields planting were completed on 03/06/2011 and 6/27/11, respectively. After each of the fields were planted completely no additional planting activities have been made. At Riverside field petioles and leaves were sampled, on 9/26/2011 and 10/17/2012, xylem sap was sampled on 4/2/2012 and stems samples were sampled on 5/10/13. Samples were not collected at Riverside field during 2014 due to decline all transgenic and control grapevines. At Solano Field petioles and leaves were sampled on 9/14/2011, 10/4/2011 and 10/22/2012, xylem sap was sampled on 4/30/2012 and stems were sampled on 4/26/13, 9/6/2013 11/5/13 and 7/22/14.

Figure 6.**Transgenic grapevines Riverside County (left) and Solano County (right) fields, Spring 2014.**

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

This research aims to provide a transgenic solution to the Pierce's Disease problem for the grower community of California. The objectives described in this proposal directly address the number 1 RSAP priority outlined in the, "Accelerate regulatory process". Establish and facilitate field trials of current PD control candidate vines / endophytes / compounds in multiple locations" handout released in the December 2009 Pierce's Disease Research symposium that outline the "Top 5 to 10 Project Objectives to Accelerate Research to Practice". This document updates the priority research recommendations provided in the report "PD/GWSS Research Scientific Review: Final Report" released in August 2007 by the CDFA's Pierce's Disease Research Scientific Advisory Panel.

Layperson summary of project accomplishments.

Four hundred and forty (440) transgenic grapevine plants expressing either polygalacturonase-inhibiting protein (PGIP; 192 plants) or a chimeric antimicrobial protein (NE-CB; 192) and 56 control untransformed vines have been planted in two locations, one in Riverside County (220 plants) and the other in Solano County (220 plants). Exactly half of these transgenic grapevines are being evaluated as plants on their own roots and as rootstocks grafted with untransformed Thompson Seedless (TS) scions to demonstrate the field efficacy of two strategies to control Pierce's Disease (PD) in California grapevines. The first strategy uses transgenic rootstocks to control the movement of the bacterium *Xylella fastidiosa (Xf)* in the water-conducting xylem of the vine through expression of PGIP. The second strategy tests whether transgenic rootstocks can clear *Xf* infections in xylem tissue by expressing NE-CB. At the Riverside County site, natural *Xf* infection has been confirmed in petioles, stem and xylem sap by ELISA and appeared to be uniform. Unexpectedly, during the 2014 spring season we observed that all of our 224 Thompson seedless vines at Riverside site were in decline; most of them had no new growth. Those that

had growth did not follow any particular genotypic pattern with transgenic and controls appearing to be equally affected, in all cases the root zone was limited to the first six inches of soil and was heavily infected with root knot nematode. Four soil samples were taken and all four were positive for Root Knot. Roots taken had four knots per inch whereas normally it would be one knot/gall per four inches. Nematodes population data in soil and root samples, already submitted for analysis, will be available in the next progress report. Since the Riverside site was previously planted with Chardonnay for about 15 years and the vineyard was removed and replanted without fumigation. Our initial conclusion is that the field location had selected for an aggressive population of Root Knot nematode during the previous grapevine planting that then attacked our vines once they were planted in that same location, resulting in a lethal infection. At the Solano County site, about 25% of the plants were mechanically inoculated in 2011 and re-inoculated in 2012, another 25% in 2012 and the remainder 50% in 2013. The presence of *Xf* was confirmed in petiole and stem extracts of grapevines using the ELISA assay. We have used various phenotyping scoring techniques and while these are not perfect we can clearly see lines that consistently score better than the control for both strategies and those that do not. We also observe that those lines that show resistance are also able to transmit their resistance from the rootstock. However, the resistance transmitted from the rootstock is weaker when compared to that obtained with a transformed plant. On May 28, 2014, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four current year canes per grafted transgenic and control plants at the Solano Field were mechanically inoculated with *Xf*. On July 22, 2014 one cane per plant was harvested for *Xf* quantification. During the summer of 2014 two more set of canes will be harvest at two different sampling dates and one more in the spring of 2015. Quantification of *Xf* will be measured by qPCR. Severity or absence of PD symptoms will be recorded at each sampling date on all inoculated canes.

The permit holder Prof. Alan Bennett of the current Solano and Riverside field permit #12-340-102r was changed to Prof. Abhaya Dandekar in January 2014. The permit was extended by APHIS and the new end date is April 1, 2016. During the transition period personnel from the Dandekar's Laboratory worked closely with PIPRA personnel to obtain all of the documentation and records necessary to maintain the regulatory oversight of the field trial. The issues that require regulatory oversight are listed in the permit and timely reporting and inspections has been conducted to maintain compliance with USDA-APHIS.

Status of funds.

We have expended all funds from July 2013 to June 2014 and have begun to expending the July 2014 through June 2015 funding..

Summary and status of intellectual property associated with the project.

The intellectual properties issues connected with the specific constructs and approach have not been investigated in any formal investigation. However, this needs to be done when the elite lines are identified and need to be patent protected. Disclosures will be made at that point to the UC innovation Access, which could develop these further as a US patent variety.

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