IDENTIFICATION OF FACTORS MEDIATING COLD THERAPY OF *XYLELLA FASTIDIOSA* INFECTED GRAPEVINES.

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ABSTRACT
Pierce's disease (PD) is currently found in many regions of California and the southeastern United States. One factor that has been shown to be associated with the observed limited geographical distribution of PD in North America is the severity of winter temperatures in those regions. For example, PD does not occur in New York, the Pacific Northwest or at high altitudes in South Carolina, Texas and California where the winter temperatures on average drop below zero degrees Celsius (Hopkins & Purcell, 2002). Purcell (1977, 1980) and Feil's (2002) research suggested that some factor(s) expressed in the intact plants helps eliminate *Xyllela fastidiosa* (*Xf)* from grapevines.

To elucidate the mechanism(s) of the "cold curing" phenomenon, it is necessary to determine the cold curing temperature threshold that maximizes PD recovery and minimizes vine mortality. This research should also allow us to generate projection maps to determine if vineyards in cold boundary areas (i.e., foothills of the Sierra and northern-most California) are at risk for developing PD. The information obtained from these experiments will facilitate basic research on mechanisms causing cold therapy and provide data that could be used by grape growers for risk assessment and management purposes. It has been well documented that xylem sap contains many metabolites such as mono- and disaccharides, organic acids, plant growth regulators and other organic compounds (Andersen et al., 1989; Bollard, 1960; Pate, 1976; Wormall, 1924; reviewed by Seyedbagheri & Fallahi, 1994). Though it is well known that these compounds are in the sap, little is known about the effects of cold temperatures on the synthesis or quantity of these compounds in sap. Also, little is known about the effect of cold temperatures on factors such as pH and osmolarity of xylem sap and how these factors may be contributing to the cold curing phenomenon. Assessing the effect of pH and osmolarity on the viability of *Xf* cells *in vitro*, could provide insight into the factors that contribute to the cold curing phenomenon.

Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as abscisic acid (ABA), which induce the synthesis of a number of cold shock proteins (Guy, 1990; Bravo, et al., 1998; Thomashow, 1998). Kuwabara et al. (2002) elicited cold-shock proteins at 23°C in winter wheat using an exogenously applied 100ppm ABA solution. The ABA treated plants elicited the synthesis of proteins that inhibited the *in vitro* growth of a wheat fungal pathogen. Although nothing is known about the effects of these cold-induced proteins on the growth of *Xf*, if they were antagonistic the application of ABA could lead to a potentially novel approach for managing Pierce's disease.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-limited, gram-negative bacterium that causes Pierce's disease (PD) in grapevines. The *Xf* strains that cause PD in grapevines also cause alfalfa stunt and almond leaf scorch, while other strains of *Xf* cause citrus variegated chlorosis, oleander leaf scorch, phony peach, and several other diseases (Purcell, 1997). Little is known about host specificity of strains or the mechanisms by which *Xf* causes plant disease (Purcell & Hopkins, 1996). Symptoms of this "mysterious disease" were first described by Newton Pierce in 1882. Today, typical symptoms of PD in grapevines include leaf margin necrosis, leaf blade drop, irregular lignification of canes, "raisining" of fruit clusters, dieback and death of grapevines (Hopkins & Purcell, 2002; Varela, et al., 2003).

PD is currently found in many regions of California and the southern United States. One factor that has been shown to be associated with the observed limited geographical distribution of PD in North America is the severity of winter temperatures in those regions. For example, PD does not occur in New York, the Pacific Northwest or at high altitudes in South Carolina, Texas and California where the winter temperatures on average drop below zero degrees Celsius (Hopkins & Purcell, 2002). Purcell (1977, 1980) demonstrated that relatively brief exposures to sub-freezing temperatures eliminated *Xf* in cold treated *Vitis vinifera* grapevines. Purcell also found that moderately susceptible 'Cabernet Sauvignon' had a higher curing rate following cold treatment compared to the PD-susceptible variety 'Pinot Noir'. More recently, Purcell's group also showed that whole, *Xf* infected potted vines that were exposed to low temperatures had a higher rate of recovery than PD-affected detached bud sticks exposed to the same cold temperatures (Feil, 2002). This implies that some factor(s) expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants.
Despite documentation of the cold curing phenomenon, little is known about the physiological/biochemical basis that mediates cold therapy. To further understand the basis of this phenomenon, we are conducting several studies to identify the

physiological/biochemical factor(s) that occur or are expressed in cold treated vines that contribute to the elimination of *Xf*. If such a factor(s) is/are found, it may be possible to induce their expression under non-freezing temperatures and potentially provide a novel approach for managing PD.

OBJECTIVES

1. Develop an experimental, growth chamber temperature regime that can consistently cure Pierce's disease affected grapevines without causing unacceptable plant mortality.
2. Analyze chemical changes such as pH, osmolarity, total organic acids, proteins and other metabolites that occur in the xylem sap of cold-treated versus non-treated susceptible and less susceptible *Vitis vinifera* varieties.
3. Assess the viability of cultured *Xf* cells growing in media with varying pH and osmolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with cold-induced plant growth regulators, such as abscisic acid (ABA), as a possible therapy for PD.

RESULTS AND CONCLUSIONS

Objective 1: The results described in previous reports show that our field plots and cold chamber plants showed lower disease ratings and higher curing rates in the colder temperature treatments. In 2005-2007 sites, vine mortality was minimal due to better cold acclimation of the grapevines prior to establishing the plots in the fall.

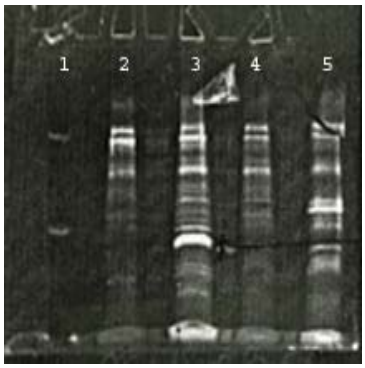
The data collection for the field and cold chamber studies is complete and analysis of the data to determine the critical temperature thresholds for inducing the cold curing phenomenon is underway. We are continuing to work with Len Coop from the University of Oregon to generate a cold temperature model to determine if vineyards in cold boundary areas (i.e., foothills of the Sierra and northern-most California) are at risk for developing PD. The information obtained from these models could provide data that could be used by grape growers for risk assessment and management purposes.

Objective 2: Xylem sap was extracted from vines from each field location and cold chamber treatment using a pressure bomb. The samples were then tested for potential changes in pH, osmolarity, protein profiles, total sugars, and calcium and magnesium concentrations in xylem sap. The results for the 2005-2006 field and cold chamber grapevine xylem sap pH, osmolarity, and calcium and magnesium concentrations can be found in previous reports. The 2006-2007 xylem sap samples were analyzed for sugars, calcium and magnesium levels. The results of these analyses will be reported in our 2008 Pierce's Disease Meeting poster in December once the statistical analyses are completed.

Xylem sap protein profiles were analyzed for the 2005-2007 samples. The sap proteins were concentrated by acetone precipitation and the proteins were electrophoresed in a 12% Tris-HCl 1-dimensional polyacrylamide gel (PAGE). Protein profiles of the PAGE gels were compared for each treatment. Unique protein bands that were found in the cold treated plants were cut from the gel, and end terminally sequenced by the UCD Molecular Structure Facility. Some of the constitutively expressing xylem sap proteins were also sequenced to determine their identity.

Cold Chamber Experiment Results:

The results reported in previous reports show that the pH of Cabernet Sauvignon (CS) xylem sap was significantly higher than Pinot Noir (PN) sap overall. Sugar and select ion concentration analysis of CS grapevines showed greater amounts of glucose and fructose in –5ºC cold chamber vines, whereas Ca^{+} levels were greater in the warmest treatments. Osmolarity was greatest in the coldest treatments and decreased with increasing temperature. Conversely, in PN grapevines, glucose and fructose levels were the lowest in the coldest treatments. Ca^{+} levels showed a similar trend with CS vines, with increased Ca^{+} levels in the warmer temperature treatments. Temperature appeared to have a less direct effect on osmolarity in Pinot Noir grapevines. ABA concentrations in the spring xylem sap collections were the lowest in the coldest cold chambers.
Protein profiles of grapevine xylem sap exposed to various temperatures were determined by PAGE (**Figure 1**). Most of the proteins were similar for the various temperatures, but a few unique proteins were found in the cold stressed and/or *Xf*-inoculated plants and these proteins were end terminally sequenced by the UCD Molecular Structure Facility. Sequencing of xylem proteins from cold-treated vines identified proteins that had high sequence homology with stress proteins that are produced by Cabernet Sauvignon berries under water deficit stress conditions, proteins that are similar to proteins produced in Pinot Noir roots, tryptase inhibitors and a thaumatin-like protein which is reported to have anti-fungal properties.



Lane 1: Dual Color SDS-ladder.
Lane 2: CS +5ºC xylem sap from control vines.
Lane 3: CS -5ºC xylem sap from control vines.
Lane 4: CS +5ºC xylem sap from inoculated vines.
Lane 5: CS -5ºC xylem sap from inoculated vines.

Figure 1. Protein profiles of grapevine xylem sap as determined by PAGE analysis. 150 uL of xylem sap was precipitated with cold acetone. Proteins were suspended in 30 uL of SDS-loading buffer and electrophoresed in a 12% Tris-HCl polyacrylamide gel.

Field Experiment Results:
ABA concentrations in the spring xylem sap collections were the lowest in the coldest field locations. ABA levels were higher in the late winter sap collections than in the spring collections for the field locations. Osmolarity, pH, calcium and magnesium levels show similar trends to those seen in the cold chamber experiments.

Objective 3: The solutions used for these viability experiments included: water, extracted *V. vinifera* ('Pinot Noir' and 'Cabernet Sauvignon' varieties) xylem sap, PD3, HEPES, sodium and potassium phosphate buffers. All buffers and media were adjusted to pH 6.8. *Xf* cells suspended in the various buffers and media were exposed to various temperatures (28ºC, 5ºC, 2.2ºC, 0ºC, -5ºC, -10ºC and -20ºC). Potassium phosphate buffer at various pH values (5.0-6.8) was also used to determine the effects of pH on the survival of *Xf*. 10^4 *Xf* cells were suspended in 1ml of the various test solutions which were then incubated at various temperatures. Aliquots of the suspensions were plated on PD3 medium and *Xf* CFUs were counted seven days post plating.

The results of these experiments were reported in detail in the 2007 progress report. To summarize the results, these experiments indicate that *Xf* can survive at 28ºC in most media except water. The mortality rate was the lowest in PD3 medium in the 5˚C and 2.2˚C temperature treatment. The deionized water treatment had the highest mortality rate followed by potassium phosphate at pH 6.2. The highest survival at 0˚C occurred with PD3 media and in xylem sap collected from grapevines growing in a cold climate (Placer County, CA). These experiments showed that *Xf* can survive at -5ºC in all buffers at pH 6.8, media and xylem sap for at least four days. No cultivable *Xf* was recovered from any of the media, buffers or xylem sap after 24 hours at -10˚C or at -20˚C.

Objective 4: To assess the possibility of using a plant hormone to artificially induce cold curing, we contacted Valent Bioscience Corporation who has an active research and development program on the use of ABA on agricultural crops. In November of 2005, 2006, and 2007, healthy and *Xf*-inoculated Cabernet Sauvignon and Pinot Noir vines grown and inoculated with *Xf* as described in Objective 1 were foliar sprayed or soil drenched with solutions of ABA in the fall. The 2005-2006 results showed interesting trends and were repeated in the 2006-2007and 2007-2008 seasons.
Our applications of ABA in the 2005-2006 season appeared to have a curing effect in PD-infected grapevines. ABA application that was the most effective was VBC-30030 applied as a drench, but some of the other forms and concentrations of ABA also had some curing effect. For this first application in 2005-2006, there was no rain until a week following the application. In 2006-2007, this experiment was replicated with some modifications to the treatments used in 2005-2006. The resulting curing rates were not the same as in the 2005-2006 treatments. The only treatment that seemed to have more curing than the control treatment was the VBC-30030 drench in Pinot Noir grapevines, but the curing rate was not as high as in the 2005-2006 season. This difference could possibly be due to a rain event that occurred a few hours after the ABA application, possibly diluting, washing off, or leaching out the applied ABA.

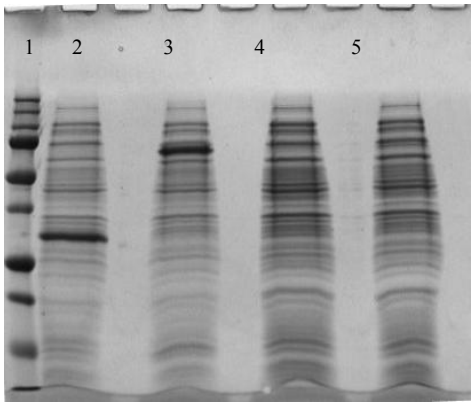
To evaluate the reproducibility of the 2005-2006 results, a third ABA trial was conducted in the 2007-2008 season. In 2007, there were four treatments with *Xf*- infected vines and healthy controls. The curing rates for the various treatments are currently being evaluated by IC-PCR.

To examine the mechanism behind the possible curing due to ABA application, the xylem sap of the grapevines was extracted using a pressure bomb four days after the application of the ABA treatments. To examine the proteins produced when grapevines are exposed to ABA, protein profiles were made of each treatment. The 150 ul of xylem sap was precipitated with cold acetone to concentrate the proteins. The proteins were resuspended in 30 uL of SDS-loading buffer and electrophoresed in a BioRad 12% Tris-HCl gel. Some of the proteins that were sequenced in the ABA treated vines were similar to those found in our cold treated vines.

2008-2009:
To evaluate the reproducibility of the 2006-2007 Chardonnay results a third ABA spray trial is being prepared for the 2008-2009 season. In the fall, treatments will be applied to healthy and *Xf*- infected Chardonnay vines as follows:

Control:	16 Chardonnay plants sprayed with water
2000 ppm spray:	16 Chardonnay plants sprayed with VBC-30054
100 ppm spray:	16 Chardonnay plants sprayed with VBC-30030
100 ppm drench:	16 Chardonnay plants drenched with VBC-30054
10 ppm drench:	16 Chardonnay plants drenched with VBC-30030

Our first unique cold expressed protein found in Objective 2 has been cloned and expressed (**Figure 2**). Expressed and purified xylem sap proteins will be used to determine if the proteins demonstrate any anti-*Xf* activity *in vitro*. If anti- *Xf* activity is shown, future work would focus on expressing the anti-*Xf* proteins in transgenic rootstocks as a possible Pierce's disease control method.



1. Molecular size markers.
2. Cloned and expressed Cabernet TLP protein.
3. Cloned and expressed PG protein (positive control).
4. Cloned, non-induced Cabernet TLP protein.
5. Cloned, non-induced PG protein.

Figure 2. Cloning and expression of an *Xf* polygalacturonase and *Vitis vinifera* thaumatin-like protein in *E. coli*.

CONCLUSIONS
The results of our field and cold chamber experiments show lower disease ratings and higher curing rates in the colder temperature treatments. The coldest treatments had the highest rate of recovery from PD, but also the highest grapevine mortality. These findings will be used to determine a Pierce's disease Risk Assessment Model based on curing rates and winter temperatures.

Analysis of the biochemical factors in sap revealed some interesting results. For the cold chamber experiments, the pH of CS xylem sap was significantly higher than PN sap overall. Sugar and select ion concentration analysis of CS grapevines showed greater amounts of glucose and fructose in –5ºC cold chamber vines, whereas Ca^{+} levels were greater in the warmest treatments. Osmolarity was greatest in the coldest treatments and decreased with increasing temperature. Conversely, in PN grapevines, glucose and fructose levels were the lowest in the coldest treatments. Interestingly, the osmolarity of PD3 media is 113 mmol/kg, whereas the osmolarity of xylem sap was 25-45 mmol/kg. Ca^{+} levels showed a similar trend with CS vines, with increased Ca^{+} levels in the warmer temperature treatments. Temperature appeared to have a less direct effect on osmolarity in Pinot Noir grapevines.

ABA concentrations in the spring xylem sap collections were the lowest in the coldest field locations and coldest cold chambers. ABA levels were higher in the late winter sap collections than in the spring collections for the field locations. PAGE protein profile analysis showed that most of the proteins we found were similar for the various temperatures, but a few unique proteins were found in the cold stressed and/or *Xf*-inoculated plants. Sequencing results of xylem proteins from cold-treated vines showed proteins that are similar to stress proteins that are produced by Cabernet Sauvignon berries developing under water deficit stress conditions, proteins that are similar to proteins produced in Pinot Noir roots, and tryptase inhibitors. One of the proteins that was expressed at comparatively high concentrations in cold-exposed vines is a thaumatin-like protein which has been reported to have anti-microbial activity. We will assess the potential anti-*Xf* properties of this protein by cloning, expressing, purifying and using this protein in *Xf* growth inhibition assays in the future.
The *in vitro* culture experiments indicate that *Xf* can survive at 28ºC in most media except water. At 28˚C, the survival rate was the highest in PD3 media followed by potassium phosphate at pH 6.8, sodium phosphate, and xylem sap. At the coldest temperatures, the highest survival at 0˚C occurred with PD3 media and in xylem sap collected from grapevines growing in a cold climate (Placer County, CA), whereas survival was the lowest in deionized water and potassium phosphate at pH 6.2. Interestingly, *Xf* can survive at -5ºC in all buffers at pH 6.8, media and xylem sap for at least four days. No cultivable *Xf* was

recovered from any of the media, buffers or xylem sap after 24 hours at -10˚C or at -20˚C. *Xf* in potassium phosphate buffers with pH values at 5.0, 5.4 and 5.8 died rapidly at all temperatures.

The results the ABA application experiments in the 2005-2006 season indicate that ABA appears to have a curing effect when applied as a drench. The synthetic ABA had the most interesting result with 100% curing in Pinot Noir vines. Disease ratings for both ABA drench treatments were significantly less than untreated controls.

In 2006-2007 this experiment was replicated with some modifications to the 2005-2006 treatments. Curing rates were not as high as what we saw in the 2005-2006 treatments. The only treatment that seemed to have more curing than the control treatment was the VBC-30030 drench in Pinot Noir grapevines. The difference observed in the 2006-2007 ABA application could possibly be due to a rain event that occurred a few hours after the ABA application, possibly diluting, washing off, or leaching out the applied ABA. We are repeating the ABA experiment this season to determine if ABA applications could be used as a possible tool in the management of Pierce's disease.

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