IDENTIFICATION OF MECHANISMS MEDIATING COLD THERAPY OF *XYLELLA FASTIDIOSA*-INFECTED GRAPEVINES

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ABSTRACT
We are attempting to characterize the biochemical factors that mediate the cold curing phenomenon of grapevines infected with *Xylella fastidiosa (Xf)*. We are working towards completing project objectives that were initiated in July of 2004. To better understand the cold therapy phenomenon, we examined Pierce's Disease (PD) disease severity, curing rates and biochemical changes in control and *Xf* infected Pinot Noir and Cabernet Sauvignon grapevines grown in four locations in Northern California and four cold chamber temperatures. After the cold treatments, xylem sap was extracted using a pressure bomb and the sap analyzed for pH, osmolarity, abscissic acid (ABA), glucose, sucrose, fructose, calcium and magnesium ion concentrations. Differences between varieties and between temperature treatments were observed. In the field and cold chamber experiments, pH and osmolarity of xylem sap from cold treated vines was lower than what is found in PD3 culture medium used to grow *Xf*. PD severity was lowest and curing rates were highest for the coldest temperatures.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-limited, gram-negative bacterium that causes (PD) in grapevines. 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 0 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 a higher percentage of grapevines that were moderately susceptible to PD such as 'Cabernet Sauvignon' (CS), were cured by cold therapy treatments compared to susceptible varieties such as 'Pinot Noir' (PN). More recently, Purcell's group also showed that whole, *Xf* infected potted vines 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. Our objective is to elucidate the physiological/biochemical basis that mediates cold therapy and to identify the physiological/biochemical factor(s) that occur or are expressed in cold treated vines that eliminate *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 unacceptable plant mortality.
2. Analyze chemical changes such as pH, osomolarity, total organic acids, proteins and other constituents that occur in 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 osomolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with plant growth regulators, such as abscisic acid (ABA), as a possible therapy for PD.

Objective 1
All experimental work used PN (PD-susceptible) and CS (moderately resistant to PD) grapevines, the same varieties used by Purcell (1977, 1980) and Feil (2002) in their previous cold therapy studies.

Temperatures inside of the growth chambers were recorded using HOBO data loggers (Onset Computer Co. Borne, MA). In addition, temperature data is also being monitored by sensitive chart recorders on the cold chambers and by adjacent weather stations at all of our field plots. In the -5C cold chamber, custom thermocouplers were made and inserted into the xylem of 10 grapevines to determine if there were differences between the ambient chamber temperature and the internal xylem temperature. No significant differences in xylem temperature were detected.

To evaluate the cold curing phenomenon under different field temperatures we established the following experiment. Twenty-two vines of Cabernet Sauvignon and twenty-two vines of Pinot Noir for each of the four sites (176 vines total), were grown during the spring and summer in five gallon pots at UC Davis. One half of the vines were mechanically inoculated with *Xf* in June, the other half were mock inoculated with water for comparison and proper statistical analysis. In 2005-2006 (1st replicate) and 2006-2007 (2nd replicate), the vines were transported to the four field sites (in order of warmest site to coldest site), UC Davis (Yolo County), UC Hopland Research Station (Mendocino County), McLaughlin Reserve (Lake County) and Foresthill (Placer County) with the onset of cool fall temperatures in early November. In the 2005-2006 season, dormant canes were collected from the vines in February and April, xylem sap was extracted using a custom-made pressure bomb, and the expressed sap was subjected to the tests described in the objectives below. The vines were returned to UCD in the spring and planted in the field. The vines were then rated for PD symptoms and the presence of *Xf* in the fall. (The 2005-2006 results can be found in the 2006 Pierce's Disease Symposium Proceedings). Disease ratings were the lowest and percent curing was the highest in the coldest field locations (Table 1).

Grapevines, using the same varieties and inoculated as described for the field studies but grown in six" standard pots, were exposed to different temperature regimes in cold rooms located at the Department of Pomology, UC Davis during the winter months. Plants were subjected to one of four temperature regimes.

Regime 1: -5°C Regime 2: +0°C
Regime 3: +2.2°C Regime 4: +5°C

There were 40 plants per treatment regime, 10 *Xf*-inoculated plants and 10 control plants for both varieties (160 plants total). After three months of treatment, xylem sap was extracted from the plants, and the vines were planted in the Plant Pathology field at UC Davis. Late in the summer, the plants were evaluated for PD symptoms to determine the most effective temperature regime for curing PD without causing unacceptable plant mortality. Supporting the findings of the field study, disease ratings were the lowest and percent curing was the highest in the coldest cold room temperature regimes for both replicates (Table 1).

Table 1. PD-cured plants compared to the number of *Xf*-inoculated grapevines that survived until spring after winter chilling at the field locations or after spending 3 months in the cold chambers.

	Field Locations				Cold Room			
	Davis	Hopland	McLaughlin	Foresthill	+5C	+2.2C	0C	-5C
Pinot Noir	3/11	9/11	8/11	8/11	3/9	3/10	4/10	6/6
Cabernet Sauvignon	1/11	8/11	7/11	10/11	0/9	4/10	5/10	7/7

Objective 2

As described in the 2005 and 2006 PD Symposium Reports, we conducted similar analyses on canes collected from our field sites as well as the vines that were placed at various cold temperatures in growth chambers. Each potted vine was sampled in February and in April when the potted vines were returned and planted at UCD. The volumes of xylem sap that were expressed from individual canes from the potted 1-year old vines used in the field and growth chamber experiments were small, ranging from 1 to 200ul/cane. Our hypothesis is that changes in the pH and osomolarity of xylem sap in vines that undergo cold treatment may have significant effects on *Xf* viability. The results of this study show that the pH of xylem sap from both cold chamber and field cold treated vines is lower than culture media used to grow *Xf* (Table 2). Osmolarity of PD3 media is 113 mmol/kg in comparison to the osmolarity of xylem sap, 25-45 mmol/kg. For the cold chamber experiments the pH of CS xylem sap was significantly higher than PN sap overall (Table 3). Sugar and select ion concentration analysis of CS grapevines showed greater amounts of glucose and fructose in –5C 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.

We also determined the relative water content of canes from cold-stressed and UC Davis vines by measuring the fresh weight of the canes, dehydrating them in an oven, and measuring the dry weight. Freezing temperatures are known to dehydrate plant tissue and this dehydration could affect the ability of *Xf* to overwinter in xylem tissue. No significant differences between fresh and dry weights were found.

Previous research on a number of plant species has shown that several plant genes are expressed in response to freezing temperatures (reviewed by Thomashow, 1998), and in some plants these low temperature-induced proteins are structurally

related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related (PR) proteins (Kuwabara, et al, 2002). Thus it may be possible that cold-stressed grapevines could produce proteins that are deleterious to *Xf*. To investigate this possibility, xylem sap was extracted from healthy and *Xf*-inoculated, cold-stressed and control CS and PN vines using the pressure bomb, proteins were concentrated by cold-acetone precipitation, and protein profiles determined by 1-dimensional polyacrylamide gel electrophoresis (PAGE). We found that good PAGE profiles were obtained by concentrating 150ul of sap to 30ul of sample each sample. Thus we are able to have multiple PAGE profiles from any particular temperature/variety/disease that can be compared to healthy control vine sap. While most of the proteins were similar for the various temperatures, 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. The potential effect of these unique proteins on *Xf* viability will be assessed in Objective 3.

Table 2. pH of grapevine xylem sap collected from cold chamber treated vines.

pH	+5 C	+2.2 C	0 C	-5 C
Pinot Noir	5.94	5.45	5.78	6.00
Cabernet Sauvignon	6.31	5.84	5.86	5.78

Table 3. Osmolarity of grapevine xylem sap collected from cold chamber treated vines.

Osmolarity	+5 C	+2.2 C	0 C	-5 C
Pinot Noir	85.0	39.5	52.7	34.7
Cabernet Sauvignon	61.9	39.5	44.8	31.6

Objective 3

In this objective we assessed the effect of many of the physical, physiological and biochemical parameters we determined in Objectives 1 and 2 on *Xf* viability *in vitro*. In 2004-2005 we assessed the effect of various buffers on the viability of *Xf* cells *in vitro* using media such as PD3 and various buffers such as sodium phosphate and potassium phosphate. *Xf* cells were placed in potassium phosphate buffer with the pH of: 5.0, 5.4, 5.8, 6.2, 6.6 and 6.8 to assess the effect of pH on the survival of *Xf*. Viability was assessed by plating the exposed cells on PD3 medium. Results of these studies are presented in the 2004, 2005 and the 2006 PD Research Symposium Reports Interestingly, the osmolarity of PD3, a common media used for growing *Xf,* is approximately 113mmol/kg, whereas the osmolarity of dormant xylem sap averages between 25-60 mmol/kg. This suggests that *Xf* is able to survive at various osmolarities.

Objective 4

Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as ABA, that induces the synthesis of a number of cold shock proteins (Bravo, et al., 1998; Thomashow, 1998;). Some of these cold-shock proteins have been shown to inhibit the growth of certain fungal pathogens (Kuwabara, et. al 2002), but no work has been done on their effect on bacterial pathogens. These same cold-shock genes can be induced under non-freezing temperatures by the exogenous application of ABA (Kuwabara, et al. 2002).

We determined the concentration of ABA in cold-stressed and control vines from the four field sites and the cold room experiments. ABA concentration was determined using an immunological assay (Phytodetek ABA Test Kit, Agdia) that has a sensitivity approximately 0.0064-0.16 picomoles ABA/ml and only requires a small volume of sap. We found that ABA concentrations in the April xylem sap collections were the lowest in the coldest field locations. ABA levels were higher in the February sap collections than in the April collections for the field locations. ABA concentrations in the spring xylem sap collections were the lowest in the coldest cold chambers.

In 2005 CS and PN grapevines were grown in one-gallon pots in the screen house to determine the effect of applying exogenous ABA on the development and/or severity of PD. At the suggestion of Sue Abrams, an ABA expert at Plant Biotechnology Institute, National Research Council Canada, we contacted Valent Bioscience Corporation who has an active research and development program on the use of ABA on agricultural crops. We met Valent representatives at UCD and described our proposed research. They were interested in the project and agree to provide us with two types of ABA, one of which (VBC-30030) is a proprietary material. One set of treatments used regular ABA (VBC-30054) as a spray (1000ppm) and a soil drench (100ppm). The second material (VBC-30030) is an ABA analog that persists longer (1 week verses 1 day) and is more active than VCB 30054. VBC-30030 was used as a spray (100ppm) and soil drench (10ppm). There were 16 healthy and 16 *Xf* -infected Cabernet Sauvignon and 16 healthy and 16 *Xf*-inoculated Pinot Noir vines used in each of the treatments plus a set used as untreated controls.

For the 2006-2007 season, vines were prepared as described above and were subjected to slightly different applications. One of the treatments used VBC-30054 as a spray at 2000ppm at one week intervals for 3 weeks. The other treatments were the same as described for the 2005-2006 season. VBC-30054 was applied as a drench at 100ppm. VBC-30030 was applied as a

drench at 10ppm. A set of plants of unsprayed plants are being used as the untreated control to allow for meaningful comparisons.

Vines were inoculated with *Xf* in June using a standard pinprick inoculation method (Hill and Purcell, 2000) and the presence of *Xf* infection confirmed by IC-PCR later in the summer. ABA treatments were applied in November when the vines still had leaves but ambient temperatures were cooling off. The pH, osmolarity, and proteins profiles of xylem sap extracted from the treatments will be determined as described above in Objective 2. Unique proteins expressed in ABA-treated vines will be removed from the gels and end terminally sequenced as previously described. PD symptoms will be rated in late summer and *Xf* infection, or lack thereof, will be confirmed by IC-PCR.

CONCLUSIONS

This study begins to document some of the biochemical/physiological changes that occur within control and *Xf*-inoculated grapevines that are exposed to various cold temperatures and attempts to better understand the cold curing phenomenon.

This study has documented that some of the temperatures examined in this study are able to induce cold curing of Pierce's disease-infected grapevines and cause significant changes in the chemistry of the xylem sap. Further studies could potentially utilize the associations between biochemical changes documented here and PD-curing to induce their expression under non-freezing temperatures and provide a novel approach for managing Pierce's disease.

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