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

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ABSTRACT
Pierce's disease does not occur in colder regions of North America. Although the "cold curing" phenomenon has been well documented, little is known about the physiological mechanisms that mediate cold therapy. To better understand the cold therapy phenomenon, we planted control and *Xylella fastidiosa (Xf*) infected *Vitis vinifera* "Pinot Noir" (PN) and "Cabernet Sauvignon" (CS) grapevines across 4 locations in Northern California and exposed control and infected grapevines across 4 temperature conditions in cold rooms. After treatment periods, xylem sap was extracted using a pressure bomb and the composition of the sap was analyzed for pH, osmolarity, glucose, sucrose, fructose, Ca^{2+}, and Mg^{2+}. Differences were found across the different field locations and cold rooms. Similar results for CS inoculated vines were found for the cold room experiments. Results for the PN field plots revealed differences in pH for both inoculated and control vines across the field plot locations. Osmolarity values of PN control vines were different across the field plot locations and in the cold room treatments.

Effects of buffer and xylem sap on the survival of *Xf* and various cold temperatures were reported in the 2004 PD/GWSS Proceedings. Abscisic acid (ABA) levels are elevated in many cold-treated plants and ABA has been shown to induce the synthesis of certain pathogenesis related (PR) proteins that in some case possess anti-fungal properties. However, we are proceeding with experiments to determine if exogenous applications of ABA on non-chilled grapevines can elicit PR proteins.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-limited, gram-negative bacterium that causes Pierce's disease (PD) in grapevines. 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. 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', were cured by cold therapy treatments compared to susceptible varieties such as 'Pinot Noir.' 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 PD affected grapevines without causing unacceptable plant mortality.
2. Analyze chemical changes such as pH, osomolarity, total organic acids, proteins and other constituents 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 osomolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with cold plant growth regulators, such as abscisic acid (ABA), as a possible therapy for PD.

RESULTS
Objective 1
Using the same varieties used in our 2004-2005 field and cold room studies (PD vs. temperature treatment results were reported in the 2005 Pierce's Disease Symposium Proceedings), Pinot Noir (PD-susceptible) and Cabernet Sauvignon (moderately resistant to PD) grapevines were prepared as described in the 2005 Pierce's Disease Symposium Proceedings. In October/November 2005, prepared grapevines were transported to 4 sites that were selected because of their varying winter temperatures. Sites included: UC Hopland Research Station (Mendocino County), McLaughlin Reserve (Lake County), Foresthill (Placer County), and UC Davis (Yolo County).

Due to the unacceptably high mortality rate at the Fall River plot in 2004, we established the 2005 plot in Lake County at the Donald and Sylvia McLaughlin Reserve. The plants for the Blodgett plot (El Dorado County) were initially planted in the beginning of November 2005. However, due to an unforeseen problem, the plot was moved to Foresthill (Placer County) in December of 2005. The Placer County site is at an elevation similar to Blodgett and we monitored the temperature data at both locations using HOBO dataloggers. Plants were picked up from the four locations in April and planted in the field at UC Davis. Plants are being rated for PD symptoms using the same symptom severity index as in the 2004-2005 season and the presence of Xf is being detected with IC-PCR. In the spring of 2006, new Pinot Noir and Cabernet Sauvignon vines prepared as described above. The grapevines will be transported to the 4 field locations in October/November 2006.

Grapevines, using the same varieties and prepared as described for the field studies but grown in 6" standard pots, were exposed to different temperature regimes in cold rooms located at the Department of Pomology, UC Davis during the winter of 2005-2006. Plants were prepared as described in the 2005 Pierce's Disease Symposium Proceedings. Once in the cold rooms, plants were not moved daily, as in year 1, to attempt to see if we could achieve greater differences between treatments (i.e., all year 1 vines were exposed to the same -5 night temperature, thus differences between treatments were not that great). Plants prepared in 2005 were subjected to one of 4 temperature regimes:

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

After 3 months of treatment, xylem sap was extracted, and the vines were planted in the Plant Pathology field area. The grapevines for the 2005-2006 cold room experiments are currently being evaluated with ICPCR and being rated for PD symptoms to determine the most effective temperature regime for curing without causing unacceptable plant mortality.

Objective 2

2a) El Dorado vs. Yolo County (2004)

Preliminary work using 'Pinot Noir' and 'Cabernet Sauvignon' field materials collected from El Dorado and Yolo counties showed some differences in xylem sap pH and osmolarity (Table 1). These results were obtained from Pinot Noir and Cabernet Sauvignon vines growing in El Dorado County and at the Foundation Plant Services vineyard at UC Davis. Both varieties were grown in the same manner at each site; however, management practices at the two sites were not identical and the clones were not the same. In 2004, dormant cuttings were collected in late February and xylem sap was extracted using a custom-made pressure bomb. These same parameters were examined again in 2005 from grapevines growing at the same two locations in late March, approximately one month later than in 2004. 2004 and 2005 data can be found in 2005 Pierce's Disease Symposium Proceedings. Differences in pH and osmolarity could possibly be explained by differences in the sampling date, management differences, or weather differences at the time of sampling. To try to elucidate these differences, xylem sap samples were collected in late February and again in late March/ early April of 2006 (Tables 1and 2).

Table 1: Osmolarity and pH of xylem sap collected from grapevines from El Dorado County and Yolo County on February 26, 2006.

		El Dorado	Yolo
pH	Pinot Noir	5.74	5.70
	Cabernet Sauvignon	6.24	5.83
Osmolarity mmol/kg	Pinot Noir	60.4	39.5
	Cabernet Sauvignon	52.0	77.0

Table 2: Osmolarity and pH of xylem sap collected from grapevines from El Dorado County and Yolo County on March 25, 2006 and April 7, 2006.

		El Dorado	Yolo
pH	Pinot Noir	5.53	5.57
	Cabernet Sauvignon	5.39	5.50
Osmolarity mmol/kg	Pinot Noir	34.3	16.6
	Cabernet Sauvignon	26.4	23.5

2b) Xylem sap analysis of field and growth chamber plants

In 2004-2005 and 2005-2006 seasons, field grown and growth chamber plants prepared as described in Objective 1, were sampled for potential changes in pH, osmolarity, protein profiles, total sugars, and calcium and magnesium concentrations in xylem sap (Table 3). In both the field experiments and the cold chamber experiments, pH and osmolarity of xylem sap from cold treated vines was lower than found in PD3 media used to grow *Xf.* Sugar and select ion concentration analysis of Cabernet Sauvignon grapevines show greater amounts of glucose and fructose in the –5ºC cold room treatment; where as Ca^{2+} levels are greater in the warmest treatments. Very little sucrose was found in the samples (data not shown). Osmolarity of collected xylem sap is greatest in the coldest treatments and decreases with increasing temperature and shows an interesting relationship with total sugars, calcium, and magnesium concentrations. Conversely, in Pinot Noir grapevines glucose and fructose levels are the lowest in the coldest treatments. Ca^{2+} levels show a similar trend with Cabernet Sauvignon vines, with increased Ca^{2+} levels with the warmer temperature treatments. Temperature appears to have a less direct effect on osmolarity in Pinot Noir grapevines.

Our hypothesis is that changes in xylem sap components in vines that undergo cold treatment may have significant effects on *Xf* viability. Previous research on several plant species has shown that a number of plant genes are expressed in response to freezing temperatures (reviewed by Thomashow, 1998). In some plants, these freeze-induced proteins are structurally related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related proteins (Hon, et al. 1995; Kuwabara, et al, 2002). Thus it may be possible that cold-stressed grapevines could produce proteins that are deleterious to *Xf*.

We are continuing to concentrate proteins in xylem sap by acetone precipitation and electrophoresing the proteins on 1-dimensional polyacrylamide gels (PAGE). Unique protein bands that were found in the cold stressed plants were cut from the gel, and end terminally sequenced. Initial sequencing results of xylem proteins from cold-treated vines showed proteins that are similar to stress proteins that are produced by Cabernet Sauvignon berries and Pinot Noir roots. Understanding the role of these proteins is limited by the *Vitis vinifera* genome not being fully sequenced to date. The appropriate databases will be checked periodically to compare our sample protein sequences against new sequencing information. Due to constraints in xylem sap sample volume, the total organic acids and peroxidase analyses were delayed until the 2005-2006 samplings and will be tested soon.

Table 3: 2005-2006 Mean osmolarity and pH of xylem sap from grapevines growing at 4 field locations around California. 1st sampling = early February 2006; 2nd sampling = late March 2006.

			Davis		Hopland		Foresthill		McLaughlin	
			1st	2nd	1st	2nd	1st	2nd	1st	2nd
pH	Pinot Noir	Control	6.14	5.56	6.17	5.74	6.17	6.10	6.06	5.96
		Inoculated	6.08	6.03	6.34	5.99	5.92	6.08	6.09	6.22
	Cabernet Sauvignon	Control	5.92	5.84	6.63	6.02	6.21	6.27	6.41	5.92
		Inoculated	6.07	5.96	6.32	5.96	6.24	6.23	6.19	6.06
Osmolarity mmol/kg	Pinot Noir	Control	91.1	42.6	72.7	44.2	179.5	67.5	118.7	37.9
		Inoculated	51.4	31.0	41.5	58.0	67.2	57.8	89.5	56.8
	Cabernet Sauvignon	Control	189.8	26.8	64.7	38.2	134.7	61.1	78.9	44.4
		Inoculated	213.1	30.7	67.2	22.0	86.6	53.1	84.3	28.1

Objective 3

We have assessed the effect of pH and osmolarity on the viability of *Xf* cells *in vitro* using various buffers and media and then exposed to various temperatures (28ºC, 5ºC, 2.2ºC, 0, -5ºC, and -20ºC). Everyday, samples were collected and diluted and plated out onto PD3 and allowed to grow for seven days. After seven days, colonies were counted to determine the potential effect each treatment had on the viability of *Xf* cells

Results reported in the 2004 Pierce's Disease Research Symposium Proceedings demonstrated that *Xf* can survive at 28ºC in most media except water and at lower temperatures, our results were similar to those found by Feil (2002). The 2004 proceedings demonstrated that *Xf* survived best in potassium phosphate buffer at 6.8. Survival at pH 6.6 quickly tapered off after a few days. With all other pH buffers (5.0-5.8) *Xf* rapidly died after 1 day. Interestingly, this data supports what has previously reported by Davis (1978), but the pH at which *Xf* survives in culture is higher than what was found in xylem sap samples from our field and cold chamber experiments. We used the same temperatures that were used in the cold room experiments for comparison. *Xf* grew best in PD3 at all temperatures.

Objective 4

Kuwabara et al. (2002) elicited cold-shock proteins at 23°C in winter wheat with an ABA concentration of 100ppm. ABA treated plants elicited proteins that were able to inhibit fungal growth when exposed to exogenous applications of ABA. Though the mechanism is not thoroughly understood, endogenous ABA has shown to induce pathways that are involved in induced resistance to plant pathogens (Ton & Mauch-Mani, 2004; reviewed in Bostock, 2005).

4a) ABA concentration in xylem sap

ABA concentrations in xylem sap have been measured for the four field sites and the cold chamber experiments. The concentration of ABA in the sap was determined using a commercially available immunoassay that has a sensitivity of 0.0064-0.16 picomoles ABA/ml (Phytodetek ABA Test Kit, Agdia) and only requires a small volume of xylem sap. ABA concentrations of xylem sap are being determined for the vines in the cold chambers and in the field. Experiment will be replicated in the 2006-2007season.

4b) ABA applications to grapevines

In November 2005, healthy and *Xf*-inoculated Cabernet and Pinot vines prepared as stated in Objective 1 were sprayed with solutions of ABA. Valent Biosciences representatives provided us with 2 types of ABA. There were 5 treatments with *Xf*-infected vines and healthy controls:

Control: 8 Pinot/ 8 Cabernet plants sprayed with water
1000ppm spray: 8 Pinot/ 8 Cabernet plants sprayed with VBC-30054
100 ppm drench: 8 Pinot/ 8 Cabernet plants sprayed with VBC-30054
100 ppm spray: 8 Pinot/ 8 Cabernet plants sprayed with VBC-30030
10 ppm drench: 8 Pinot/ 8 Cabernet plants sprayed with VBC-30030

To determine effectiveness of ABA and synthetic ABA treatments, vines are currently being evaluated for PD symptoms and being tested with ICPCR. Experiments will be replicated in the 2006-2007 season.

REFERENCES

Bostock, R.M. 2005. Signal Crosstalk and Induced Resistance: Straddling the line between cost and benefit. Annual Review of Phytopathology 43:545-580.

Davis, M.J., A.H. Purcell, and S.V. Thomson. 1980c. Isolation media for the Pierce's disease bacterium. Phytopathology. 70:425-429.

Feil, H. 2002. Effect of sub-freezing temperature on the survival of *Xylella fastidiosa* in vitro and in plants. In Ph.D. dissertation, University of California, Berkeley.

Guilhabert M.R and B.C. Kirkpatirck. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute a biofilm maturation to *X. fastidiosa* and colonization and attenuate virulence. Molecular Plant Microbe Interact. 18(8):856-68.

Hill, B.L. and A.H. Purcell. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. Phytopathology 85:1368-1372.

Hon, W.C., M. Griffith, A. Mlynarz, Y.C. Kwok, and DSC Yang. 1995. Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiology 109:879-889.

Kuwabara, C., D. Takezawa, T. Shimada, T Hamada, S. Fujikawa, and K. Arakawa. 2002. Abscisic acid- and cold-induced thaumatin-like protein in winter wheat has an antifungal activity against snow mold, Microdochium nivali. Physiolgia Plantarum 115: 101-110.

Purcell, A.H. 1977. Cold therapy of Pierce's disease grapevines. Plant Dis. Reptr. 61:514-518.

Purcell, A.H. 1980. Environmental therapy for Pierce's disease of grapevines. Plant Disease 64:388-390.

Purcell, A.H. and Hopkins, D.L. 1996. Fastidious xylem-limited bacterial plant pathogens. Annual Rev. Phytopath. 131-151.

Purcell, A.H. 1997. *Xylella fastidiosa*, a regional problem or a global threat? J. Plant Pathology. 79:99-105.

Purcell, A.H. and Saunders, S.R. 1999. Fate of Pierce's disease strains of *Xylella fastidiosa* in common riparian plants in California. Plant Disease 83:825-830.

Ton, J. and B. Mauch-Mani. 2004. β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA- dependent priming for callose. The Plant Journal 38:119-130.

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USE OF THE *E. COLI* α-HEMOLYSIN SECRETION SYSTEM IN BACTERIA DESIGNED FOR SYMBIOTIC CONTROL OF PIERCE'S DISEASE IN GRAPEVINES AND SHARPSHOOTERS

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ABSTRACT
Strains of *Alcaligenes xylosoxidans denitrificans* (*Axd*) that secrete anti-*Xylella* factors are being developed for use in a strategy to prevent the spread of *Xf* or possibly to cure infected grapevines. We built constructs that fused the last 60 amino acids of the autotransporter α-hemolysin from *E. coli* to test proteins. These were two different forms of anti-BSA single chain antibodies (scFvs) which are surrogates for anti-*Xylella* effector proteins. These proteins were efficiently secreted from *E. coli* when co-expressed with the proteins HlyB and HlyD. HlyB, HlyD, and TolC together form the membrane structure used by α-hemolysin to cross both the inner and outer membrane of the cell. We report here on efforts to move this system into *Axd*, a species not closely-related to *E. coli*, but that can survive in both grapevine and sharpshooters.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is the principal vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapes. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission or by interfereing with the replication of *Xf* in the plant may stop the spread of PD. These endpoints can be accomplished by genetically modifying bacteria that live in the sharpshooter, the plant, or both in a method called symbiotic control. Symbiotic control seeks to modify the phenotype of an organism indirectly by modifying its symbiotic bacteria.

Symbiotic control approaches to disrupt pathogen infection of humans are being developed by several groups. These include interference with the ability of triatomid bugs to transmit pathogens causing Chagas' disease (Beard et al., 2001), interference with HIV attachment to its target cells in the reproductive tracts of humans (Chang et al., 2003; Rao et al., 2005), and the elimination of persistent *Candida* infections from biofilms in chronically infected human patients (Beninati et al., 2000). Symbiotic control has also been applied to deliver cytokines mammalian guts to relieve colitis (Steidler et al., 2000; Steidler, 2001). Thus, the method has wide applicability.

Alcaligenes xylosoxidans denitrificans (Axd) is Gram negative pseudomonad-like species that can colonize the GWSS foregut and cibarium, as well as various plant tissues, including grape xylem. It is non-pathogenic in insects, plants and healthy humans. Given these characteristics, *Axd* has become the focus of our symbiotic control efforts to control PD in grapes. Over the past several years we developed the technology to stably modify *Axd* by inserting genes into its chromosome via transposition, have developed methods to suppress horizontal gene transfer, and have isolated a single chain antibody that recognizes an epitope on the surface of the PD strain of *Xf*. (Bextine et al., 2004). We are currently engaged in combining these systems in order to produce strains of *Axd* that are suitable for environmental release in a practical strategy symbiotic control strategy for PD.

One way to deliver anti-*Xylella* protein factors from *Axd* in grapevine is by secretion. Secreted anti-*Xylella* factors might circulate throughout the plant, reaching foci of infection across physical xylem boundaries. Secretion from Gram-negative bacteria, however, is complicated by the fact that these species have two membranes that a protein must cross before appearing outside the cell. Gram negatives contain at least 6 identified types of secretion systems. Unfortunately, many of these systems are unpredictable when expressed heterologously. One system that seems to have wide applicability is the a-hemolysin autotransporter from *E. coli* (Fernandez et al., 2000). This protein is secreted in a single energy-dependent step across both membranes of Gram negative bacteria when the other components of the system are also present (the proteins HlyB, HlyD, and TolC). Fusion of the last 60 amino acids of the protein is sufficient to target any N-terminal passenger protein for secretion.

We report here the evaluation of the *E. coli* α-hemolysin system for use in *Axd* to secrete soluble anti-*Xylella* protein effectors in grapevine and insects.

OBJECTIVES
1. Test the *E. coli* α-hemolysin secretion system in *E. coli* and *Axd*.