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

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ABSTRACT
During October/November 2004, 11 control and 11 *Xylella fastidiosa* (*Xf*) infected Pinot Noir and Cabernet Sauvignon grapevines grown in five gallon pots were transported to four locations in Northern California with different winter severities. Another set of similar healthy and *Xf*-infected vines were placed in four different cold rooms with varying temperatures. These vines were rated for mortality and remission of Pierce's disease (PD) symptoms in fall 2005. A high level of mortality occurred at the coldest location (Fall River, Shasta County), moderate mortality occurred at UC Blodgett Research Station, while little mortality occurred at the UC Davis and Hopland sites. Disease ratings were lower in vines from the three cold temperature sites compared to vines grown at UC Davis, however the large discrepancy in the number of vines surviving at each location prevented meaningful comparisons between the field sites. Unexpectedly, mortality of vines in the warmest and coldest chamber regimes was greater than the two intermediate temperatures. Disease severity was also greatest in the warmest temperature which may have contributed to the observed high mortality when the vines were subsequently planted in the field. All three cold chamber regimes had lower disease ratings than the warmest temperature.

Comparisons of xylem sap pH and osmolarity in Cabernet vines growing in a vineyard in Placer County and UC Davis were not consistent with results obtained in 2004. Differences in the date of collection may have influenced these results. Effects of buffer and xylem sap on the survival of *Xf* and various cold temperatures were reported in the Proceedings of the 2004 Pierce's Disease Research Symposium. 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. ABA concentrations were lower in xylem sap collected from vines growing in El Dorado County compared to UC Davis, which suggests ABA is probably not directly mediating the cold therapy phenomenon. However, we are proceeding with experiments to determine if exogenous applications of ABA on non-chilled grapevines can elicit PR proteins.

INTRODUCTION
The geographical distribution of PD in North America is strongly associated with the severity of winter temperatures, i.e. PD does not occur in New York, the Pacific Northwest nor at high altitudes in South Carolina, Texas, and California (Hopkins and Purcell, 2002). Sandy Purcell demonstrated that relatively brief exposures to sub-freezing temperatures can eliminate *Xf* in some percentage of cold treated *Vitis vinifera* grapevines; however some of the coldest temperatures he used killed the vines (Purcell 1977, 1980). He also found that a higher percentage of vines that were moderately susceptible to PD such as Cabernet Sauvignon, were cured by cold therapy treatments compared to susceptible varieties such as Pinot Noir. Purcell's group also showed that whole, 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).

Clearly, some factor(s) expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants. 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 factor(s) 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, osmolarity, total organic acids, proteins and other constituents that occur in the xylem sap of cold-treated versus non-treated susceptible and less susceptible *V. 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 plant growth regulators, such as ABA, as a possible therapy for PD.

RESULTS AND CONCLUSIONS

Objective 1

Using the same varieties used by Purcell (1977, 1980) and Feil (2002) in previous cold therapy studies, Pinot Noir (PD-susceptible) and Cabernet Sauvignon (moderately resistant to PD) grapevines grafted onto 101-14 rootstock were inoculated with *Xf* in the spring using a pinprick inoculation procedure (Hill and Purcell, 1995; Purcell and Saunders, 1999). The vines were grown in five gallon pots in a greenhouse using a nutrient-supplemented irrigation regime. Treatment vines were inoculated with the Stagg's Leap strain of *Xf*, whereas control vines were inoculated with water. During late summer and fall, the plants were moved into a screen house in order to acclimatize them to decreasing temperatures. While in the screen house, plants were watered by drip irrigation and supplemental fertilizer application until the first week of October 2004. Twelve weeks after inoculation, the plants were rated for symptom development.

In the spring of 2005, new plants of Pinot Noir and Cabernet Sauvignon grafted on 101-14 rootstock were planted in 5-gallon pots, inoculated by the same procedure used in the spring of 2004 mentioned above. Plants were placed in the same greenhouse, subjected to a similar temperature regime, and were watered using the same nutrient-supplemented regime. Plants were moved to the same screen house as the 2004 plants and will continue to be watered by drip irrigation and receive supplemental fertilizer applications until the first week of October 2005.

During October/November, 2004, 11 inoculated and 11controls of each variety (44 plants total) were transported to three sites that were selected because of their relatively cold winter temperatures, as well as UC Davis, which was the control. Plot sites include: Fall River (Shasta County), UC Hopland Research Station (Mendocino County), and UC Blodgett Forest Research Station (El Dorado County). Potted grapevines were planted in the ground to the top of the pot in order to maintain uniform soil type, prevent roots in the pots from exposure to abnormally cold temperatures, and to prevent the plants from falling over. Plants were irrigated as needed until rain provided adequate moisture for the vines. Vines were allowed to undergo natural dormancy during the fall and experience ambient temperatures during the winter. Temperature, ETo, and other weather data for each plot was monitored using CIMIS weather data (http://wwwcimis.water.ca.gov/cimis/data.jsp). The plants prepared in 2005 will be used to replicate the 2004 study. This data, and previous temperature profiles at these sites, will be used to determine a growth chamber temperature regime that can consistently cure PD affected grapevines without causing unacceptable plant mortality.

Grapevines, using the same varieties and inoculated as described above, 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. Plants prepared in 2004 were subjected to one of four temperature regimes:

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

There were 40 plants per treatment regime (10 inoculated plants and 10 control plants for both varieties). In regimes where there were differences in day and night temperatures, plants were moved twice daily by carts to simulate daily temperature fluctuations. After three months of treatment, xylem sap was extracted from the plants, and then the plants were moved and planted in the Plant Pathology field at UC Davis. Late in the summer of 2005, the plants were evaluated for symptoms to determine the most effective temperature regime for curing without causing unacceptable plant mortality. The field plant evaluation shows higher disease ratings for the warmer temperature treatments, Davis and Hopland, when compared to the colder treatments, Fall River and Blodgett (Table 1). As we expected, plant mortality was the highest at the colder locations (Table 2). Fall River vines had very high mortality when compared to the other treatments. To try to reduce the mortality in Fall River vines, plants for the 2005-2006 trials will be planted later in the fall to allow the plants to acclimate prior to planting. Cold room treated vines show a similar relationship with the exception of the mortality rate of the +5 day/ -5 night treatment (Tables 3 and 4). This high rate of mortality could be due to rabbits burrowing under the fence and feeding on these plants.

Table 1: Mean PD ratings[z] for PD-infected plants

	Davis	Hopland	Fall River	Blodgett
Pinot Noir	2.17	1.45	1.00	1.33
Cabernet Sauvignon	2.33	2.33	2.00	1.40

[z]Vines were rated for the severity of disease symptoms, 0= healthy to 5= dead.

Table 2: Plant mortality rate

	Davis	Hopland	Fall River	Blodgett
Pinot Noir	0%	2%	91%	41%
Cabernet Sauvignon	0%	2%	55%	36%

Table 3: Mean PD ratings[z] for PD-infected plants

	+5°C Day/ -5°C Night	2.2°C Day/ -5°C Night	0°C Day/ -5°C Night	-5°CDay/ -5°C Night
Pinot Noir	3.00	1.17	1.40	1.00
Cabernet Sauvignon	2.50	1.56	1.29	1.00

z. Vines were rated for the severity of disease symptoms, 0= healthy to 5= dead.

Table 4: Plant mortality rate

	+5°C Day/ -5°C Night	2.2°C Day/ -5°C Night	0°C Day/ -5°C Night	-5°CDay/ -5°C Night
Pinot Noir	45%	27%	32%	59%
Cabernet Sauvignon	14%	9%	0%	14%

Objective 2

Preliminary work from Pinot Noir and Cabernet Sauvignon field materials collected from El Dorado County and Yolo County showed some differences in xylem sap pH and osmolarity (Tables 5 and 6). These results were obtained from Pinot Noir and Cabernet Sauvignon vines growing at Clos des Knoll vineyard in El Dorado County and at the Foundation Plant Services (FPS) vineyard at UC Davis (Yolo County). Both varieties were grown in the same manner at each site; however management practices at the two sites were not identical. It is also important to note that the El Dorado County vines and the Yolo County vines were not the same clones. In 2004, dormant cuttings were collected in late February and xylem sap was extracted using a custom-made pressure bomb. Differences were noted in xylem sap pH, ABA concentration, and osmolarity. These same parameters were examined again in 2005 from grapevines found at the same two locations in late March.

Although only preliminary findings, we found that the pH of xylem sap collected in 2004 in late February was lower, 5.37 for Pinot and 5.23 for Cabernet vines in El Dorado County (colder winter temperatures) than vines growing at FPS (UC Davis), 6.35 and 6.06, respectively. Small differences in osmolarity were also noted in xylem sap from Placerville, 55.2 and 55.5, versus the osmolarity of xylem sap from UC Davis vines, 58.3 and 60.8 respectively. This is different from the xylem sap collected in late March of 2005. The pH of sap from El Dorado County was higher than Yolo County vines. The osmolarity was again similar, but lower at both sites than in 2004. Differences in pH and osmolarity could possibly be due to the difference in timing of collection. The significance and reproducibility of these differences needs to be confirmed with sampling in late February and again in late March of 2006.

In 2004 and 2005, field grown and growth chamber plants prepared as stated in Objective 1, were sampled for potential changes in pH, osmolarity, protein profile and other constituents that occur in xylem sap. 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 maybe possible that cold-stressed grapevines produce proteins that are deleterious to *Xf*.

To investigate this possibility, after three months of treatments, xylem sap was extracted from plants from each location/ treatment, for both growth chamber and field plants, using the pressure bomb. Xylem sap osmolarity and pH were determined for each location/treatment (Tables 7 and 8). We are in the process of concentrating the proteins by acetone precipitation and running the protein precipitate using a 1-dimensional polyacrylamide gel electrophoresis (PAGE). If unique proteins are found in the cold stressed plants, these proteins will be cut from the gel, end terminally sequenced by the UC Davis Molecular Structure Facility and their sequences will be compared to others in the database. The potential effect of these proteins on *Xf* viability will be assessed as described in Objective 3. After sampling, the plants were moved and planted in the Plant Pathology field at UC Davis. Plants are currently being evaluated for symptoms to determine the most effective temperature regime for curing. This process will be repeated for the plants prepared for growth chamber and field studies in the fall/winter of 2005.

Table 5: Osmolarity and pH of xylem sap collected from grapevines in El Dorado County (Clos de Knoll Vineyard) and Yolo County (FPS) in 2004 (late February).

		El Dorado	Yolo
pH	Pinot Noir	5.37	6.35
	Cabernet Sauvignon	5.23	6.06
Osmolarity mmol/kg	Pinot Noir	55.2	58.3
	Cabernet Sauvignon	55.5	60.3

Table 6: Osmolarity and pH of xylem sap collected from grapevines in El Dorado County (Clos de Knoll Vineyard) and Yolo County (FPS) in 2005 (late March).

		El Dorado	Yolo
pH	Pinot Noir	5.87	5.79
	Cabernet Sauvignon	5.81	5.55
Osmolarity mmol/kg	Pinot Noir	34.80	37.50
	Cabernet Sauvignon	27.17	30.61

Table 7: Osmolarity and pH of xylem sap from grapevines from four locations around California- Field.

			Davis		Hopland		Fall River		Blodgett	
			1st*	2nd**	1st*	2nd**	1st*	2nd**	1st*	2nd**
pH	Pinot Noir	Control	5.81	5.79	5.96	5.73	4.94	5.97	5.88	5.23
		Inoculated	5.95	5.77	5.65	5.53	5.29	6.14	5.49	5.36
	Cabernet Sauvignon	Control	6.23	5.43	5.84	5.73	6.38	5.93	5.90	5.52
		Inoculated	6.16	5.58	5.93	5.61	6.99	5.92	6.12	5.57
Osmolarity mmol/kg	Pinot Noir	Control	44.91	37.50	42.30	54.67	59.11	35.36	67.20	69.91
		Inoculated	59.60	36.56	49.10	43.17	73.33	50.00	71.33	41.73
	Cabernet Sauvignon	Control	45.11	40.00	61.40	68.09	94.33	55.44	79.45	53.45
		Inoculated	33.33	34.80	88.30	76.00	61.00	51.00	76.33	34.64

*1st collection occurred between 2/24/05 and 3/6/05.
**2nd collection occurred between 4/15/05 and 4/22/05.

Table 8: Osmolarity and pH of xylem sap from grapevines treated with four different cold regimes- Growth Chamber.

			-5°C day; -5 °C night	+0°C day; -5°C night	+2.2°C day; -5°C night	+5°C day; -5°C night
pH	Pinot Noir	Control	5.41	5.46	5.44	5.11
		Inoculated	5.42	5.42	5.45	5.19
	Cabernet Sauvignon	Control	5.51	5.33	5.66	5.34
		Inoculated	5.54	5.66	5.59	5.72
Osmolarity mmol/kg	Pinot Noir	Control	36.5	45.3	58.5	37.6
		Inoculated	38.3	33.0	49.9	34.6
	Cabernet Sauvignon	Control	42.3	38.9	41.6	33.7
		Inoculated	45.8	45.1	37.2	25.5

Objective 3

We have assessed the effect of pH and osmolarity on the viability of *Xf* cells *in vitro* using various buffers and media such as PD3 and new chemically defined media (Leite, et al., 2004). The liquid solutions used for these viability experiments included: water, extracted xylem sap, PD3, HEPES, sodium and potassium phosphate buffers.

In order to further examine these conditions, cultures of *Xf* Stagg's Leap strain were grown at 28°C on PD3 for 11 days. Cells were scraped from the culture plates and suspended at concentrations of 1.5 x 10^7 bacteria per milliliter of liquid medium. One milliliter of the suspension was then placed into each 1.5 mL micro-centrifuge tubes and placed at various temperatures. Samples were 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 of these experiments indicate that *Xf* can survive at 28ºC in most media (except water). The results also indicate that *Xf* can survive at -5ºC for 8 weeks. At lower temperatures, our results were similar to those found by Feil (2002). *Xf* survived the best in HEPES and sodium phosphate buffers and the worse survival occurred in water and xylem sap at –5ºC. At –10ºC and –20ºC, *Xf* rapidly died in all liquid media tested.

Potassium phosphate buffer was used to determine the effects of pH on the survival of *Xf*. Samples were prepared like above, the cells were placed in potassium phosphate buffer at the pH levels of: 5.0, 5.4, 5.8, 6.2, 6.6 and 6.8. The cells were placed at -5ºC for up to seven days. 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 for Objective 3 are reported in the 2004 Pierce's Disease Research Symposium Proceedings.

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, which induces the synthesis of a number of cold shock proteins (Bravo, et al., 1998; Thomashow, 1998). Preliminary studies, involving samples of Pinot Noir and Cabernet Sauvignon field materials collected from El Dorado County and Yolo County in February 2004, and again in March 2005, showed ABA concentrations were lower in El Dorado County, cold-exposed vines, than in vines from Yolo County. ABA concentrations were lower in Pinot Noir than Cabernet Sauvignon for both El Dorado County and Yolo County vines.

We are in the process of determining ABA concentrations of xylem sap in cold-stressed and control vines growing both in the growth chamber and in the field-grown plants in the four sites using the temperature regimes described in Objective 1.

This fall, Cabernet and Pinot vines prepared as stated in Objective 1, will be sprayed with 100µM solutions of ABA, a concentration that elicited cold-shock proteins at 23°C in winter wheat (Kuwabara, et. al 2002). Additional concentrations up to 500µM may also be evaluated if no response is noted at 100µM. The pH and osmolarity of xylem sap from the treated vines will be determined as described above. The concentration of ABA in the sap will be determined using a commercially available immunoassay that has a sensitivity of 0.02-0.5 picomole/0.1 ml (Plant Growth Regulator Immunoassay Detection Kits, Sigma Chemical Co.). Preliminary work has shown that ABA concentrations in grapevine xylem sap are detectable using this kit. Xylem sap proteins will be collected, concentrated and analyzed by 1- and 2-dimensional PAGE as previously described. Unique proteins expressed in ABA-treated vines will be removed from the gels and end terminally sequenced and analyzed as previously described. We will also determine the pH, osmolarity and protein profiles of xylem sap from ABA-treated vs. non-treated vines and assess the potential of this sap for anti-*Xf* activity.

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