Project Title:
IDENTIFICATION AND UTILIZATION OF COLD TEMPERATURE INDUCED GRAPEVINE METABOLITES TO MANAGE PIERCE'S DISEASE

Sub-Title of Report: Final Report for CDFA Agreement Number 04-0486

Second title of research project: Identification of mechanisms mediating cold therapy of *Xylella fastidiosa*- infected grapevines

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Abstract and Layperson Summary

The results of 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 were 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 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. 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.

Numerous xylem sap compounds such as sugars, organics acids, divalent cation and phenolic compound concentrations were compared between warm and cold-exposed grapevines. There was considerable variation in the concentrations of these compounds but there was no consistent, significant differences between the compounds analyzed in CS versus PN xylem saps.

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 *X. fastidiosa*-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 under water deficit stress conditions, proteins that are similar to proteins produced in Pinot Noir roots, and tryptase inhibitors. The protein similarity that is most interesting is a thaumatin-like protein (TLP), which has been reported to have anti-microbial activity that appears to be produced in greater quantities under the coldest conditions. We assessed the potential anti-*X. fastidiosa* properties of this protein by cloning, expressing and purifying this protein. Some inhibition of Xf growth was observed *in vitro*, but the level of inhibition was low.

The *in vitro* Xf culture experiments showed that *X. fastidiosa* 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, *X. fastidiosa* can survive at -5ºC in all buffers at pH 6.8, media and xylem sap for at least 4 days. No cultivable *X. fastidiosa* was recovered from any of the media, buffers or xylem sap after 24 hours at -10˚C or at -20˚C. *X. fastidiosa* in potassium phosphate buffers with pH values at 5.0, 5.4 and 5.8 died rapidly at all temperatures.

The results of ABA application made to potted grapevines growing in a screen house in 2005-2006 indicated that ABA had 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 drench treatments decreased or were eliminated after application of drench treatments.

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.

Dr. Meyer's research showed elevated levels of the plant hormone abscisic acid (ABA) in xylem sap extracted from cold exposed grapevines. She showed that exogenous application of ABA greatly increased the PD curing rates of potted grapevines exposed to the comparatively mild winter temperatures in Davis (Meyer and Kirkpatrick, 2011). In 2010 and 2011 we applied ABA in the fall to mildly PD-infected Riesling vines growing in a vineyard in Napa. The severity of PD symptoms in the ABA-treated vines was compared to non-treated controls in October, 2011 and 2012. No statistically significant differences in the severity of PD symptoms was found between ABA-treated and non-treated vines.

Based on apparent low levels of inhibition when Xf was incubated with cloned and purified thaumatin (TLP)we cloned the TLP gene in an Agrobacterium binary vector and with the assistance of the UC Davis Plant Transformation facility produced 13 independent Thompson seedless lines that were propagated in the greenhouse. qRT PCR analysis of the transgenic lines showed good levels of TLP mRNA expression while non-transgenic Thompson seedless had very low levels of TLP expression. These vines were further propagated and 12 reps of each transgenic line were mechanically inoculated with wild type Xf in the greenhouse. The severity of PD symptoms in the TLP transgenic vines was compared with the severity of symptoms in non-transgenic controls and Xf cells were quantified by culture and qPCR. PD disease severity was the same in TLP-transgenic vines and controls 17 weeks following inoculation with wild-type *X. fastidiosa*. Likewise, Xf populations were not significantly different in TLP-trangenic vines versus control grapevines. These results indicated that elevating levels of TLP in the transgenic grapevines did not provide any significant protection against Xf infection as disease severity was similar in both transgenic and non-transgenic control vines.

Introduction

The geographical distribution of Pierce's disease (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 *Xylella fastidiosa* 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 Pierce's disease 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 *X. fastidiosa* 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.

5) a) Over express the grapevine thaumatin-like protein (TLP) in transgenic grapevines. Characterize the levels of TLP expression in the TLP-transgenic lines using qRT-PCR.

b) Inoculate TLP-expressing grapevines with Xf and determine the incidence and severity of PD in transgenic versus non-transgenic *V. vinifera*.

Results and Discussion

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 *Xylella fastidiosa* (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 *Xylella fastidiosa*, 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 Sauvingnon 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 screenhouse as the 2004 plants and a watered by drip irrigation and received 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 3 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 were used to replicate the 2004 study. This data, and previous temperature profiles at these sites, were 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 4 temperature regimes.

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

There were 40 plants per treatment regime (10 inoculated plants and10 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 3 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 (Table 1) when compared to the colder treatments, Fall River and Blodgett. 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 were planted later in the fall to allow the plants to acclimate prior to planting. Cold room treated vines showed 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 Plant Ratings 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

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 Plant Ratings for PD-infected plants

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

Table 4: Plant Mortality Rate

	+5C Day/ -5C Night	2.2C Day/ -5C Night	0C Day/ -5C Night	-5CDay/ -5C 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 and Yolo counties showed some differences in xylem sap pH and osmolarity (Tables 5 and 6). These results were obtained from Pinot Noir and Cabernet Savignon 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, abscisic acid concentration, and osmolarity. These same parameters were examined again in 2005 from grapevines found at the same two locations in late March.

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 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.

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.

Physiological and Biochemical Analyses. Grapevine xylem sap samples were tested for pH, osmolarity, total phenolics, abscisic acid, organic acids, sugars, and calcium and magnesium ion concentrations. The pH of each xylem sap sample was measured using a pH meter (pH meter 140, Corning, Lowell, MA) and micro-combination pH microelectrode (MI-710, Microelectrodes, Inc., Bedford, NH). Osmolarity was measured using a vapor pressure osmometer (5500 Model, Wescor, Logan, UT). Abscisic acid (ABA) levels were measured using an ABA ELISA kit (Phytodetek ABA Test Kit, Agdia, Elkhart, Indiana) according to the manufacture's protocol. Sucrose, glucose, fructose, calcium and magnesium concentrations were measured by the Agriculture and Natural Resources analytical laboratory on the University of California, Davis campus. Xylem sap samples (20ul for total sugars and 30ul for calcium and magnesium) were diluted with deionized water and then analyzed using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Organic acids were measured by first filtering the xylem samples through 10,000-MW filters prior to analyses. Separation and quantification of organic acids was accomplished by cation exchange chromatography. Samples (20 ul injection volume) in 0.015 normal sulphuric acid buffer were run isocratically at 37°C through an Ion-300 polymeric column on a Waters 600 HPLC system.

Total phenolics were measured using the Folin-Ciocalteu procedure. Ten microliters of xylem sap were added to 90ul of sterile-deionized water in a 1.5ml microcentrifuge tube. 500ul of a 1:10 dilution of Folin-Ciocalteau reagent were added to each sample. After 30 seconds, but within 8 minutes, 400ul of 7.5% anhydrous Na_2CO_3 were added to each tube. Sample tubes were mixed by inversion and incubated at room temperature for one hour. Two hundred microliters of

sample from each tube were transferred to a 96-well ELISA plate (Fisher Scientific, Hanover Park, IL). Absorbance was measured at 765 nm using a microplate reader (Molecular Devices, Sunnyvale, CA) and SoftMax Pro 4.7.1 software. Total phenolic concentrations in the samples were calculated using a gallic acid standard curve.

Freezing temperatures are known to dehydrate plant tissue which could affect the ability of *X. fastidiosa* to over-winter in xylem tissue. We determined the relative water content of canes from field vines by harvesting one gram of fresh cane, dehydrating the cane section in a drying oven at 60°C for 48 hours, and then measuring the dry mass.

Table 5: Osmolarity and pH of xylem sap collected from grapevines from 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 Sauvingnon	5.23	6.06

Osmolarity	Pinot Noir	55.2	58.3
mmol/kg	Cabernet Sauvingnon	55.5	60.3

Table 6: Osmolarity and pH of xylem sap collected from grapevines from 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 Sauvingnon	5.81	5.55

Osmolarity	Pinot Noir	34.80	37.50
mmol/kg	Cabernet Sauvingnon	27.17	30.61

Table 7: Osmolarity and pH of xylem sap from grapevines from 4 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 Sauvingnon	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 Sauvingnon	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 4 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 Sauvingnon	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 Sauvingnon	Control	42.3	38.9	41.6	33.7
		Inoculated	45.8	45.1	37.2	25.5

Sugars. The early (February/March) collected grapevine xylem sap sugar analysis showed significant differences in sugar concentrations between locations. The fructose concentration was significantly higher in control 'Cabernet Sauvignon' vines from Davis than 'Cabernet Sauvignon' vines from Foresthill and Hopland. Control Pinot Noir vines from Davis in the first field collection had significantly lower fructose concentrations than the other 3 field sites. Significant differences between locations in the second field collection were also found. Davis and Hopland control Pinot Noir had higher levels of fructose than Foresthill. Both control and inoculated Cabernet vines from Hopland were significantly lower in fructose than vines from Foresthill. The cold chamber results show control Pinot Noir in the coldest chamber (-5°C) had higher levels of fructose than vines from the other chambers. The overall concentration of fructose was significantly higher in the early field collection than the late field collection.

Glucose content was significantly higher in control 'Cabernet Sauvignon' vines from Davis than Foresthill and Hopland in the early field collection. Both Cabernet Sauvignon control and inoculated vines in Lake were significantly higher than Hopland. In the late field collection, control and inoculated Pinot Noir grapevines from Foresthill had significantly lower glucose concentrations than grapevines from Davis and Hopland, while only control Pinot Noir was significantly lower in Lake County. In the cold chamber, control Pinot Noir vines from the coldest temperature treatment (-5°C) had significantly higher levels of glucose than the other cold chamber vines.

Calcium and Magnesium. Early field collected control 'Cabernet Sauvignon' vines from Davis had significantly higher calcium levels than vines from Foresthill and Hopland. Control Pinot Noir vines from Lake County showed significantly higher levels of calcium than Hopland and Foresthill in the early field collection. For the late collection period (late March/early April), Hopland control Cabernet Sauvignon vines had significantly higher levels of calcium than Lake and Foresthill. Late collected (late March/early April) Davis and Hopland control Pinot Noir vines had significantly higher calcium levels than Foresthill and Lake County. Calcium levels from the cold chamber showed significantly higher concentrations between the -

5°C chamber and the 0°C chamber in the Cabernet inoculated vines. Pinot Noir control vines at -5°C had significantly higher calcium concentrations than the 0°C and 2.2°C vines. No significant differences in magnesium concentrations were found between field locations or cold chamber temperatures.

Abscisic Acid. ABA concentrations in the early xylem sap collections showed no significant differences between locations. In the late collections, ABA levels were significantly lower in inoculated Cabernet Foresthill vines than vines from Davis and Lake County. Davis control Pinot Noir vines had significantly higher ABA levels than vines from Foresthill and Hopland. Overall, ABA levels were significantly lower in the early collected xylem sap than the late collected xylem sap. Cold chamber ABA showed the inoculated Cabernet Sauvignon vines -5°C treatment was significantly lower than the 2.2°C and +5°C treatments. Control and inoculated Pinot Noir at 0°C had significantly lower ABA levels than the 2.2°C and +5°C treatments.

Total phenolic compounds. The total phenolic content of xylem sap was significantly different based on location. Early collected sap from Davis, Hopland and Lake County in control and *X. fastidiosa* inoculated Pinot Noir vines had significantly lower total phenolics than Foresthill. Control and *X. fastidiosa*-inoculated Foresthill Cabernet vines had significantly lower phenolics than the vines from Lake County. In late spring sap collected from control and inoculated Pinot Noir vines, Foresthill vines had significantly higher levels of total phenolics than the three other field locations. Significantly lower total phenolic levels were found in late spring collected xylem sap from Foresthill control 'Cabernet Sauvignon' grapevines than Lake County vines.

The cold chamber vines also showed significant differences in total phenolic concentrations between treatments. The control and inoculated Cabernet vines for the coldest -5°C treatment had significantly higher total phenolic content than the warmest treatment (+5°C). The control and inoculated Pinot Noir vines had significantly higher total phenolic content in the coldest treatment (-5°C) than the other three cold chamber treatments. The total phenolic concentrations in the +5°C treatment were significantly lower for both control and inoculated varieties except inoculated Cabernet.

Previous work showed that grape vines infected with *X. fastidiosa* show significant recovery when exposed to cold winter temperatures, such as those experienced in this Placer county vineyard. As a control, we also collected sap from Davis grown vines where curing, due to warmer temperatures, is significantly less than that observed in Placerville. Sap was expressed by placing canes in a "pressure bomb", allowing one end of the cane to protrude from the cylinder, and then pressurizing the chamber with air to pressures between 300 and 400 psi, to collect the xylem sap exudate. These samples were kept frozen at -80C until they were analyzed by High Performance Liquid Chromatography/Mass Spectrometry by Mauri Anderson of the Waterhouse Lab.

The xylem sap samples were chromatographed using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with electrospray ionization (ESI) mass spectrometry (MS), which produced good resolution. Phenolic compounds were identified based on retention time, UV spectra from diode array detection, and MS using commercially available reference standards. In the Placerville (cold) Pinot Noir samples, a number of phenolic compounds were identified: B procyanidins, catechin, epicatechin, trans-resveratrol, caftaric acid, and a resveratrol tetramer. Cabernet Sauvignon samples produced an identical polyphenol profile except that the resveratrol tetramer was not present. Interestingly, the warm Pinot Noir

sap lacked characteristic peaks for trans-resveratrol as well as the resveratrol tetramer. The fact that resveratrol is present in vines that experience "cold curing" while it is absent in vines that do not undergo "cold curing" suggests that resveratrol may play a role in the curing process. We previously reported on the anti-*Xf* activity of trans-resveratrol *in vitro*. These results have been subsequently supported by Maddox et al (2009) who published their results before we had an opportunity to prepare our results for publication.

In 2010 we expanded our efforts to identify and quantify different polyphenolic compounds in xylem sap over the winter months. We collected sap from Pinot Noir and Cabernet Sauvingon vines (not infected with PD) in Winters, Ca (warm environment) and Placervlle, Ca (cold environment) during the months of January, February, March, and April. The phenolic compounds were similar on the various collection dates. In the cold samples from Pinot Noir vines we found B procyanidins (flavanoids), Catechin, Epicatechin, Caftaric acid, Coutaric acid, and Quercetin 3-glucuronide. The warm sap had the same polyphenolic profile. The amount of each phenolic compounds in these sap xylem samples is very difficult to absolutely quantify, but based on relative signal strength, quercetin 3-glucuronide is present at concentrations seven fold higher in cold sap than in warm sap.

Research conducted in this project demonstrated a correlation between increased levels of phenolic compounds in the xylem sap of cold-exposed grapevines compared to phenolic levels in sap extracted from vines exposed to moderate winter temperatures. Several of these phenolic compounds were shown to be toxic to Xf in *in vitro* studies. However it is difficult to envision a method of elevating phenolic compound levels to sufficient levels to be toxic to Xf without potentially changing the characteristics of juice extracted from berries to make wine.

Organic acids. Differences in organic acid composition between field locations were found. Significantly higher concentrations of oxalic acid levels were found between early collected control Cabernet Sauvignon vines from the Davis and Foresthill locations. For the inoculated Cabernet vines, significantly lower oxalic acid concentrations were found between the Davis location and the three other locations in the early collections. No significant differences in oxalic acid were found between locations for control or inoculated Pinot Noir vines in the early collection. No significant differences in oxalic acid were found between control and inoculated Cabernet vines in the late March/early April time point. Oxalic acid concentrations were significant higher in Lake County than in Foresthill in the late collected control Pinot Noir.

No significant differences were found in citric acid concentrations between early sap collected from control and inoculated Cabernet Sauvignon grapevines. Citric acid concentrations were significantly higher in early collected Lake County inoculated Pinot Noir xylem sap than sap collected at the three other locations. In the late collection, citric acid concentrations in control Cabernet Sauvignon vines from Hopland was higher than at the three other field locations. Late collected Davis control Pinot Noir had higher levels of citric acid than Foresthill and Lake County. Inoculated Davis Pinot Noir also had significantly higher citric acid levels than vines in Lake County.

Significant differences between locations were found in tartaric acid concentrations in the early collected xylem sap. Early collected inoculated Pinot Noir xylem sap had significantly higher concentrations in Hopland than the three other locations. Significantly higher levels of tartaric acid were found in late collected control Cabernet Sauvignon xylem sap from Hopland than in sap collected from the three other locations. In late collected inoculated Cabernet Sauvignon, Davis had significant higher concentrations than Lake County and Foresthill. In late

collected Pinot Noir, tartaric acid levels were significantly higher in Lake County than in sap collected from the other three locations in the control vines. The inoculated Pinot Noir vines in Lake County and Hopland vines had significantly higher tartaric acid levels than Foresthill.

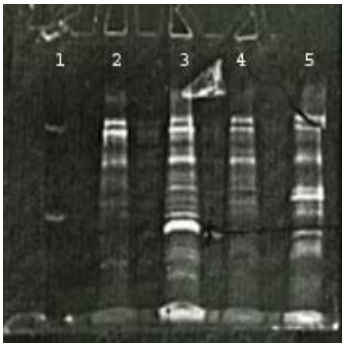
Significant differences between locations were found in malic acid concentrations in the early collected xylem sap. In early collected inoculated Cabernet Sauvignon xylem sap, Lake County had significantly higher levels than the three other locations. In early collected control and inoculated Pinot Noir, Lake County had significantly higher concentrations than the three other locations. In late collected Cabernet Sauvignon, significantly higher levels of malic acid were found in the control Lake County vines than the vine in Davis. In the *X. fastidiosa*-inoculated Cabernet vines, significantly higher levels of malic acid were found in Lake County than in Hopland and Foresthill. No significant differences were found between late collected Pinot Noir.

Analysis of organic acids from cold chamber treated grapevine xylem sap showed that there were no significant differences in oxalic acid concentration between chamber treatment or variety. Citric acid analysis showed that non-inoculated Cabernet Sauvignon vines at 0°C had significantly lower levels than the -5°C and 2.2°C treated vines. Inoculated Cabernet Sauvignon vines showed significantly higher concentrations of citric acid in the -5°C treatment than the 0°C and +5°C treatments. Both inoculated and control Pinot Noir vines showed significantly higher amounts of citric acid in the -5°C treatments than the 0°C, 2.2°C and +5°C treated vines.

No significant differences in tartaric or malic acid were found in the non-inoculated Cabernet Sauvignon treatment. The inoculated Cabernet Sauvignon treatment showed significantly higher levels of both tartaric and malic acid content in the -5°C treatment than the +5°C treatment. The Pinot Noir control treatment showed significantly higher tartaric acid content in the 2.2°C treatment than the three other treatments. The Pinot Noir inoculated treatment showed significant higher concentrations in the 2.2°C treatment than the 0°C treatment. Both the control and inoculated Pinot Noir showed significantly higher malic acid content in the 2.2°C treatment than the 0°C and +5°C treatment.

Water content and xylem sap protein profiles. No significant differences between fresh and dry masses were found between any of the treatments. The protein profiles indicate that there were many proteins of similar molecular weight for the four cold chamber temperatures treatments (Fig. 1). Fifteen to 30 protein bands were typically resolved for each treatment sample. Some differences in banding profiles were observed between varieties, but the main difference that was observed was between temperature treatments. Few differences were found between control and inoculated vines. A few protein bands that were unique or found in higher quantity were found in the cold stressed and/or *X. fastidiosa*-inoculated plants and these proteins were end terminally sequenced by the UCD Molecular Structure Facility. Sequence analysis 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, a homolog of which is reported to have anti-fungal properties. None of the proteins that were analyzed from inoculated vines were *X. fastidiosa* proteins.

Figure 1: Protein profile of grapevine xylem sap. 150 uL of xylem sap was precipitated with cold acetone. Proteins were resuspended in 30 uL of SDS-loading buffer and loaded in to a BioRad 12% Tris-HCl gel.



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.

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 *Xylella fastidiosa* 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 1.5×10^7 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 *X.f.* can survive at 28ºC in most media (except water). The results also indicate that *X.f.* 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, *X.f* rapidly died in all liquid media tested.

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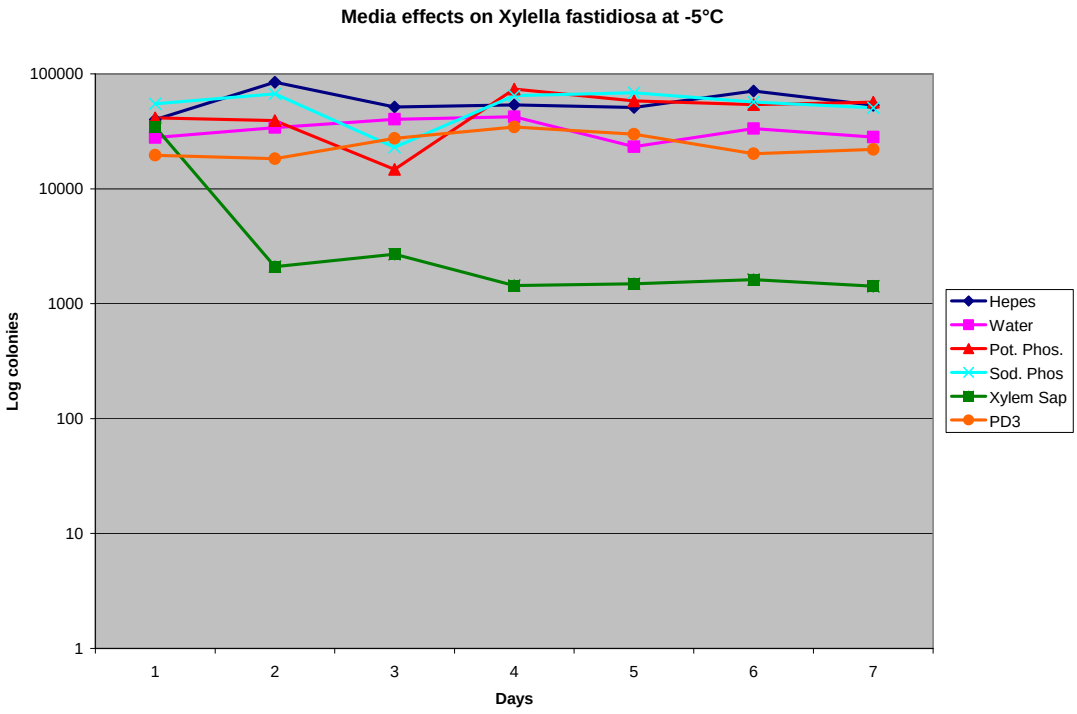


FIGURE 2: Media, buffers and xylem sap effects on X. fastidiosa growth following incubation at -5 C.

Potassium phosphate buffer was used to determine the effects of pH on the survival of *Xylella fastidiosa*. Samples were prepared like above, the cells were placed in potassium phosphate buffer at the pH 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.

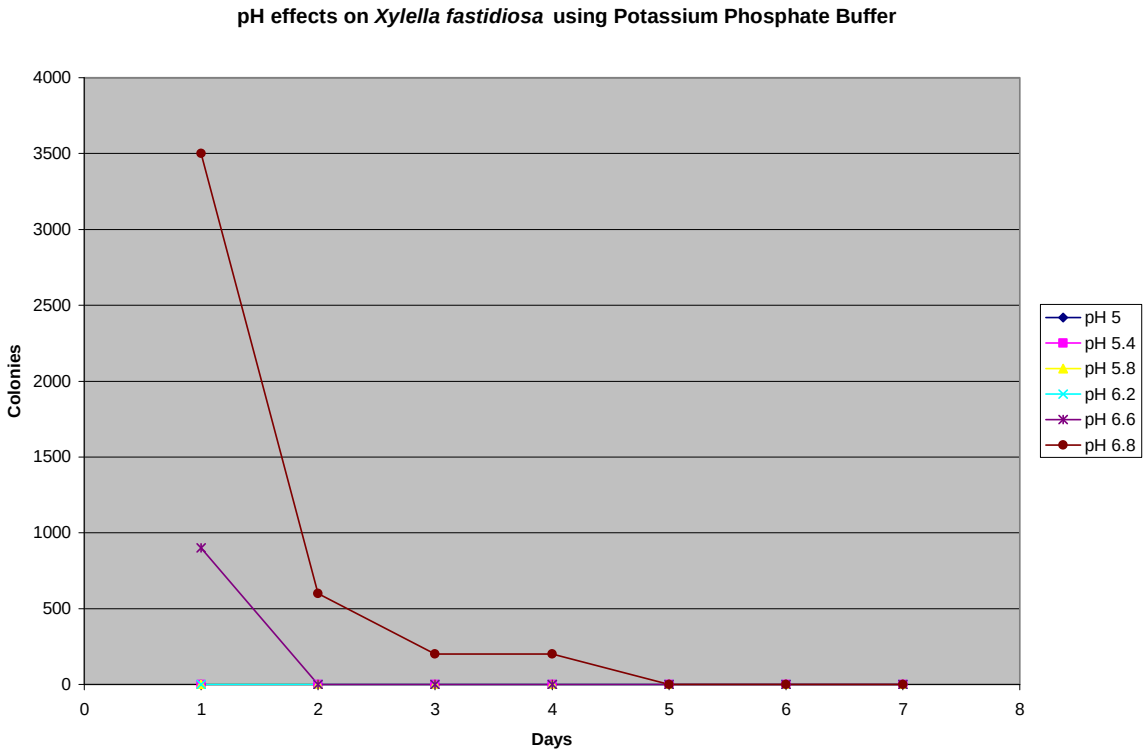


FIGURE 3: Effects of pH on growth of Xf in potassium buffers at various pH values.

Objective 4) Determine if foliar or drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezing conditions.

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 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 Sauvingnon field materials collected from El Dorado and Yolo counties in February 2004, and again in March 2005, showed abscisic acid concentrations were lower in the El Dorado County, cold-exposed vines, that vines from Yolo County. ABA concentrations were lower in Pinot Noir than Cabernet Sauvignon for both El Dorado and Yolo County vines.

We determined 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.

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 *X.*

fastidiosa-inoculated Cabernet Sauvignon and Pinot Noir vines grown and inoculated with Xf as described in Objective 1 were sprayed with solutions of ABA in the fall. The 2005-2006 results showed interesting trends and were repeated in 2006-2007.

2006-2007 Results:

To evaluate the reproducibility of the 2005-2006 results a second ABA spray trial was conducted in the 2006-2007 season. In fall 2006 there were 4 treatments with *X. fastidiosa*- infected vines and healthy controls:

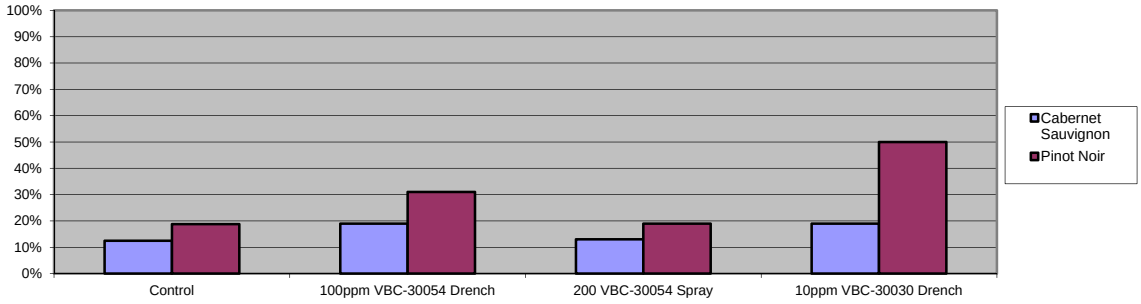
Control: 16 Pinot/ 16 Cabernet plants sprayed with water
2000ppm spray: 16 Pinot/ 16 Cabernet plants sprayed with VBC-30054
100 ppm drench: 16 Pinot/ 16 Cabernet plants drenched with VBC-30054
10 ppm drench: 16 Pinot/ 16 Cabernet plants drenched with VBC-30030

To determine effectiveness of ABA and synthetic ABA treatments on *X. fastidiosa*-infected grapevines, the vines were evaluated for PD symptoms and tested by IC-PCR in the late summer, 2007.

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 as seen above. Curing rates were not the same 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, but the curing rate was not as high as in the 2005-2006 season (Figure 4). 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.

Figure 4: Percent curing of *Xylella fastidiosa* infected grapevines in the 2006-2007 experiment.



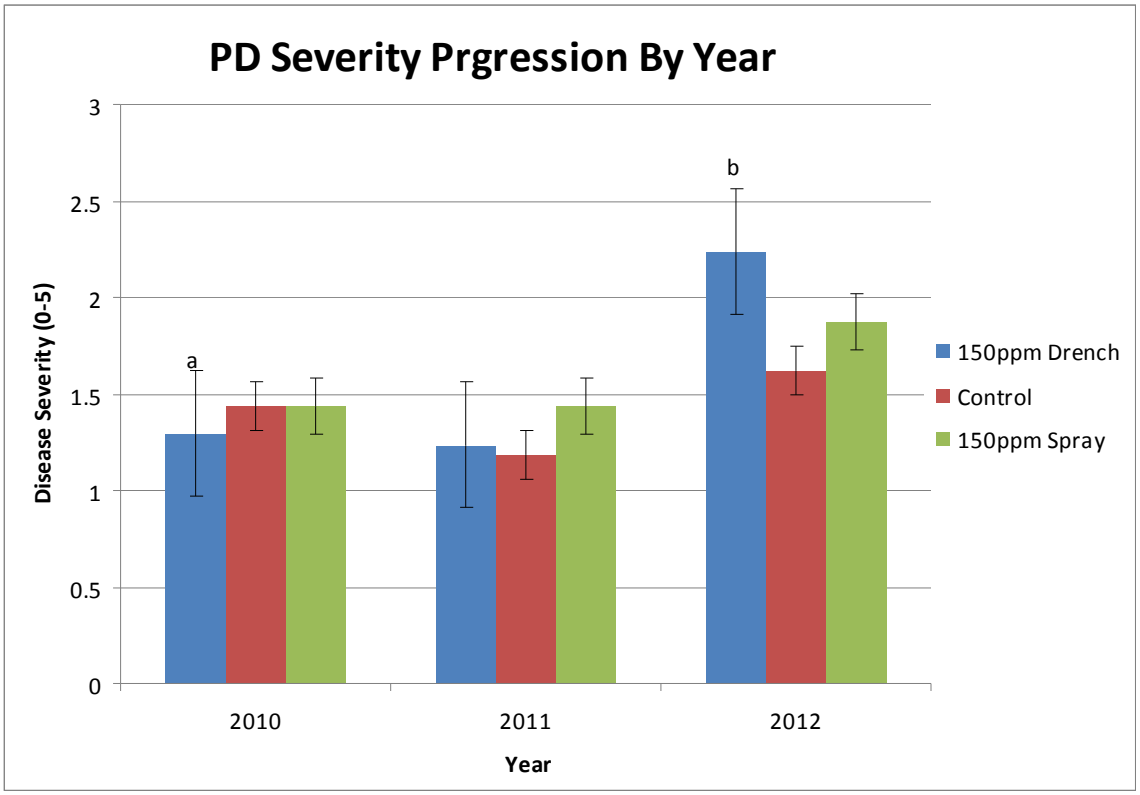
ABA Field Evaluations

In August 2010 we mapped and rated PD disease symptom severity of 45 Reisling vines, approximately 4 years old, that were growing in a vineyard near the Napa river. The majority of these vines had mild symptoms of PD, rated as a disease severity of 1 on a scale of 0 (healthy) to 5 (dead); approximately 3 to 4 vines in each group of 15 treatment and control vines were rated as a symptom category of 2.

15 of those vines were sprayed to run off with a 150ppm ABA solution prepared from a commercially available ABA product (Protone, marketed and kindly provided by Valent Chemical company). Approximately 2.5 L of ABA solution was sprayed with a backpack sprayer on each vine. A separate group of 15 PD-affected vines were drenched with 3 gallons each of a 150ppm solution ABA. The drench solution was applied into a shallow depression made on either side of the trunk of the vine. 15 diseased vines were left as untreated controls.

In August 2011 all of the vines were rated for PD symptom severity and the spray and drench applications were repeated on the same vines that were treated in 2010. In 2012 all of the vines were rated for PD symptoms and ABA applications were not repeated as analysis of PD disease severity showed no significant differences between the spray or drench treatments and the non-treated PD-affected control vines as shown in Figure 5 below. Actually the only significant difference that occurred was an INCREASE in the disease severity on the drench treatment rated in 2012.

FIGURE 5

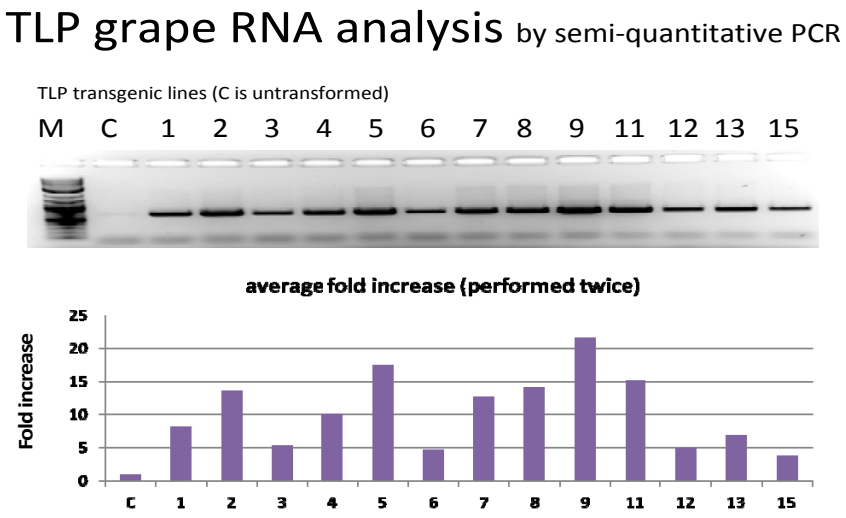


Field applications of ABA to Reisling grapevines with mild symptoms of PD, showed that vines treated by spraying or drench 150ppm of ABA did not result in any reduction in PD symptoms which we previous observed in experiments using 1 gallon potted grapevines (Meyer and Kirkpatrick, 2011). This is most likely due to the much larger mass of roots and canopy in the field vines compared to potted vines. Pressure injections of ABA into field vines may be one

option for increasing the efficacy of ABA application, but it is unlikely that a grower would be willing to go with the extra cost of vine trunk injections.

Objective 5a: Over express the grapevine thaumatin-like protein (TLP) in transgenic grapevines. Characterize the levels of TLP expression in the TLP-transgenic lines using qRT-PCR.

The wt TLP gene from Thompson seedless grapevines was cloned into an Agrobacterium transformation vector that was developed in the Dandekar lab. This vector has signal peptide sequences that facilitate the expression and translocation of the TLP into grapevine xylem. We received 15 independently transformed TLP transgenic lines from the UC Davis Plant Transformation facility, 2 of those lines died before they could be vegetatively propagated. RNA was extracted from the remaining 13 lines as well as non-transformed Thompson seedless grapevine controls using standard procedures. Equal amounts of total RNA were analyzed by a semi-quantitative RT PCR (qRT PCR) using primers specific for the grapevine TLP. **Figure 6** below show the results of this analysis:



All of our lines showed over-expression of TLP mRNA as compared to non-transgenic, wild type plants. We were please to find that TLP expression in greenhouse grown non-transgenic grapevines was quite low compared to the transgenic lines so if there is any benefit to over expressing TLP in "warm" temperature grown vines we should be able to see some phenotypic differences especially in the 7 lines whose TLP expression was 10 fold or greater than the non-transgenic control vines.

Objective 5b) Inoculate TLP-expressing grapevines with Xf and determine the incidence and severity of PD in transgenic versus non-transgenic *V. vinifera*. Quantify Xf populations in transgenic lines and non-transgenic controls.

Ten reps of the TLP transgenic vines (13 distinct lines) and wild-type Thompson Seedless grapevines were mechanically inoculated using two 20µl drops of 10^7 (as measured by optical

density) *Xf* Fetzer cells suspended in PBS. Control Thompson Seedless and TLP vines were inoculated with PBS. These vines were kept in the greenhouse and were observed for PD symptom development over 17 weeks. When symptoms first became apparent, two of the TLP lines appeared to develop symptoms more rapidly than the Thompson Seedless plants. These differences proved to be not statistically significant. The Thompson Seedless control vines and TLP-transgenic vines progressed through PD symptom development with no significant differences (Figures 7 and 8). At 15 weeks bacterial cells were isolated from the point of inoculation (POI) as well as 25cm above POI. There was no significant difference in Xf populations in TLP transgenics versus non-transgenic controls.

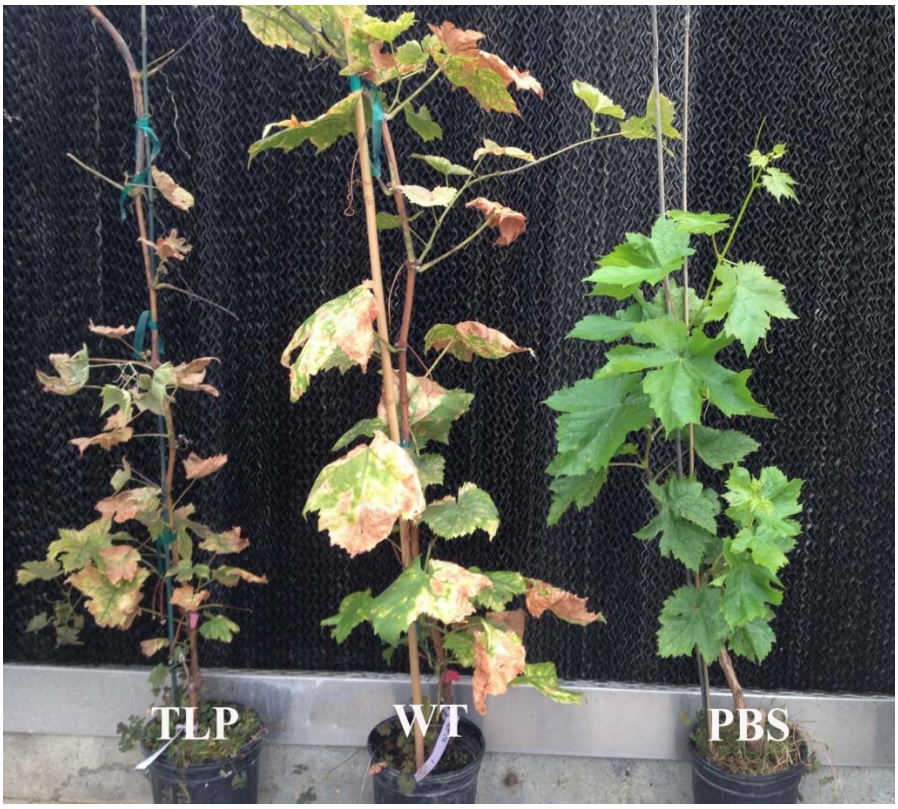


Figure 7: Representative PD symptoms in TLP-transgenic and non-transgenic Thompson seedless vines mechanically inoculated with wt Fetzer Xf or PBS controls 15 weeks post inoculation.

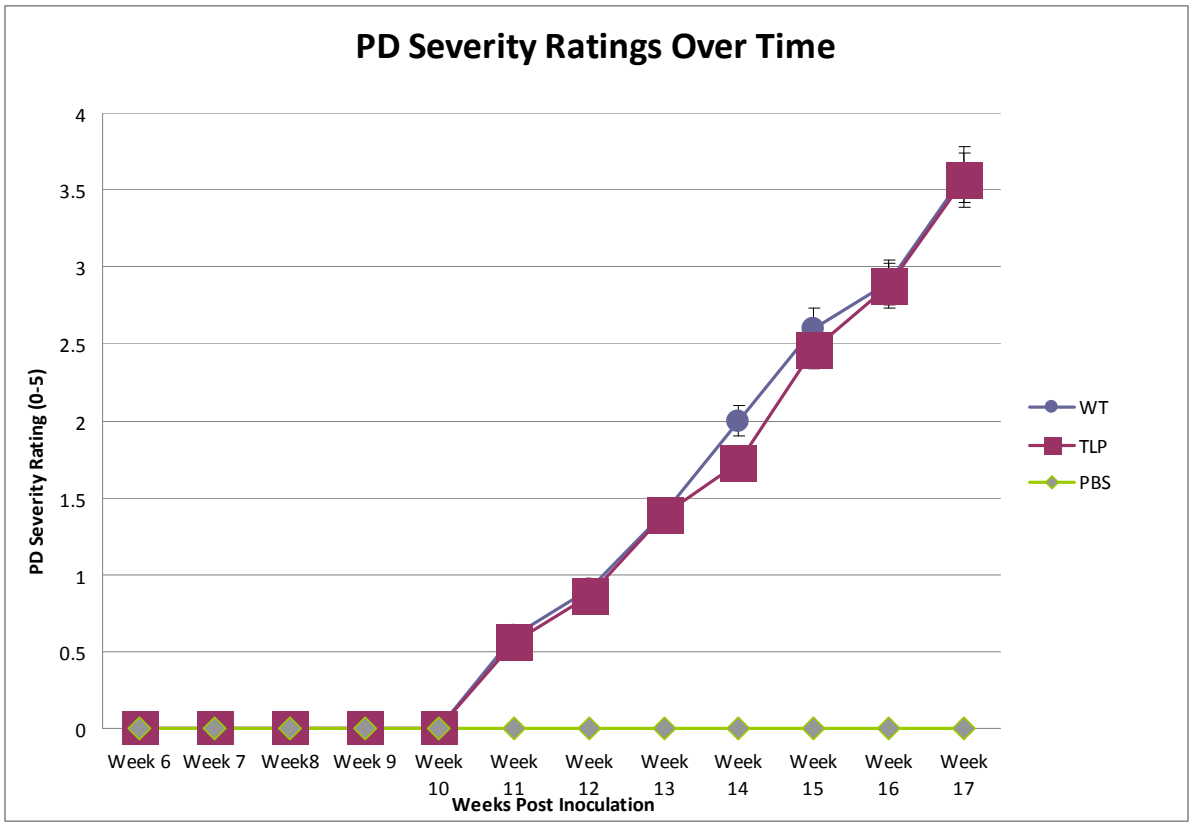


Figure 8: PD symptom severity following mechanical inoculation TLP-expressing and non-transgenic grapevines inoculated with wt Fetzer *X. fastidiosa* or PBS buffer.

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Kirkpatrick, B. 2012 identification and utilization of cold temperature induced metabolites to manage Pierce's disease. Pierce's disease Research progress Reports, California Department of Food and Agriculture. pp. 137-141

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