

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

Principal Investigator:

Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA 95616
bekirkpatrick@ucdavis.edu

Cooperators:

Melody Meyer
Department of Plant Pathology
University of California
Davis, CA 95616
mmmeyer@ucdavis.edu

Rodrigo Almeida
ESPM
University of California
Berkeley, CA 94720
rodrigo@nature.berkeley.edu

Richard Bostock
Department of Plant Pathology
University of California
Davis, CA 95616
rmbostock@ucdavis.edu

Abhaya Dandekar
Department of Plant Sciences
University of California
Davis, CA 95616
amdandekar@ucdavis.edu

Dean Gabriel
Department of Plant Pathology
University of Florida,
gabriel@biotech.ufl.edu

Andrew Waterhouse
Department of Enology and Viticulture
University of California
Davis, CA 95616
alwaterhouse@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2009 to October 2009.

ABSTRACT

This work builds on discoveries made in the past six years of research on better understanding the mechanism(s) responsible for the PD(PD) cold curing phenomenon. A thaumatin-like (TLP) grape protein was found in elevated levels in the xylem sap from cold-exposed vines. We have cloned and expressed TLP in *E. coli* and our preliminary finding show that crude TLP protein extract possesses anti-*Xylella fastidiosa* (*Xf*) activity *in vitro*. Greater amounts of total phenolics were measured in xylem sap extracted from cold-exposed vines. We are beginning to characterize these phenolic compounds, and assess their potential anti-*Xf* activity *in vitro*.

LAYPERSON SUMMARY

Previous work on "cold curing" of Pierce's disease (PD) affected grapevines led to the identification of thaumatin-like protein (TLP) in grapevine xylem sap. TLP is expressed in greater amounts in vines that have been exposed to cold temperatures and may be associated with the cold curing phenomenon. Currently we have cloned and expressed TLP in *E. coli*. Producing TLP in *E. coli* should allow us to produce enough protein to better evaluate the role of TLP in curing of PD. Crude extracts of *E. coli* expressing TLP were applied to PD3 medium plates and initial results showed TLP-amended medium plates greatly inhibited the growth of one strain of *Xylella fastidiosa*. We are currently working on producing purified and biologically active TLP. Previous work also identified polyphenolic compounds as a possible mediator of the "cold curing" phenomenon. Here, we show that a specific polyphenolic compound, resveratrol, is produced in vines that experience cold curing, while it is absent from grapevines grown in warmer environments.

INTRODUCTION

In our previous project we characterized many biological parameters of xylem sap from cold-exposed (freezing temperatures) and "warm", (non-freezing) temperatures in both field grown and cold chamber exposed grapevines. We found that *Xylella fastidiosa* (*Xf*) infected potted grapevines that were exposed to freezing temperatures at several sites in northern California and vines exposed to -5C in cold chamber emerged pathogen free the following summer (Meyer and Kirkpatrick, 2004-2008). We measured many different biological parameters, such as pH, organic acid, sugar and ion concentrations, and osmolarity in Pierce's disease (PD) susceptible *Vitis vinifera* 'Pinot Noir' and PD-less susceptible *V. vinifera* 'Cabernet Sauvignon' grapevines over three winters.

One of the parameters determined in these previous studies was the protein profiles of cold- and warm-treated xylem sap. One of these proteins, a thaumatin-like protein (TLP), was significantly up regulated in cold exposed vines. We have cloned and expressed the *V. vinifera* TLP protein and showed some inhibition of *Xf* growth when crude protein extracts from TLP-expressing *E. coli* were applied to PD3 medium plates. Work is currently being conducted to purify and demonstrate the biological activity of recombinant TLP protein.

We have also been assessing the potential role that xylem sap phenolic compounds may play in the "cold curing" process. In collaboration with the Waterhouse lab at UC Davis, we have characterized the phenolic compounds in cold and warm xylem sap by HPLC/MS, and identified that the major polyphenol in cold-exposed xylem sap is trans-resveratrol. We have also begun to evaluate the potential toxicity of trans-resveratrol to *Xf*.

OBJECTIVES

1. Over-express the grapevine thaumatin-like protein (TLP) in transgenic grapevines. Prepare anti-TLP antibodies to quantify TLP in transgenic xylem sap using ELISA and western blot analyses..
2. Inoculate TLP-expressing grapevines with *Xf* and determine the incidence and severity of PD in TLP-transgenic versus non-transgenic *V. vinifera*.
3.
 - a. Fractionate and chemically characterize the phenolic compounds that are present in xylem sap from cold-exposed grapevines.
 - b. Compare the phenolic compound composition and concentration in xylem sap extracted from cold- and warm-exposed *V. vinifera* grapevines as well as grapevines treated with abscisic acid (ABA) under non-freezing conditions.
 - c. Determine if these compounds affect *Xf* growth/survival *in vitro*.
4. Determine if foliar and drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezing conditions.

RESULTS AND DISCUSSION

We have cloned and expressed *Vitis vinifera* thaumatin-like protein in *E. coli* (**Figure 1**). We have used various methods of protein expression and purification to attempt to produce TLP protein that is both relatively pure and biologically active. The presence of disulfide bonds in the protein structure has presented some challenges as much of the recombinant TLP protein is found as inclusion bodies in *E. coli*. We are continuing to try different approaches to overcome some of the problems we have experienced.

Xylem sap total phenolics from ABA-treated and non-treated controls have been measured. Trends showing that total phenolics were found in higher concentrations in cold-exposed vines were also seen in the ABA-treated vines (Meyer and Kirkpatrick, 2008). In addition, the phenolic content in ABA-treated vines was higher than non-treated vines (**Figure 2**).

Xylem sap was expressed from dormant ‘Cabernet Sauvignon’ and ‘Pinot Noir’ grape vine canes obtained from the Chateau Leidigh Estate Winery located in Placer County in February, 2009. Previous work has shown that grape vines infected with *Xf* 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 decided to analyze trans-resveratrol’s potential anti-*Xf* activity *in vitro* because it was the most abundant polyphenol in cold xylem sap based on the RPHPLC/MS analyses. Resveratrol has also been shown to have positive effects on human cardiovascular health, and negative effects on several diverse microbes including certain bacteria (Chan 2002, and Frémont 2000), life, which further justified our interest in evaluating its effects on *Xf*.

Commercially available Trans-Resveratrol from Sigma-Aldrich (product number R5010) was used in a plate based assay. A 7.5 µg/ml stock solution of Trans-Resveratrol was prepared by dissolving 7.5 mg of trans-resveratrol in 100 ml of water overnight, and in the dark to prevent isomerization to the cis-resveratrol form (Bonnefont-Rousselot *et al.* 2009). Solid PD3 media was autoclaved for 15 min and allowed to cool to approximately 50-60C (comfortable to the touch). 100 µl and 250 µl of the trans-resveratrol stock solution were added to 500 mls of PD3 media to achieve concentrations of 15 ng/ml and 38ng/ml respectively. These values were chosen based on the concentrations of trans-resveratrol detected in xylem sap from cold exposed grapevines. The plates were allowed to solidify in the dark for two hours. After solidification, approximately 10 µl of a 10⁸ CFU/ml suspension of *Xf* ‘Fetzer’ or *Xf* ‘Temecula’ were spread onto each plate using a plastic inoculating loop. These plates were then placed in a crisper which had been wrapped with aluminum foil, to prevent light from causing isomerization of the trans-resveratrol, and incubated for 10 days at 28C. Both strains grew well on PD3 plates which contained no trans-resveratrol, while there was no apparent growth of *Xf* Temecula on the reserveratrol supplemented PD3

plates. Surprisingly, similar growth inhibition was not observed with the 'Fetzer' strain (**Figure 3**). This experiment has only been performed once and it is now being repeated with the same, as well as additional, strains of *Xf*.

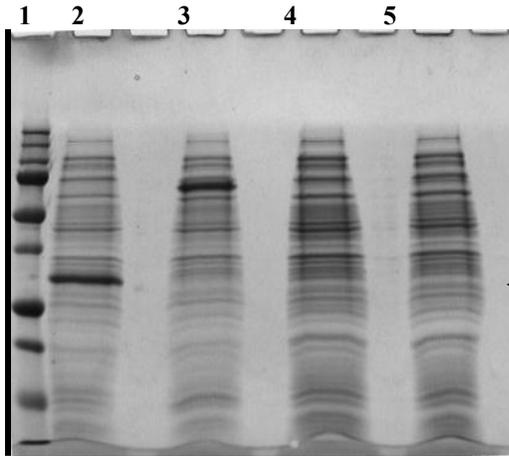


Figure 1: Thaumatin-like Protein (TLP) that was cloned and expressed using a *E. coli* expression vector. Note that arrow points to the correct size of grapevine TLP protein (~35 kD).

Lane 1: Dual color SDS ladder.
Lane 2: Cell lysate from IPTG induced *E. coli* with TLP construct.
Lane 3: Cell lysate from IPTG induced *E. coli* with a polygalacturonase (PG) construct (positive control).
Lane 4: Cell lysate from *E. coli* with TLP construct, not induced.
Lane 5: Cell lysate from *E. coli* with PG construct, not induced.

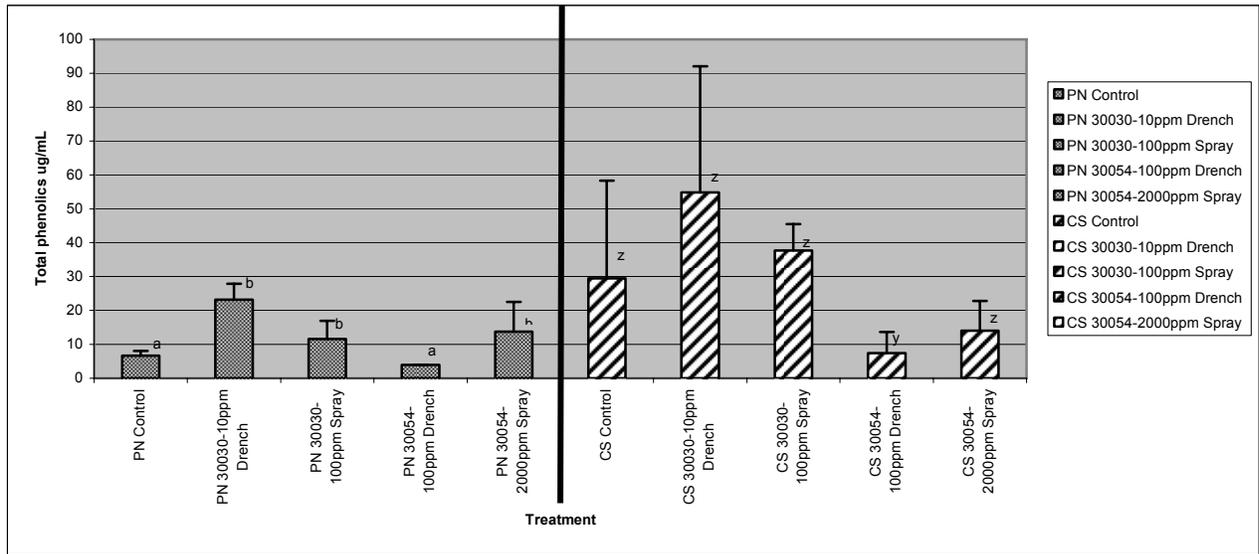


Figure 2. Total phenolic content of xylem sap from ABA treated vines as measured by a gallic acid colorimetric assay. PN = Pinot noir xylem; CS = Cabernet sauvignon xylem sap. 30054 = a natural ABA, 30030 a chemically modified ABA. Different letters are significantly different by unpaired t-test with a 2-tailed p-value ≤ 0.05

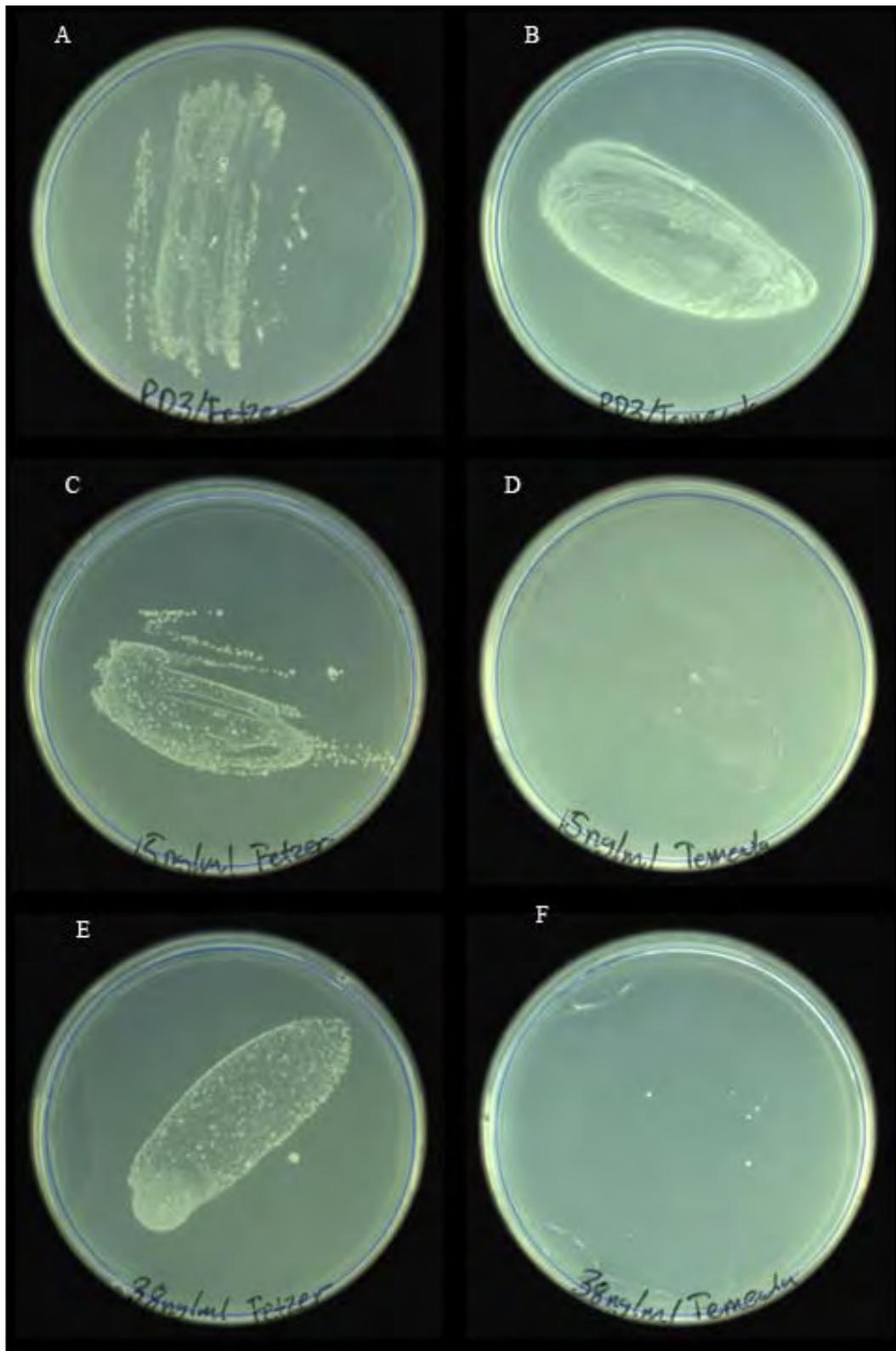


FIGURE 3. Results of plating Xf Fetzer and Xf Temecula on PD3 media (A and B) and PD3 media containing 15 ng/ml Resveratrol (C and D) and 38 ng/ml Resveratrol (E and F). The PD3 plates containing Resveratrol and Xf Temecula showed much less growth than Xf Fetzer at the same Resveratrol concentrations

CONCLUSIONS

Though the production of a purified and biologically active TLP protein has had its challenges due to the formation of TLP inclusion bodies, we believe that TLP protein in the inclusion bodies can still be used in the production of good quality anti-TLP antibodies. Efforts using other expression systems and methods to denature and renature the TLP inclusion bodies are

underway. The anti-TLP antibodies will be used to quantify TLP in xylem sap from cold and ABA-treated grapevines and detect TLP in transgenic grapevines. Assuming that TLP expressed in grapevine sap has increased anti-*Xf* activity, it is possible that TLP-expressing transgenic grapevines could decrease the incidence and/or severity of PD.

Phenolic compounds, specifically trans-resveratrol, show promise as agents that are harmful to the growth of at least one strain of *Xf*. The results of our plate assay are supported by the fact that we detected no resveratrol in warm winter sap collected in Davis, where we observe significantly less overwinter curing than in Placerville. It has been previously reported that resveratrol production in *Vitis vinifera* can be up-regulated by several diverse factors such as plant injury, UV light exposure, and pathogen invasion (Pryce *et al.* 1976, and Gautheron *et al.* 1991). It is possible that cold temperatures may serve as an external stress that increases the production of trans-resveratrol.

REFERENCES CITED

- Chan, Marion Man-Ying. Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. *Biochemical Pharmacology*, January 2002, p. 99-104.
- Cowan, Marjorie Murphy. "Plant Products as Antimicrobial Agents" *Clinical Microbiology Reviews*, October 1999, p. 564-582, Vol. 12, No. 4.
- Frémont, Lucie. "Biological effects of resveratrol" *Life Sciences* Volume 66 No.8, January 2000, p. 663-673.
- Jeandet, Phillipe; Bessis, Roger; and Bernard Gautheron. The production of resveratrol (3,5,4'-trihydroxystilbene) by grape berries in different developmental stages. *Am. J. Enol. Vitic.* 42:1:41-46 (1991).
- Kantz, K. and V.L. Singleton. 1990. Isolation and determination of polymetric polyphenolics using Sephadex LH-20 and analysis of grape tissue extracts. *Am. J. Enol. and Viticul.* 41:223-228.
- Langcake, P and Pryce, RJ. "The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury *Physiological Plant Pathology*) p. 77-86 (1976).
- Meyer, M. and B. Kirkpatrick. Identification of mechanisms mediating cold therapy of *Xf*-infected grapevines. *Proceedings of the PD Research Symposium, Calif. Dept. Food and Agriculture*; 2005 pp242-246; 2006 pp. 236-239; 2007 pp. 208-211; 2008 pp. 167-171.
- Kuwabara, C., D. Takezawa, T. Shimada, and K. Arakawa. 2002. Abscisic acid and cold-induced thaumatin-like protein in winter wheat has an antifungal activity against snow mold, *Microdochium nivale*. *Physiolgia Plantarum* 115: 101-110.
- Purcell, A.H. 1980. Environmental therapy for PD. *Plant Disease* 64:388-390.
- Toshitsugu Taguri, Takashi Tanaka and Isao Kouno, "Antimicrobial Activity of 10 Different Plant Polyphenols against Bacteria Causing Food-Borne Disease", *Biol. Pharm. Bull.*, Vol. 27, p. 1965-1969 (2004).
- Waterman, P.G. and S. Mole. 1994. *Analysis of phenolic plant metabolites*. Blackwell Scientific Publications, pp. 238.

FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

ACKNOWLEDGMENTS

We would like to thank Mauri Anderson and George Kasun for their work on the phenolics and contributions to this report. We would also like to thank Bob Leidigh of Chatuea Leidigh Estate Winery for allowing us to use grapevine materials from his vineyards.