FINAL REPORT FOR CDFA AGREEMENT NUMBER 15-0215-SA.

PROJECT TITLE: HIGH-THROUGHPUT LIVE CELL SCREEN FOR SMALL MOLECULES TARGETING TOLC EFFLUX PUMP OF *XYLELLA FASTIDIOSA*.

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REPORTING PERIOD: The results reported here are from work conducted July 1, 2015 to June 30, 2017.

ABSTRACT

Type I secretion (T1S) by *Xylella fastidiosa* (Xf) is required for multidrug efflux, a pump critical for survival of Xf in grapevines. In Xf, T1S depends on a very limited number of genes, possibly making this system more vulnerable to inhibition by small molecule treatments than T1S found in most bacterial pathogens, which typically carry redundant T1S systems. Xf single gene mutations in the T1S system are much more sensitive to the surfactant Silwet L-77 than wild type Xf. High throughput screening assays of Xf cell viability were developed using fluorescence and optical density measurements both with and without 200 ppm Silwet L-77. GFP-marked Xf strain Temecula1 was used to screen two Prestwick combinatorial small molecule libraries (phytochemical and FDA approved drugs; 1600 chemicals in total) for Xf cell growth inhibition. Significant (>50%) inhibition of Temecula1 growth was observed in presence of 50 µM of 215 different chemicals, 6 of which exhibited even higher (24% - 40%) stronger inhibition in the presence of Silwet L-77, indicating these 6 chemicals possibly target T1S efflux. Forty-six chemicals reduced growth >100%, indicating Xf cell lysis. Seven chemicals, including four phytochemicals, reproducibly lysed Xf at 25 µM levels. Four of these chemicals were eliminated from further consideration because they have pharmaceutical uses and would likely face severe regulatory hurdles. Three chemicals were further evaluated as potential treatments for PD by both soil drench and spray applications and for phytotoxicity to grape and tobacco leaves. One was strongly phytotoxic to grape leaves at 25 mM levels and was eliminated from further consideration; one appears only slightly toxic at 50 mM levels, based on chlorophyll degradation assays. Two chemical treatments identified in this screen showed showed significant potential for chemical control of PD when applied as soil drenches and/or foliar sprays, and significantly reduced progression of disease symptoms (54-59% compared to untreated controls) over a period of three months.

LAYPERSON SUMMARY OF ACCOMPLISHMENTS

- **Two significant leads from the high-throughput bioassay were identified as (a) lytic at 25 µM concentration, (b) low cost, and (c) likely of low regulatory concern.**
- **Treatment A was significantly phytotoxic to grape leaves at 25 mM concentration, but treatment B was only slightly phytotoxic at 50 mM levels.**
- **Foliar spray treatments of both A and B resulted in only marginal phytotoxicity. Soil drench application had no visible phytotoxic symptoms for either treatment.**
- **Treatments A and B were evaluated as potential treatments for PD applied either as soil drench or foliar spray. PD disease severity was reduced by approximately 40% by both treatments and both application methods.**

INTRODUCTION

This is a new project that is based on two discoveries made during the course of two earlier CDFA funded projects. The first discovery is our demonstration that the Type I multidrug resistance (MDR) efflux system of *X. fastidiosa* (Xf) is absolutely required for both pathogenicity and even brief survival of the Pierce's Disease (PD) pathogen in grape (Reddy et al., 2007). Knockout mutations of either *tolC* or *acrF* (manuscript in preparation) render Xf nonpathogenic, and in addition, the *tolC* mutants were so highly sensitive to grape chemicals that the mutants are not recovered after inoculation. Inoculation of very high titers of Xf strain Temecula *tolC* mutants in grape results in rapid, 100% killing of inoculated bacteria. These results demonstrated a critical role for Type I efflux in general and TolC and AcrF in particular for defensive efflux by Xf of plant antimicrobial compounds, such as phytoalexins.

In the process of investigating the increased sensitivity of the MDR efflux mutants to plant-derived antimicrobial chemicals, we also discovered that even wild type Xf, with its lone MDR efflux system, is much more sensitive to plant-derived antimicrobial chemicals than most other plant pathogens, which carry multiple efflux systems. Both *tolC* (encoding the outer membrane and periplasmic tunnel component of Type I secretion) and *acrF* (encoding the inner membrane pump component of Type I secretion) are essential for MDR efflux in Xf, which has only one copy of each gene and only one such MDR efflux system. By contrast, most plant pathogens have redundant MDR efflux systems and multiple *tolC* genes. These results suggest that Xf should be much more vulnerable to chemical treatments affecting Type I efflux than other bacterial plant pathogens.

MDR efflux mutants in other systems have provided proven, highly sensitive and quantitative screening methods for antimicrobial chemicals (Tegos et al., 2002). The goal of this project is to exploit the increased vulnerability of Xf and our knowledge of particular chemicals that require efflux in a high throughput assay that screens small molecule combinatorial libraries and Xf-resistant grapevines for chemicals that may disable Type I secretion directly or indirectly. A highly sensitive live cell assay that is well suited for high throughput screening was developed and used for this screening.

OBJECTIVES

The specific objectives of this one year proposal are:
1. Screen two Prestwick combinatorial libraries for chemicals affecting Type I efflux from Xf.
2. Screen sap and crude extracts from *V. vinifera* grape plants subjected to freezing treatments (sufficient to cure PD) for potential effects on Type I efflux from Xf.
3. Determine if sap and crude extracts from PD resistant *Muscadinia rotundifolia* contain more and/or more effective chemicals affecting Type I efflux from Xf than susceptible *V. vinifera* plants.

RESULTS AND DISCUSSION

Objective 1: Screen two Prestwick combinatorial libraries for chemicals affecting Type I efflux from Xf.

Initial experiments focused on Xf culturing conditions (starting optical density and cell volumes) that would be adequate to obtain reproducible results in a chemical screen for Xf growth using a 96 well microtiter plate format. Two day old cultures of GFP-marked Temecula1 cells (OD600= 0.25) were diluted to starting OD=0.05 and used for seeding 96-well microtiter plates for high throughput screening of the chemical libraries. Cell volumes of 100, 150, and 200 µl / well were tested at 28° C. Overall, 150 µl / well volumes were determined to be practical and reproducible for observing growth (refer Fig. 1). Bacterial growth was measured at 48 hours after inoculation, both as (correlated) increase in optical density (OD_{600}) and GFP fluorescence (excitation at 485/20 nm and emission at 528/20 nm) (Steff et al., 2001).

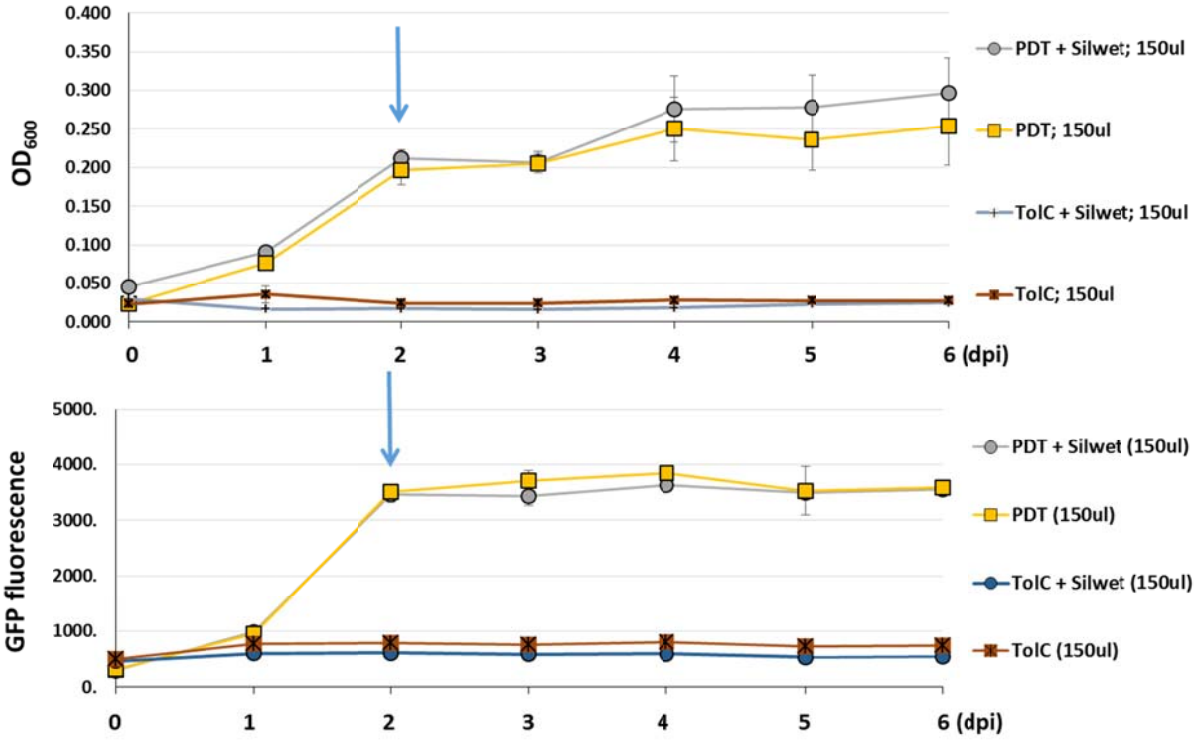

Fig. 1. Growth of Xf cells at a cell volume of 150 µl / well in a 96 well format. PDT, wild type PD strain Temecula-1. TolC, a *tolC* mutant of PDT. Silwet L77 (Silwet) was added at 200 ppm to both PDT and TolC for evaluation purposes.

As can be observed from Fig. 1, maximum growth and fluorescence emission was observed at 48 hrs after seeding the plates using 150 µl volumes, and therefore chemical treatments were added at the time of plate seeding, and effects of the treatments were evaluated 48 hrs later. Silwet L77 at 200 ppm had no effect on growth of the wild type strain Temecula-1.

For the primary chemical screens, plates were preloaded with Temecula-1 cells with or without 200 ppm Silwet L-77 and with each tested chemical loaded at a concentration of 50 µM. Each chemical in the Prestwick Phytochemical and Chemical libraries was screened in two separate experiments per library. The statistical parameter (Z′) was used to evaluate the quality of the assays exactly as described (Zhang et al., 1999). The overall Z′ value for the Prestwick Phytochemical library was 0.76 and the overall Z′ for the Prestwick Chemical library was 0.78; these values are within the statistically "excellent" reproducibility range (Z′ > 0.75; Zhang et al., 1999).

Significant growth inhibition (>50%) of Temecula1 was observed with 22 phytochemicals (Fig. 2), 8 of which exhibited strongly significant growth inhibition (>90%). Greater than 100% inhibition occurred when the optical density (data not shown) and the fluorescence emitted (Fig. 3) was reduced to below that of the starting cell values, and indicated lysis. None of the 320 phytochemical library compounds was found to enhance growth. None of the 320 phytochemical library compounds exhibited enhanced inhibition in the presence of 200 ppm Silwet L-77, indicating that none of these compounds directly affected T1S. Eleven phytochemicals, including some natural antibiotics, were identified as strongly inhibitory (> 80%) at 50 µM, including the phytoalexin gossypol and the alkaloids remerine and olivicine.

Significant growth inhibition (>50%) of Temecula1 was observed with 193 chemicals from the Prestwick Chemical library (Fig. 3), 121 of which exhibited strongly significant growth inhibition (>90%). Greater than

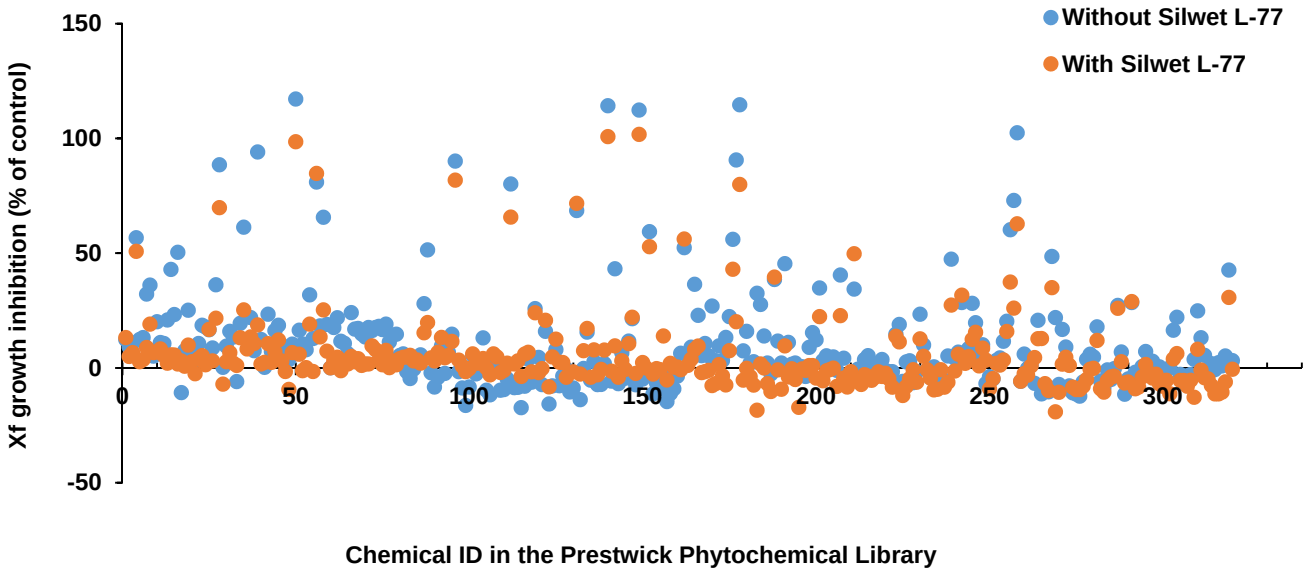

Fig 2. Screening of the Prestwick Phytochemical Library of 320 compounds for growth inhibition of Xf, both with and without Silwet L-77. Growth of PDT in the presence of 320 chemicals (numbered along the horizontal axis) both with (orange dots) and without (blue dots) 200 ppm Silwet L-77. Both OD and GFP fluorescence were measured. Plates were incubated at 28° C for two days, and both OD and GFP fluorescence again measured. Growth inhibition was calculated as the difference between the change in OD (not shown) or GFP fluorescence between treatments and the respective untreated control. Chemicals exhibiting at least 50% of growth inhibition relative to the respective untreated control were selected for additional screening at different concentrations (dose effect).

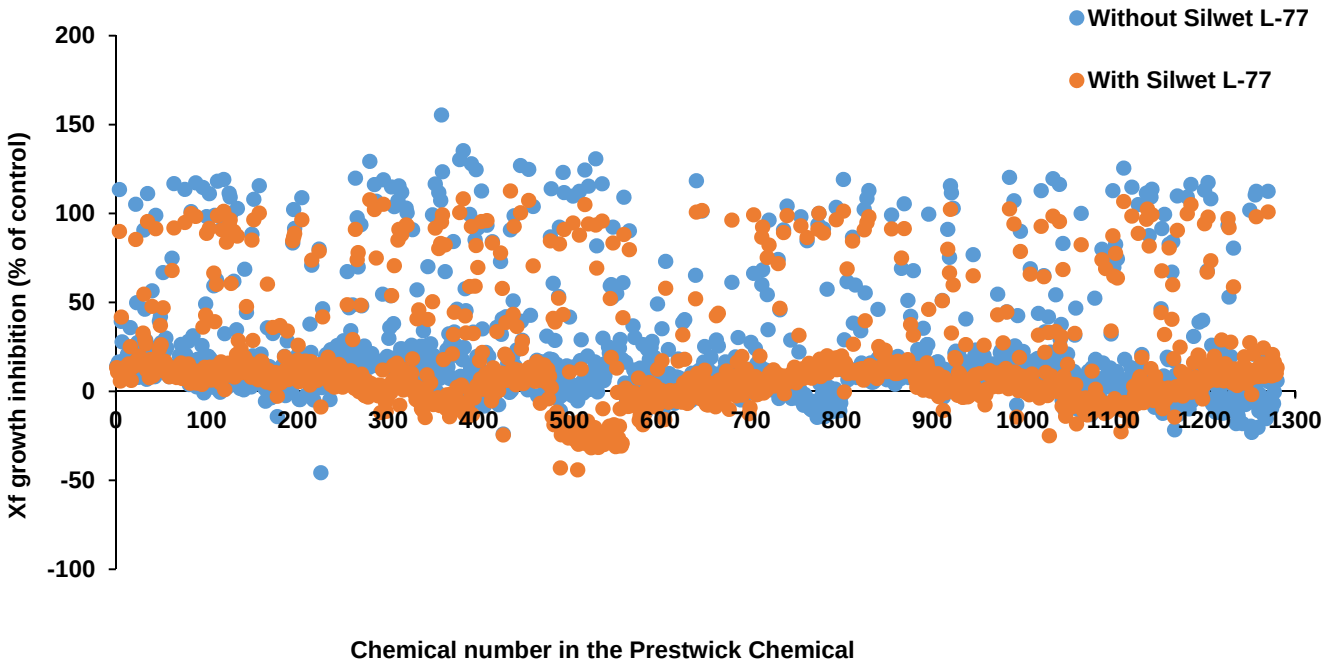

Fig 3. Screening of the Prestwick Chemical Library of 1,280 compounds for growth inhibition of Xf, both with and without Silwet L-77. Legend as in Fig. 2.

100% inhibition occurred when the optical density (data not shown) and the fluorescence emitted (shown in Fig. 3) was reduced to below that of the starting cell values, and indicated lysis. Notably, 6 chemicals exhibited not only direct growth inhibition (ranging from 53% - 90%), but this inhibition was enhanced (>24% more) by Silwet L-77, indicating that these chemicals possibly target T1S efflux. These chemicals include a thiazolide antiparasitic agent, several antibiotics and a calcium antagonist.

Following the primary screen at 50 µM, the effect of different dose levels (25 µM, µM, and 100 µM) were evaluated using 3 replications of each level, in each case with and without Silwet L-77. This evaluation was performed both for confirmation purposes and to determine if a threshold level effect was present for some chemicals. No threshold effects were observed; initial results were confirmed at all dose levels. Silwet L-77 had no effect on any of the phytochemicals. However, Silwet enhanced the inhibition of six compounds from the Prestwick chemical library in primary screen. At different dose levels, only one compound consistently inhibited Xf growth more strongly in the presence of Silwet L-77, and at all three treatment levels, indicating an effect of the chemical on multidrug efflux (Type I secretion).

Over 120 chemicals have been identified that inhibited growth of Xf by >90% @ 50 µM, including 46 chemicals that appeared to lyse Xf cells. Seven chemicals proved to lyse Xf cells at 25 µM, including four phytochemicals. Four of these chemicals were eliminated from further consideration because they have pharmaceutical uses and would likely face severe regulatory hurdles, and one due to cost considerations. Two chemicals are being further evaluated as potential treatments for PD by both soil drench and spray applications. Phytotoxicity to grape and tobacco leaves has now been evaluated using chlorophyl loss as a sensitive indicator of phytotoxicity (for example, refer Jain et al. 2012). Leaf disc punches from young fully expanded leaves were floated in water containing different levels of each phytochemical evaluated, both vacuum infiltrated and uninfiltrated (Fig. 4). Chlorophyll content in both grape and tobacco leaf discs was estimated after 3 days extracting overnight in 80% acetone and quantified spectrophotometrically according to the procedure of Arnon (1949). The results are presented in Fig 5, below:

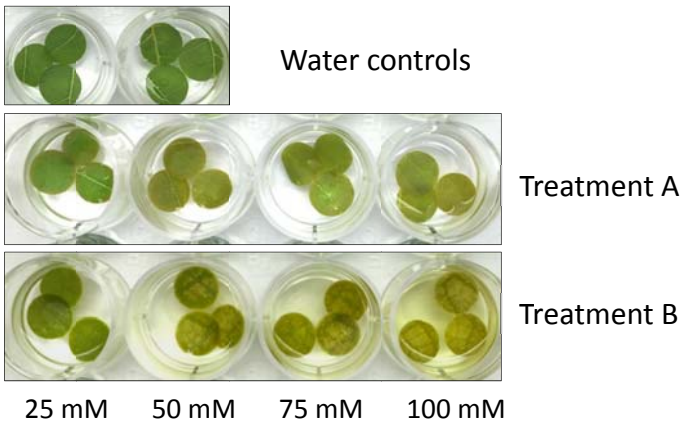

Fig. 4. Effect of two selected Prestwick chemical treatments at the indicated concentrations on chlorophyll degradation in grape leaf discs (photo taken after 18 hrs).

Clearly, treatment A was strongly phytotoxic to grape leaves at 25 mM levels and somewhat to tobacco leaves at the same level, whereas treatment B was phytotoxic to tobacco leaves at 25 mM but not to grape leaves until ca. 50 mM levels were used.

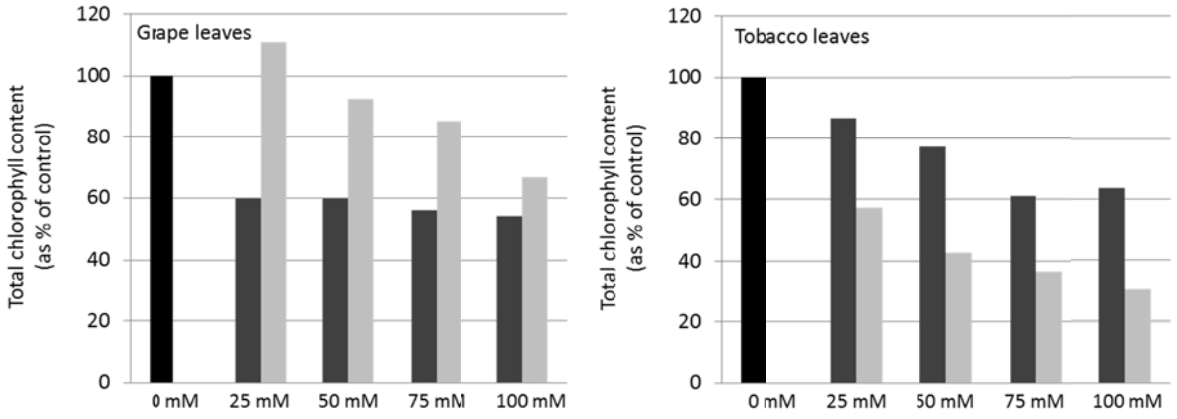

Fig. 5 Three leaf discs (10 mm diameter) were floated in H_2O for 18 hrs (for grape leaves) and 48 hrs (for tobacco leaves) containing two treatments (black bars for Treatment A and grey bars for Treatment B as indicated. Chlorophyll was extracted overnight in 80% acetone and quantified.

Treatments A and B were applied both either as a soil drench (complete soil saturation), app. 200-250 ml solution for a gallon pot) or as foliar spray for uniform coverage of leaf surface on both dorsal and ventral sides. Based on the phytotoxicity results reported above using *V. vinifera* grape leaves, the treatment concentrations selected for use in treatments were: for Treatment A, 5 mM soil drench and 15 mM foliar spray; and for Treatment B, 25 mM soil drench and 25 mM foliar spray. Preliminary treatments indicated no significant damage to grape vines when either chemical was applied as soil drench. However, highly localized leaf burn spots (treatment A) or pigmentation (treatment B) were visible after 2-3 days, as the result of liquid accumulating on leaf surface following spray treatments (Fig. 6).

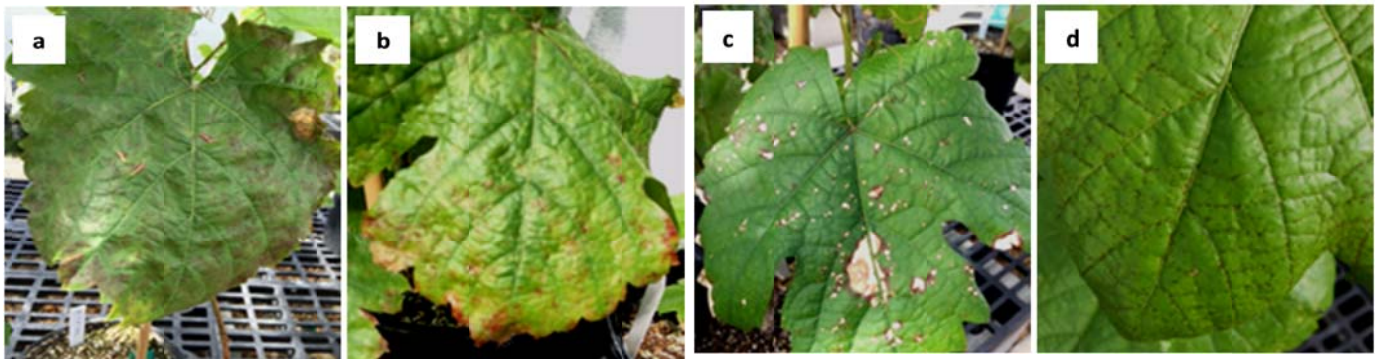

Fig. 6: Effect of chemical treatments *via* soil drench (a & b) and foliar spray (c & d) on appearance of leaves of treated *V. vinifera* cv. Carignane. (a), 5 mM chemical A drench; (b), 25 mM chemical B, drench; (c), foliar spray of 15 mM Chemical A; (d), foliar spray of 25 mM chemical B. Photos were taken three weeks-after treatment.

For Xf pathogenicity assays, *Vitis vinifera* cv. Carignane plants were inoculated by needle puncture as described (Zhang et al. 2015). Basically, five-day-old Xf cultures were grown in PD medium and resuspended in SCP buffer (trisodium citrate, 1 g/L; disodium succinate, 1 g/L; MgSO4_7H2O, 1 g/L; K2HPO4, 1.5 g/L; and KH4PO4, 1 g/L; pH 7.0) (OD600 = 0.25). Ten µL droplets of Xf bacterial suspensions were applied with a sterile tuberculin needle on opposite sides of 4–5 internodes of ca. 3 ft high grapevines in 1 gallon pots, starting with the second internode from the base. Plants were not watered for at least 36 hours prior to inoculation, resulting in the droplets of bacterial suspension being drawn quickly into the xylem stream.

Following inoculation, plants were immediately treated either by soil drench using between 200-250 ml of indicated chemical per treatment, or by spray until runoff of indicated chemical per treatment. PD symptoms appeared after about a month in the leaves of the lowest inoculated nodes.

Chemical treatments were repeated again at the end of the first and second month post-inoculation, for a total of three treatments. Disease severity was quantified by counting the total number of diseased leaves (symptomatic leaves, including bare petioles and bare nodes) and expressed as a % of total number of leaves (symptomatic and asymptomatic) (refer Zhang et al. 2015). The effect of chemical A and B treatments (soil drench and foliar spray) on the disease progression over a period of three months is summarized in Fig. 7.

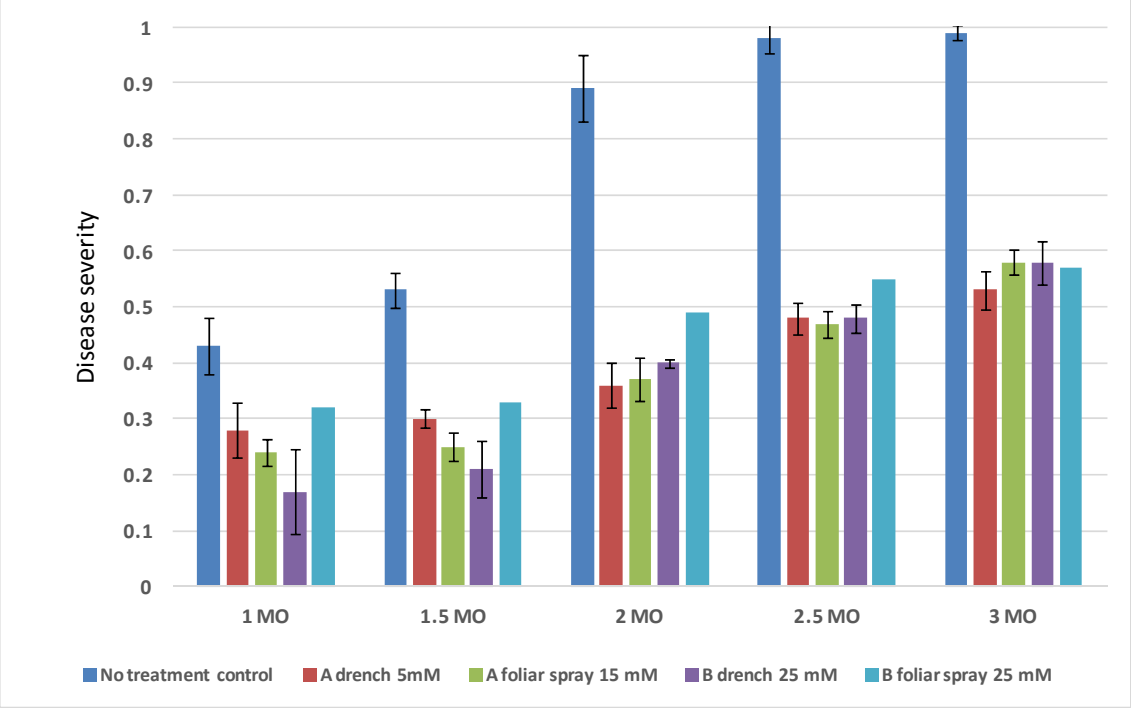

Fig. 7: Effect of chemical (A & B) treatments (soil drench or foliar spray) on progression of PD symptoms on grapevines (*V. vinifera* cv. Carignane) over a three-month period. The chemical treatments were applied immediately after Xf inoculations, and repeated twice at monthly intervals. The bars represent average ± SD for 5 replicates for the treatment controls and 3 replicates for each of the chemical treatments (except for foliar spray B, where only two plants were inoculated).

As can be observed in all treated plants, PD disease severity was reduced by approximately 40% by all treatments. Untreated controls were stunted and died after 3 months; treated plants maintained under the same conditions had longer internodes, and 25% more nodes and continued growing under root-bound conditions. The treatment concentrations were not dose optimized, nor were Xf titers determined.

Objective 2. Screen sap and crude extracts from *V. vinifera* grape plants subjected to freezing treatments (sufficient to cure PD) for potential effect on Type I efflux from Xf.

V. vinifera grape plants were cold treated. No significant amounts of sap have been extracted to date following cold treatments, despite several attempts.

Objective 3. Determine if sap and crude extracts from PD resistant *Muscadinia rotundifolia* contain more and/or more effective chemicals affecting Type I efflux from Xf than susceptible *V. vinifera* plants.

Muscadinia rotundifolia grapevines crude extracts were obtained using methodology previously reported (Reddy et al. 2007) and screened as outlined in Objective 1, but no significant effects were observed.

STATUS OF FUNDS

Fully spent.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY

A patent application is being prepared for the University of Florida.

PUBLICATIONS PRODUCED AS A RESULT OF THE PROJECT

Zhang, S., Jain, M., Fleites, L. and **D.W. Gabriel**. 2016. High-throughput assay for small molecules targeting the TolC efflux pump of *Xylella fastidiosa.* IS-MPMI XVII Congress, Portland, OR, July 17-21

Zhang, S., Jain, M., Fleites, L. and D.W. Gabriel. 2016. High-throughput assay for small molecules targeting the Type I efflux pump of *Xylella fastidiosa.* Session 3: Xylella fastidiosa. 2016 Pierce's Disease Research Symposium, San Diego, CA, Dec 13.

Zhang, S., Jain, M., Bernert, A.C., Fleites, L.A. and D.W. Gabriel. 2017. Identification of two potential small molecule chemical controls for Pierce's Disease. Poster 115P presented at the 2017 annual APS meeting, San Antonio. Phytopathology (abstract in press).

Zhang, S., Jain, M., Bernert, A.C., Fleites, L.A. and D.W. Gabriel. 2017. Identification of two potential small molecule chemical controls for Pierce's Disease. Manuscript in preparation.

LITERATURE CITED

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol 24:1–15

Jain, S., Kumar, D., Jain, M. et al. 2012. Ectopic overexpression of a salt stress-induced pathogenesis-related class 10 protein (PR10) gene from peanut (Arachis hypogaea L.) affords broad spectrum abiotic stress tolerance in transgenic tobacco Plant Cell Tiss Organ Cult (2012) 109: 19. doi:10.1007/s11240-011-0069-6.

Reddy, JD, Reddy, SL, Hopkins, DL, and Gabriel, DW. 2007. TolC is required for pathogenicity of *Xylella fastidiosa* in *Vitis vinifera* grapevines. Molecular Plant-Microbe Interactions, 20*:*403-410.

Steff, AM, Fortin, M, Arguin, C, and Hugo, P. 2001. Detection of a decrease in green fluorescent protein fluorescence for the monitoring of cell death: An assay amenable to high-throughput screening technologies. Cytometry, 45:237-243.

Tegos, G, Stermitz, FR, Lomovskaya, O, and Lewis, K. 2002. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrobial Agents and Chemotherapy 46:3133-3141.

Zhang, S, Chakrabarty, PK, Fleites, LA, Rayside, PA, Hopkins, DL, and Gabriel, DW. 2015. Three New Pierce's Disease Pathogenicity Effectors Identified Using *Xylella fastidiosa* Biocontrol Strain EB92-1. PloS one, 10: e0133796.

Zhang, JH, Chung, TD, and Oldenburg, KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomolecular Screening, 4:67-73.

Zhang, Y, Callaway, EM, Jones JB, and Wilson, M. 2009. Visualisation of hrp gene expression in *Xanthomonas euvesicatoria* in the tomato phyllosphere. European J. Plant Pathology, 124:379-390.

Zhang S, Chakrabarty PK, Fleites LA, Rayside PA, Hopkins DL, Gabriel DW. 2015. Three New Pierce's Disease Pathogenicity Effectors Identified Using *Xylella fastidiosa* Biocontrol Strain EB92-1. PLoS ONE 10(7): e0133796.

FUNDING AGENCIES.

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