Title of Report

Renewal Progress Report for CDFA Agreement Number 12-0118-SA

Title of Project

Greenhouse Evaluation of Grapevine Fungal Endophytes and Fungal Natural Products Antagonistic to *Xylella fastidiosa* for Control of Pierce's Disease.

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Introduction

Xylella fastidiosa (*Xf*) is a Gram negative, xylem-limited, insect-vectored bacterium and is the causal agent of Pierce's Disease (PD) of grapevine (Hopkins and Purcell, 2002). The recent introduction of a more effective vector, the Glassy-Winged SharpShooter (GWSS), *Homalodisca vitripennis*, to Southern California shifted the epidemiology of PD from a monocylic to a polycyclic disease. This led to a PD epidemic with severe economic consequences for the Southern California grape industry. The potential for the GWSS to move north and become established throughout the state remains a severe threat to the other major grape-growing regions (Central and Northern California). Current PD management strategies primarily involve vector management through the use of insecticides.

Control of PD with fungi or fungal metabolites is a largely unexplored research area. Fungi are receiving increasing attention from natural product chemists due to the diversity of structurally distinctive compounds they produce, together with the fact that many fungal species remain chemically unexplored. Fungi are excellent sources of interesting novel molecules that may be candidates with potential for control of bacterial diseases. Indeed, using fungi as biocontrol agents against plant disease is an active area of research (Amna 2010; Proksch et al. 2010; Xu et al. 2008).

Our objectives are to characterize the microbial diversity in grapevines that escaped PD in natural vineyard settings, and compare this population to PD-infected grapevines with the goal of identifying fungi that are unique to PD-escaped vines. We hypothesize that some of these fungal endophytes possess anti-*Xf* properties, likely due to the production of secondary metabolites. We are assessing the ability of these endophytes and their natural products (i.e. secondary metabolites) for inhibitory activity against *Xf in vitro*. Finally, we are determining in greenhouse tests if 1) fungi have potential use as prophylactic biocontrol agents for control of PD by inoculating grapevine cuttings with endophytic, *Xf*-antagonistic fungi and 2) if fungal natural products have curative properties for vines already infected with PD. If successful, we envision that these control strategies can be implemented at the nursery level (for biocontrols) or directly in the field (for natural products).

List of Objectives

Objective 1: Evaluate grapevine fungal endophytes for *in planta* inhibition of *Xf* and PD development using our established greenhouse bioassay.

Objective 2: Purify and characterize natural products produced by the inhibitory fungi.

Objective 3: Evaluate natural products for their potential as curative treatments for vines already infected with PD.

Description of Activities Conducted to Accomplish each Objective, and Summary of Accomplishments and Results for each Objective.

Objective 1: Evaluate grapevine fungal endophytes for *in planta* inhibition of *Xf* and PD development using our established greenhouse bioassay.

The goal of this objective is to provide increased tolerance to PD by inoculating grapes with natural fungal endophytes that possess anti-*Xf* properties. From 2010 to 2012 our research team collected plant tissue samples (sap, petioles, canes, spurs) from PD-escaped and PD-infected grapevines and isolated fungi inhabiting these samples. Following this, we identified these specimens to the genus level by comparing the ribosomal DNA sequences to specimens deposited in the GenBank database. We tested the ability of all the organisms recovered to inhibit *Xf* growth using an *in vitro* bioassay (Rolshausen and Roper, 2011), which allowed us to select a total of nine biocontrol candidates (eight fungi and one bacterium). Currently, we are analyzing the total microbial profile (culturable and non-culturable fungi and bacteria) inhabiting the plant tissues collected from the PD-infected and PD-escaped grapevines using a high-throughput Illumina sequencing platform. This culture independent approach is aimed at identifying other biocontrols that might have been over-looked with the culture dependent approach.

The nine putative biocontrol agents currently in our collection were subsequently re-introduced in grapevines cuttings prior to planting. To accomplish this, fungi were grown on PDA medium for two weeks and spores were harvested in sterile water and the concentration was adjusted to 50 spores/μl. Grape cuttings were vacuum infiltrated (**Fig. 1**) with the fungal spores, and planted in the greenhouse. Control plants were infiltrated with sterile water only. After a few weeks, the green shoots arising from these cuttings were inoculated with *Xf* (Temecula strain) by mechanical needle inoculation (Hill and Purcell, 1995). A sub-sample of plants were left un-inoculated with *Xf* to determine if the concentration of fungal spore used is detrimental itself to the grape cuttings. This experiment was repeated twice with 8-10 plants per treatment in 2013 on grape cvs. 'Merlot' and 'Chardonnay'. Plant symptoms were rated on a disease scale from 0 to 5 every two weeks (0= no symptoms; 5=Plant dead or dying) according to Guilhabert and Kirkpatrick (2005) (**Fig. 2**).

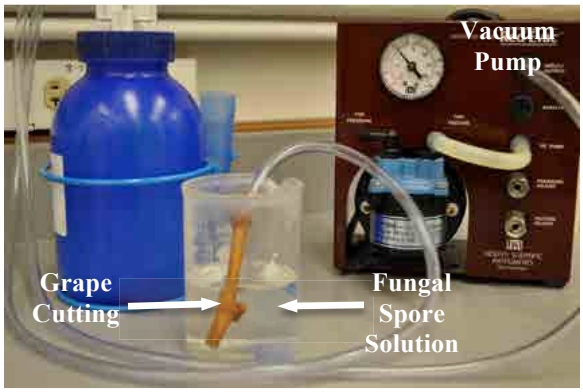

Figure 1: Technique used to vacuum infiltrate grape cuttings with spores of the fungal endophytes.

Figure 2: Pierce's Disease symptoms severity rating in grapevine cv. 'Merlot'; 0 = no symptoms (Mock inoculation); 1 through 5= grapes infected with the wild type strain (Temecula) showing an increase in the disease severity.

In 2013, PD severity was rated after 14 weeks in Merlot and Chardonnay (**Table 1**). In the buffer inoculated vines, no biocontrol treatments were lethal or caused PD-like symptoms to grapevines (data not shown). In the *Xf* inoculated grapevine cv. 'Merlot', four fungi (#5-8, **Table 1**) and the bacterium reduced PD severity and yielded higher number of relatively healthy vines in comparison to control plants (i.e., no biocontrols). However, in grapevine cv. 'Chardonnay', PD severity rating was high for all treatments making it difficult to observe differences amongst treatments. Grapevine petioles were harvested from all treatment and quantification of *Xf* titer *in planta* by qPCR is currently underway (see below). Grapevines were subsequently pruned back and kept in the greenhouse to allow new shoots to develop so we could assess PD development and *Xf* titer in second year plants. Grapevine cv. 'Merlot' are still being evaluated in the second year in part because symptoms are not expressed as quickly as in 'Chardonnay', and also because the assigned greenhouse for the experiment with grapevine cv. 'Merlot' is cooler than the greenhouse assigned for the experiment with grapevine cv. 'Chardonnay' and thus vines have been growing slowly. However, data collected from the Chardonnay showed that some treatments provided a higher number of relatively healthy grapevines in the second year. The PD severity rating was not recorded in that second year because vines inoculated with *Xf* were either dead or healthy. Petioles on those plants were or will be collected and *Xf* titer will also be quantified by qPCR.

Table 1: Results of the 2013 greenhouse evaluation of endophytic biocontrol agents for reduction of PD symptoms development in grapevine cvs. 'Merlot' and 'Chardonnay'. Average Disease Severity Rating (ADSR) and Percent of Plant Relatively Healthy (PPRH) were calculated using the disease rating scale as shown in **Fig. 2**. A plant was considered relatively healthy when the disease rating was below 3. ADSR and PPRH were recorded for the first year of the experiments on both cultivars. PPRH is only presented for the second year on Chardonnay; in the second year, vines were either dead (because of PD) or completely healthy with no PD symptoms, therefore we did not record the ADSR.

	2013 - Merlot		2013 - Chardonnay		
	ADSR	PPRH-y1	ADSR	PPRH-y1	PPRH-y2
Control	4.2 ± 0.8	10	4.6 ± 0.7	0	0
Fungus 1	3.6 ± 1.3	30	4.1 ± 1.7	13	0
Fungus 2	4 ± 1.2	20	3.9 ± 1.7	22	22
Fungus 3	3.6 ± 1.3	30	4 ± 1.7	13	13
Fungus 4	4.4 ± 0.7	0	3.8 ± 2.5	25	38
Fungus 5	2.7 ± 1.2	50	4.5 ± 0.5	0	0
Fungus 6	2 ± 0.9	80	3.4 ± 1.9	22	22
Fungus 7	1.7 ± 1.3	60	4.2 ± 0.8	0	11
Fungus 8	2 ± 1.8	70	3.5 ± 2.2	25	50
Bacterium 1	2.3 ± 1.8	50	4.2 ± 1.6	11	33

The qPCR test is a key to evaluate the biocontrol agents' ability to suppress PD in greenhouse bioassays. We first utilized an assay developed by Schaad et al. (Schaad et al., 2002). We optimized PCR conditions for the primerset, XfF1 (AAAAATCGCCAACATAAACCCA) and XfR1 (CCAGGCGTCCTCACAAGTTAC), using SyBr green fluoresce detection. However, the qPCR data coupled with sequencing of the amplicons results revealed low detection specificity in environmental samples leading to false positives. Therefore, we developed new primer sets targeting the ITS region of ribosomal genes and the RNA polymerase sigma-70 factor gene of *Xf* were developed using the PRISE software program (Fu et al., 2008). PRISE software automates the task of placing primer–template mismatches at the 3' end of the primers, which would enhance sequence selectivity. We are still optimizing the DNA extraction protocol from plant tissues and PCR conditions with these primer sets to yield optimal data. All plants samples from 2013 have been stored in the freezer and total DNA was extracted.

Objective 2: Purify and characterize natural products produced by the inhibitory fungi.

The goal of this objective is to identify natural products produced by fungi inhabiting grapevines that can be used as curative treatments for control of PD. We have thus far, identified eight fungal specimens inhabiting grapevine tissues (xylem sap, shoot, petioles and spur) that were able to inhibit *Xf* in a bioassay (Rolshausen and Roper, 2011). In brief, *Xf* liquid cultures are adjusted to OD_{600nm}=0.1 (approx. 10^7 CFU/ml); 300 µl of the *Xf* cell suspension are added to 3 ml of PD3 top agar (containing 0.8% agar) and briefly vortexed. This mixture is then overlayed onto a petri plate containing solid PD3 medium. A sterile circle of agar is drawn from the margin of an actively growing pure fungal culture and is placed onto the plates previously inoculated with *Xf.* Plates are incubated at 28ºC for seven days and then observed for an inhibition zone around the fungal colony (**Fig. 3**).

In addition, crude extracts collected from the fungal cultures showing inhibition towards *Xf* were collected for evaluation using a similar growth inhibition assay as described above. In brief, agar plugs 0.5 cm in diameter for each fungus were used to inoculate 250 mL liquid media, and the fungi were cultivated at room temperature on a shaker. After 10 days, each culture was filtered and further extracted with ethyl acetate, re-suspended in sterile methanol to an extract mass of 1mg, pipetted onto sterile paper discs and allowed to dry in a laminar flow hood. Once dry, the paper discs containing the crude extracts are placed onto the *Xf* cultures and incubated at 28ºC for eight days. Following this, plates were

observed for a halo of inhibition around the paper disc and compared to control *Xf*-only plates and plates with paper discs treated with methanol only (**Fig. 4**). We are currently fractionating the crude extracts from these fungi in order to purify and identify the inhibitory molecules. Thus far, we have purified two individual molecules (molecules 'R' and 'C') that are active against *Xf* growth *in vitro.* We have also characterized chemical structure for these two molecules. These molecules and fungi are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot be disclosed in this report.

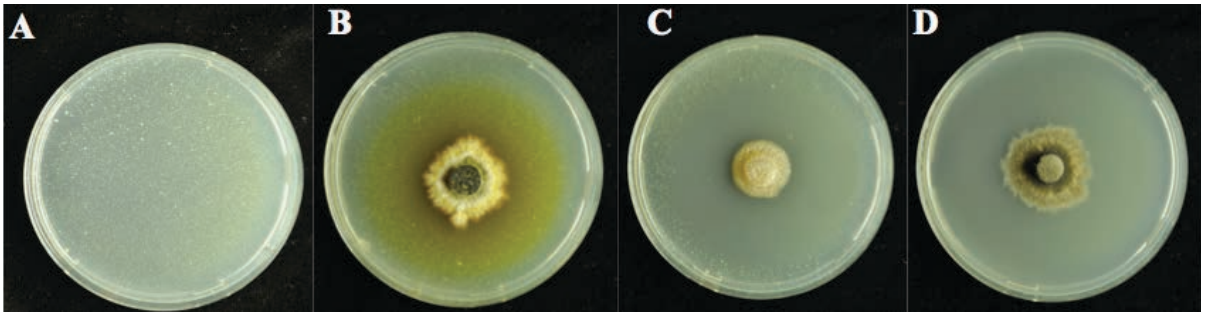

Figure 3: *In vitro* inhibition assay used to evaluate fungi. A) *Xf*-only Control; B) Fungus showing no *Xf* inhibition; C) Fungus showing moderate *Xf* inhibition; D) Fungus showing total *Xf* inhibition. *Xf* cells were plated in top agar and agar plugs containing fungi were placed on top. Fungi were evaluated after 8 days of incubation at 28˚C.

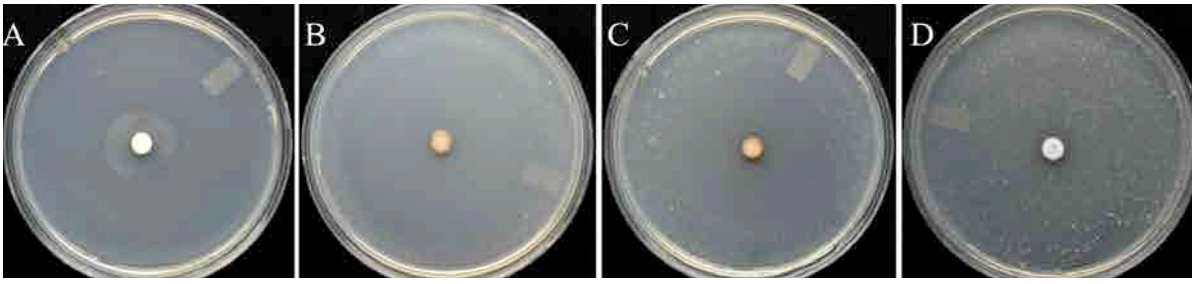

Figure 4: *In vitro* inhibition assay used to evaluate crude ethyl acetate extracts from culture supernatants of fungi. These extracts strongly inhibit *Xf* growth at concentrations of 1 mg/ml as indicated by either a complete lack of growth (Panel A) or a large inhibition zone around the paper disc containing the crude extracts (Panel B, C). Panel D indicates the negative control disc that contained methanol only. No inhibition zone was observed for the negative control.

Objective 3: Evaluate natural products for their potential as curative treatments for vines already infected with PD.

The goal of this objective is to develop a greenhouse bioassay in order to evaluate the efficacy of fungal natural compounds *in planta* as curative treatments for PD. Once this proof of concept is established in the greenhouse, the experiment will be carried over to the field. The deliverable for this objective is the development of a commercial product for the control of PD.

We have identified one fungal natural compound (molecule 'R') as an active molecule inhibitory to *Xf.* Our group has also recently identified a second molecule (molecule 'C') that inhibits *Xf in vitro*. In 2013 our greenhouse trials were designed to test the molecule 'R' on PD-infected vines and determine if

PD symptoms and *Xf* titer *in planta* were reduced after injection of the molecule 'R'. In 2013, grapevines cuttings cv. 'Merlot' and 'Chardonnay' were infected with *Xf* (Temecula strain) by mechanical needle inoculation (Hill and Purcell, 1995). The molecule 'R' was injected two weeks post-*Xf* inoculation. Molecule 'R' was extracted from a three week-old fungal culture grown in 250 ml of PD broth as previously described (Obj. 2). However, since this molecule is not water-soluble, we were forced to dissolve it in DMSO (Di-Methyl Sulfoxide) and 5 mg was injected into healthy (control) and *Xf*-infected grapevines. Mock injections of healthy and PD-infected plants consisted of pure DMSO only. Injections were made using a 1ml 16-gauge needle. Injections were performed in the xylem tissue of the grape cutting just below the shoot (**Fig. 5**). The four treatment combinations were 1) *Xf* x molecule 'R'; 2) *Xf* x buffer; 3) Buffer x molecule 'R'; 4) Buffer x buffer, with 10 plants per treatment. Disease severity was rated every two weeks following injection of molecule 'R' and up to 14 weeks, after which grapes were pruned. In the second year, grapevines were kept until expression of new PD symptoms occurred on the new shoots. The rationale was to determine if the treatment with molecule 'R' could cure the disease or at least mitigate PD severity.

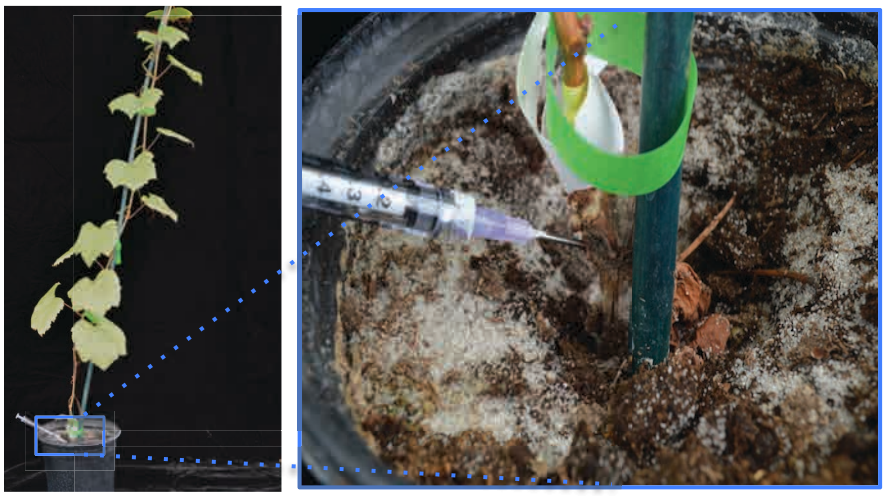

Figure 5: Needle-injection of an anti-*Xf* molecule in the xylem of PD-infected grapevine cuttings.

Our results showed no difference between treatments 1 and 2, whereby all *Xf*-inoculated grapevines expressed PD symptoms. In addition, all *Xf*-inoculated grapevines expressed PD symptoms or were dead in the second year, regardless of the treatment. We believe that the lack of water-solubility of molecule 'R' and the fact that we were forced to dissolve in DMSO likely limited its systemic properties *in planta* and its activity in the xylem where *Xf* resides.

Publications Produced and Pending, and Presentations Made that Relate to the Funded Project

Aldrich, T., Rolshausen, P.E., Roper, M.C., and Maloney, K. Progress toward the discovery of natural product inhibitors of *Xylella fastidiosa* from endophytic fungi. 2010 American Chemical Society meeting, Anaheim, CA.

Rolshausen, P.E., and Roper, M.C. Control of Pierce's Disease with fungal endophytes of grapevines antagonistic to *Xylella fastidiosa*. In Proceedings, 2010 Pierce's Disease Research Symposium, pp. 224-228. California Department of Food and Agriculture, San Diego, CA.

Rolshausen, P.E., and Roper, M.C. Control of Pierce's Disease with fungal endophytes of grapevines antagonistic to *Xylella fastidiosa*. In Proceedings, 2011 Pierce's Disease Research Symposium, pp. 166-172. California Department of Food and Agriculture, Sacramento, CA.

Rolshausen, P.E., and Roper, M.C. Greenhouse Evaluation of Grapevine Fungal Endophytes and Fungal Natural Products Antagonistic to *Xylella fastidiosa* for Control of Pierce's Disease. In Proceedings, 2012 Pierce's Disease Research Symposium, pp. 187-192. California Department of Food and Agriculture, Sacramento, CA.

Rolshausen, P.E., and Roper, M.C. Greenhouse Evaluation of Grapevine Fungal Endophytes and Fungal Natural Products Antagonistic to *Xylella fastidiosa* for Control of Pierce's Disease. In Proceedings, 2013 Pierce's Disease Research Symposium, pp. 161-168. California Department of Food and Agriculture, Sacramento, CA.

Jiue-in Yang, Caroline Roper, James Borneman, James Gloer, Katherine Maloney, and Philippe Rolshausen. Characterization of the fungal microbial community inhabiting grapevine: identification of a biocontrol agent for Pierce's Disease. 2013 American Society for Microbiology meeting, Denver, CO.

Research Relevance Statement

We are testing both prophylactic and curative measures for PD that will ultimately contribute to a sustainable PD management strategy. Practically, we envision that the biocontrol organisms could be applied into grapevine cuttings at the nursery level through vacuum infiltration of fungal propagules into the xylem tissue, thereby, providing enhanced protection against PD. We are currently testing this strategy in greenhouse trials and we have seen some promising results. As a curative strategy, we are evaluating the use of anti-*Xf* fungal natural products to provide a solution to growers that have vineyards already infected with PD. We are currently developing a xylem injection prototype that will effectively deliver a sustainable supply of the anti-*Xf* compounds into the xylem of an infected vine. We have already discovered two active anti-*Xf* compound. One of them is being tested in the greenhouse. The next step is to discover additional active natural anti-*Xf* compounds and evaluate their efficacy in greenhouse experiments with PD-infected grapevines. Once we demonstrate these compounds mitigate PD in the greenhouse, we will test their efficacy in natural vineyard settings in the future.

Layperson Summary of Project Accomplishments

Several management strategies for Pierce's Disease (PD) are currently being used, but rely primarily on vector control through the use of insecticides. Here we propose to test an alternative control strategy to complement those currently in place or those that are being developed. Our goal is to identify fungi inhabiting grapevine that are antagonistic to *Xylella fastidiosa* (*Xf*). We hypothesized that in natural field settings grapevines escape PD and remain healthy (a documented phenomenon in PD-infected vineyards) because the organisms residing in that particular vine do not allow the establishment of *Xf*. From 2009-2012 we sampled from vineyards in Napa and Riverside Counties that were under high disease pressure and identified fungi living in the xylem sap, shoots, petioles and wood spurs of diseased and PD-escaped grapevines. We have identified several fungi that inhibit *Xf* growth in culture. Eight fungi and one bacterium were re-introduced in grape cuttings that were inoculated with *Xf*. In addition, we also extracted natural compounds secreted by these fungi and identified two purified molecules inhibitory to the bacterium. In the future our goals are to; 1) complete the experiment with introduction of fungi in grape cuttings and determine which of these beneficial fungi should be further tested in natural vineyard settings; 2) elucidate the chemical structure of fungal natural products antagonistic to *Xf* and test them as a curative treatments in PD-infected grapevines in the greenhouse and later on in the field. These molecules and fungi are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot be disclosed in this report.

Status of Funds

As of March 2013, remaining funds were $14,965. An Assistant Specialist began work on the project on 12/01/2012. These funds will cover 100% of her salary and benefits until 06/30/2014.

Summary and Status of Intellectual Property Associated with the Project

The goal of this research is to identify fungi and their natural products that are antagonistic to *Xf* that could be implemented as; 1) a preventive management strategy at the nursery level during the propagation phase; 2) a curative management strategy that can be used by growers in commercial vineyards. We have identified eight microorganisms antagonistic to *Xf* either directly in culture or *in planta* or through their active natural products. The results of this research have been disclosed to the UC Riverside Office of Technology Commercialization and a case number has been allocated (UC Case No. 2011-401-1) which is currently being reviewed for patentability. For this reason we cannot disclosed the name of the fungi or compounds inhibitory to *Xf* in this report.

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