Title of Report

Interim 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.

Objective 2: 2- Identify fungal natural products and semisynthetic derivatives active against Xf.

Objective 3: 3- Evaluate fungal natural products and semisynthetic derivatives 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.

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 2013 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.

From those nine putative biocontrol agents, we narrowed the pool to 5 based on preliminary data from our greenhouse bioassay and from fungal phenotypic traits (ability to grow rapidly and form spores). Those 5 biocontrols (4 fungi and 1 bacterium) were re-introduced in grapevines cuttings prior to planting. To accomplish this, the organisms 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). This experiment was repeated twice on grape cvs 'Merlot' and 'Cabernet Sauvignon' with 10 and 20 plants, respectively. In addition, a sub-sample of plants (n=10) were left un-inoculated with *Xf* to determine if the concentration of fungal spore used is detrimental itself to the grape cuttings. Plant symptoms were rated after 14 weeks 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**), after which vines were pruned back and grown again. At this time plants were either expressing early PD symptoms (i.e., shoot stunting) or looked relatively healthy. Thus, the number of healthy plants (with PD rating < 3) was scored.

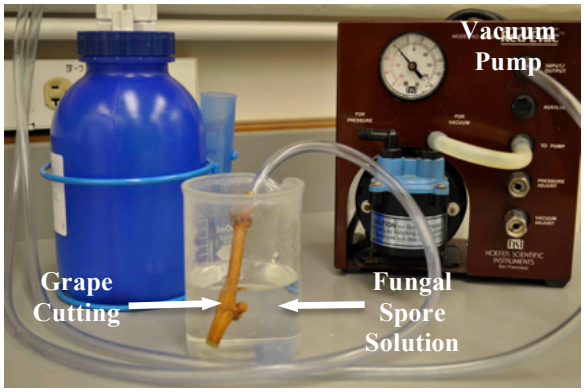


Figure 1: Technique used to vacuum infiltrate grape cuttings with spores of the fungal endophytes.

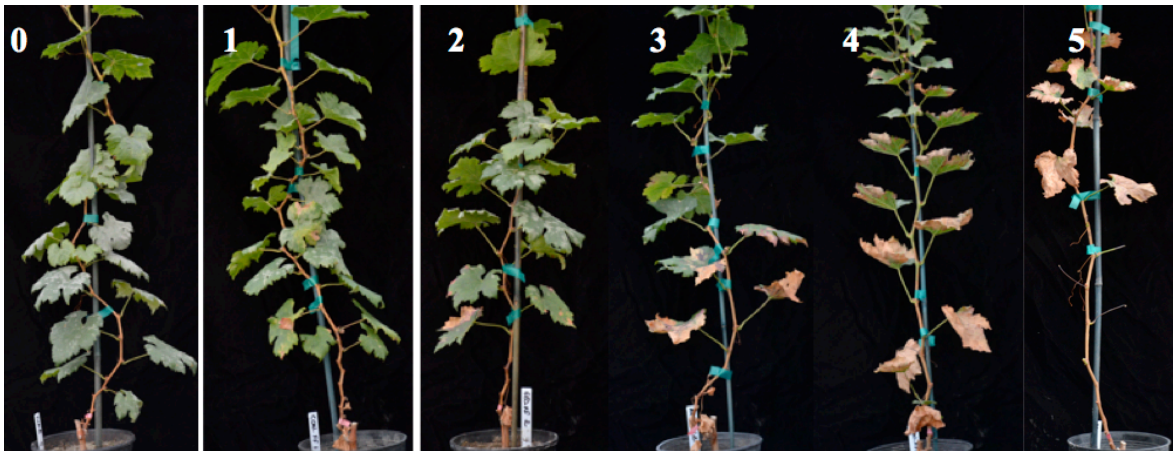


Figure 2: Pierce's Disease symptoms severity rating in grapevine cv. 'Merlot'; 0 = no symptoms (Mock inoculation); 1 through 5= grapes infected with the wild type strain (Temecula) showing an increase in the disease severity.

In the buffer inoculated vines, no biocontrol treatments were lethal or caused PD-like symptoms to grapevines (data not shown). In the *Xf* inoculated grapevine cultivars, one fungus (CRY) reduced disease severity before pruning and over 50% of the plants were relatively healthy after pruning (**Table 1**). It appeared that there was a cultivar effect whereby 'Cabernet Sauvignon' was a lot more susceptible to 'Merlot' and thus biocontrols performed better with the later than the former grape cultivar.

Table 1: Evaluation of endophytic biocontrol agents for reduction of PD symptoms development in grapevine cvs. 'Merlot' and 'Cabernet Sauvignon'. Pierce's Disease Rating (PDR), and Percent of Relatively Healthy Plant (PRHP; Plant with disease rating < 3) were calculated using the disease rating scale as shown in **Fig. 2**

	Cabernet Sauvignon		**Merlot**	
	PDR	PRHP	PDR	PRHP
Control	3.2 ± 1	5	4.2 ± 0.8	20
ACH	3.1 ± 1.2	15	2.3 ± 1.8	20
CRY	2 ± 1.3	55	2 ± 0.9	70
EUR	3.4 ± 0.8	20	1.7 ± 1.3	70
GEO	2.9 ± 1	5	2.7 ± 1.2	60
COC	3.8 ± 0.6	5	4 ± 1.2	10

Objective 2: Identify fungal natural products and semisynthetic derivatives active against *Xf*.

The goal of this objective is to identify fungal species and fungal natural products produced by endophytes that can be used as curative treatments for control of PD. We previously 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 medium containing 0.8% agar and briefly vortexed. This mixture is then overlayed onto a petri plate containing 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. 1**)

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 of 0.5 cm diameter of 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 7 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. Crude extracts showing inhibition were further processed to purify and identify the inhibitory molecules. Thus far, we have purified two molecules (radicinin and molecule 'C') that are active against *Xf* growth *in vitro* and have characterized their chemical structure. Radicinin is produced by *Cochliobolus* sp. and molecule 'C' is produced by *Dreschlera* sp. These molecules are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot always be disclosed in this report.

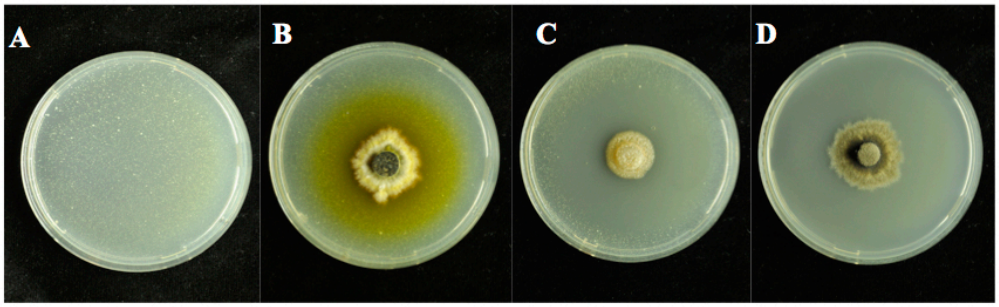


Figure 1: *In vitro* inhibition assay used to evaluate fungal activity towards *Xf*; *Xf* cells were plated in top agar and agar plugs containing fungi were placed on top. Inhibition was evaluated after 8 days of incubation at 28˚C. A) *Xf*-only control; B) No *Xf* inhibition; C) Mild *Xf* inhibition; D) Total *Xf* inhibition.

Radicinin showed great potential *in vitro* (Aldrich et al., 2015). Hence, in an *in vitro* dose response assay, where *Xf* cells are submitted to an increasing concentration of a fungal molecule, radicinin was able to inhibit *Xf* growth (**Fig. 2**). We have been developed of a more efficient procedure for isolating radicinin from *Cochliobolus* sp. This is a critical step, as it will allow us to produce substantial amount of water-soluble derivatives and further test them *in planta*. Radicinin is not commercially available, and we had been employing a multistep isolation procedure involving liquid-liquid extraction of *Cochliobolus* cultures followed by an expensive and time-consuming chromatography step to obtain pure radicinin for all our studies to date. Recently, we developed a procedure for purifying radicinin by recrystallization instead of chromatography. In this way, we were able to increase our yield of radicinin from 60.5 mg/liter of culture to 150 mg/liter of culture. This procedure also makes scaling up of the isolation for commercial use much more practical. In addition, the radicinin obtained by this new procedure is significantly more pure, as observed by NMR spectroscopy.

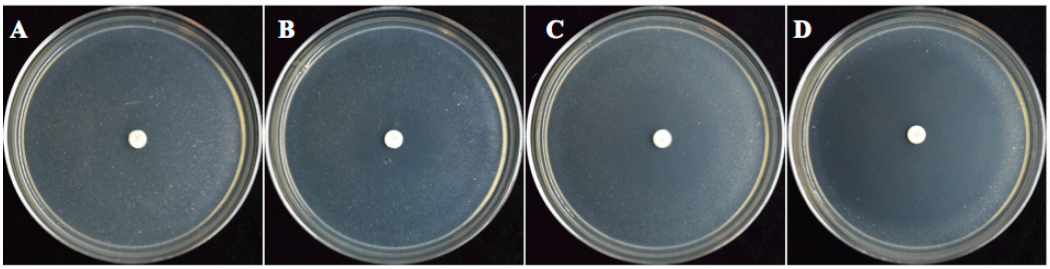
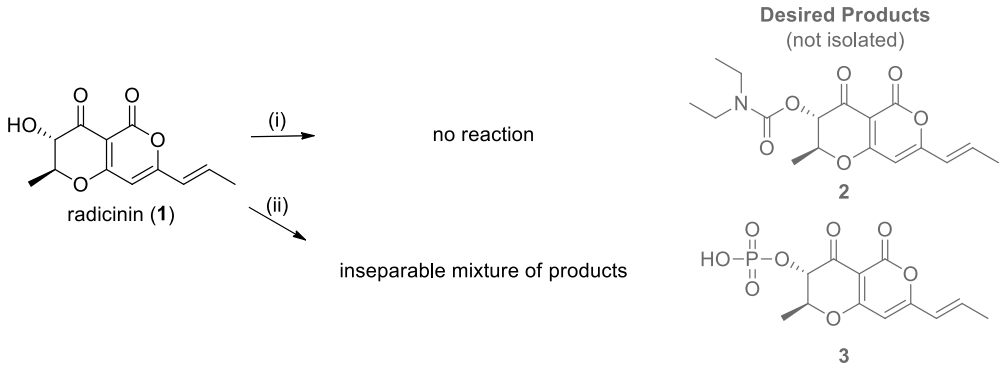


Figure 2: Dose response assay to evaluate *in vitro Xf* inhibition at increasing concentration of a fungal molecule. A) 0 µg molecule R1 (control); B) 50 µg molecule R1; C) 100 µg molecule R1; D) 250 µg molecule R1.

Now that we have figured out how to scale up radicinin production and purification, the next step was to prepare water-soluble semisynthetic derivatives of radicinin to facilitate testing *in planta*. We determined the solubility of radicinin in water to be 0.15 mg/mL, which is considered very slightly soluble. We have shown that acetylradicinin, which was modified at the hydroxyl group of radicinin, retains its anti-*Xf* activity (**Fig. 3;** Aldrich et al., 2015). This result suggests that modification of this position may provide a viable strategy for increasing the water-solubility of radicinin without loss of activity. Adding ionizable groups is a commonly employed strategy for improving the water-solubility of bioactive molecules (Kumar and Singh, 2013), so we had proposed to add two such groups at the hydroxyl position of radicinin (**Scheme 1**). The carbamate (**2**) is weakly basic and should form a water-soluble salt in low pH solutions, while the phosphate (**3**) is acidic and should form a water-soluble salt at high pH. Both carbamates and organophosphates are commonly found in pesticides, so we had good reason to believe that one or both of these compounds would be able to move into the xylem of grapevines. However, attempts to prepare the weakly basic carbamate and the acidic phosphate were unsuccessful. Specifically, the reaction with diethylcarbamoyl chloride (**i**) did not go to completion, while the phosphate reaction (**ii**) gave a mixture of products that we were unable to purify.



Scheme 1: *Xf*-inhibitory natural product radicinin (**1**), and semisynthetic derivatives (**2-4**). Reagents: (i) *N,N*-diethylcarbamoyl chloride, triethylamine (Vougogiannopoulou *et al*. 2008). (ii) 1. Cl_3CCN, 2. $(n\text{-}Bu)_4NH_2PO_4$, CH_3CN, 3. DOWEX 50WX8, NH_4HCO_3 (Lira *et al*. 2013).

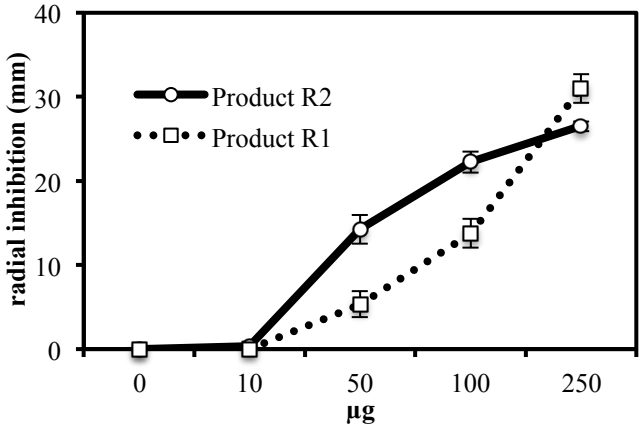
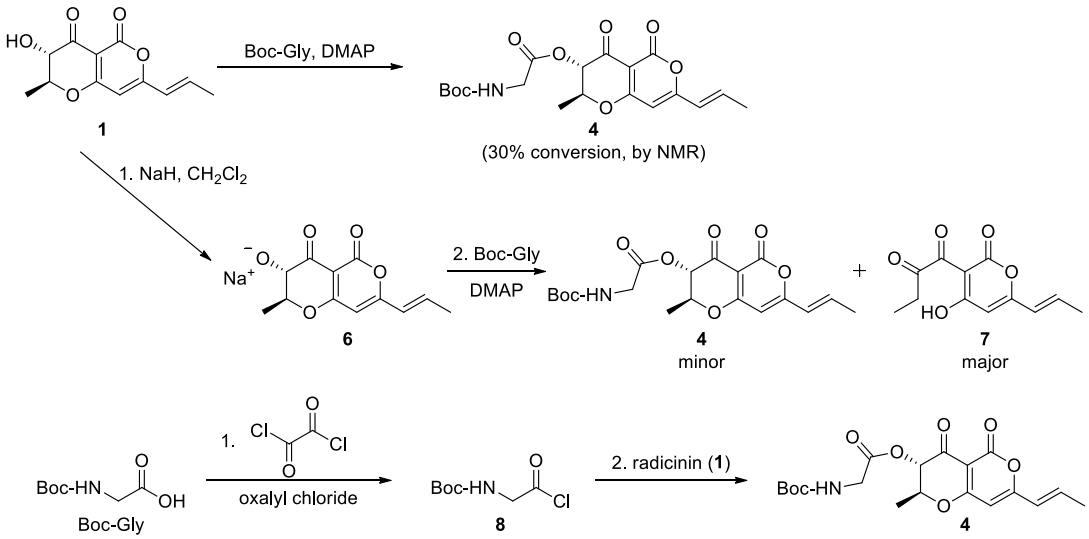
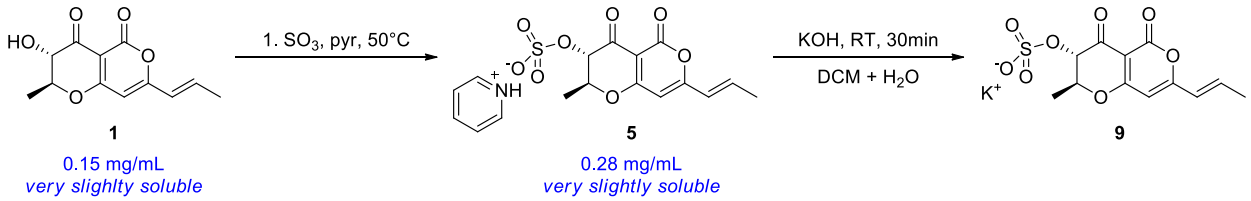


Figure 3: *In vitro* dose response assay; this lab assay quantifies inhibition of *Xf* growth as a measure of a halo around disc (mm) containing increasing concentration of 'R1' (radicinin) and molecule derivative 'R2'.

We then attempted to make two alternate ionizable radicinin derivatives: a glycine-derivative (**4, Scheme 2**), and radicinin pyridinium sulfate (**5, Scheme 3**). The failure of reactions to form either **2** or **4** suggested that the alcohol group of radicinin is much less nucleophilic than we originally expected. We attempted to increase the nucleophilicity of this group by first deprotonating with sodium hydride to give an alkoxide (**6, Scheme 2**). We isolated **6** and found it to be more than a thousand-fold more water-soluble than radicinin, at 218 mg/mL, (which is considered freely soluble). However, the high pH of the alkoxide solution leads us to be concerned about possible nonspecific toxicity. We also doubt that this high water solubility would be maintained in a cellular environment, which is buffered at neutral pH. Despite the increased nucleophilicity of **6**, we never observed any formation of carbamate **2**, and observed only minimal formation of the boc-glycine derivative **4**. Under the reaction conditions to form **4**, radicinin appeared to undergo tautomerization and ring-opening to give isomer **7** (**Scheme 2**). We successfully prepared a sulfate of radicinin, as the pyridinium salt **5**. Salt 5 maintained its activity against *Xf* in our disc assay (**Fig. 2**). This reaction proceeded to completion and the product proved easy to isolate. Unfortunately, the water solubility of **5** was only about twice that of radicinin: 0.28 mg/mL, lower than we had hoped. Recently, we were able to successfully replace the pyridinium counterion with potassium to give salt **9** (**Scheme 2**), which we hope will be more water soluble than **5**, while retaining activity. We are currently in the process of bringing up more of potassium salt **9** for solubility testing.



Scheme 2: Attempts to form the Boc-Gly derivative of radicinin using traditional peptide coupling methodology (top), or deprotonating first with sodium hydride (middle) gave the desired derivative as only a minor product, along with a ring-opened isomer of radicinin (**7**). We next plan to try activating Boc-glycine to the acid chloride (**8**) using oxalyl chloride, prior to reaction with radicinin (bottom).



Scheme 3: We prepared the pyridinium sulfate of radicinin (**5**), which was roughly twice as water-soluble as radicinin. Recently, we were able to exchange the pyridinium counterion for a more polar potassium ion in the potassium sulfate **9**.

In addition to radicinin, we had identified Cytochalasin as another natural molecule produced by *Drechslera* capable of inhibiting *Xf* growth in our laboratory bioassay (**Fig.2**). This year, we began the bioassay-guided isolation of natural products from the remaining fungi able to inhibit *Xf* in our lab bioassay (**Fig.1**), including *Cryptococcus* sp., *Ulocladium* sp., *Eurotium* sp. and *Geomyces* sp. Each fungus was grown for 14 days in potato dextrose broth. The cultures were extracted twice with ethyl acetate, and the organic extracts fractionated by column chromatography on silica gel to give 7-10 fractions. These fractions, along with the crude extracts, were subjected to the disc-diffusion bioassay to determine which inhibit growth of *Xf* (**Fig.2**). Fractions from *Eurotium* strain EUR1, *Geomyces* strain GEO1, and *Ulocladium* strain ULO1 showed activity against *Xf*. Neither the crude extract nor any of the fractions from *Cryptococcus* strain CRY1 showed activity against *Xf*. Active fractions from EUR1, GEO1 and ULO1 were examined using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Preliminary results indicated that; (i) one fraction from GEO1 looked like it could be a protein; (ii) another fraction from GEO1 suggested the absence of any major compound and that activity in this fraction may be due to trace amounts of a highly potent compound which will likely prove difficult to identify; (iii) a fraction of EUR1 revealed a few compounds, one of which showed an isotope pattern characteristic of a bromine atom; and (iv) a fraction of ULO1 revealed a relatively pure compound with an isotope pattern characteristic of two chlorine atoms. We are currently in the process of repeating the cultivation and extraction of ULO1 and EUR1 to obtain more material for purification by high-performance liquid chromatography (HPLC) and structure elucidation by two-dimensional NMR spectroscopy and MS.

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

The goal of this objective is to evaluate the anti-*Xf* efficacy of fungal natural products derivatives *in planta*. 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 cure of PD. We have currently identified two fungal natural products as an active molecule inhibitory to *Xf* (see objective 2). However, none of these products are water-soluble, and we are currently synthesizing radicinin derivatives that could be tested in our lab bioassays using grapevine vascular injection techniques and leaf-spray. We are also trying to identify other water-soluble fungal natural products that could be tested in the greenhouse. No action can be taken until we have identified such molecules.

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

Aldrich, T.J., Rolshausen, P.E., Roper, M.C., and Maloney, K.N. 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.

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Roper, M.C., Yang, J-I., Borneman, J., Jayanetti, D., Gloer, J., Maloney, K.N., and Rolshausen, P.E. Biocontrol of *Xylella fastidiosa*, the causal agent of Pierce's Disease of grapevine: Identification of the endophytic mycobiota inhabiting diseased and symptomless grapevine. 2014 international Molecular Plant Microbe Interaction meeting, Rhodos, Greece.

Yang, J-I., Roper, M.C., Borneman, J., Gloer, J., Maloney, K.N., and Rolshausen, P.E. 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.

Yang, J-I., Roper, M.C., Borneman, J., Gloer, J., Maloney, K.N., and Rolshausen, P.E. Biological control of Pierce's Disease: Identification of the endophytic mycobiota inhabiting diseased and symptomless grapevines. 2014 American Phytopathological Society meeting, Minneapolis, MN.

Aldrich, T.J., Rolshausen, P.E., Roper, M.C., Reader, J.M., Steinhaus, M.J., Rapicavoli, J., Vosburg, D.A., Maloney, K.M. 2014. 2015. Radicinin from *Cochliobolus* sp. inhibits *Xylella fastidiosa*, the causal agent of Pierce's Disease of grapevine. Phytochemistry 116:130-137.

Roper, M.C., Yang, J-I., , Borneman, Maloney, K.N., and Rolshausen, P.E. Characterization of the grapevine endophytic phytobiome and its influence on Pierce's disease development. 2015 Phytobiome Initiative meeting, Washington D.C.

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, or as a drench/foliar treatment on grapevines thereby providing enhanced protection against PD. We are currently testing these strategies in greenhouse trials and we have seen some promising results with one fungus specifically. 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 have already discovered two active anti-*Xf* compound and we are currently improving water-solubility of those compounds so they can become systemic *in planta* and be active in the xylem where the bacteria resides. We are also characterizing 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 organisms that inhibit *Xf* growth in culture, and those organisms were tested in greenhouse conditions. One fungus specifically mitigated PD development. In addition, we extracted natural compounds secreted by these fungi and identified two purified molecules inhibitory to the bacterium. In the future our goals are to; 1) evaluate the efficacy of the fungal biocontrol in natural vineyard settings; 2) increase the water solubility of the 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 June 2015 $79,054 was available to cover the expenses related to this project.

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 or as soil drench and foliar applications; 2) a curative management strategy that can be used by growers in commercial vineyards as a trunk injection or foliar application. We have identified one fungus and two fungal natural products antagonistic to *Xf* either directly in culture or *in planta*. 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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