Interim Progress Report for CDFA Agreement Number #16-0512-SA

Title of Project: Greenhouse evaluation of grapevine microbial endophytes and fungal natural products 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). PD is endemic to California but 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. GWSS has move to the San Joaquin valley and has impacted table grape production and it now threatens to become established in the heart of the wine grape production area including Napa and Sonoma Counties. Current PD management guidelines largely rely on vector control through the use of insecticides.

In this proposal we explore the use of grape endophytic microorganisms as a practical management tool for PD. Our research adds to the ongoing IPM efforts for discovery of biocontrol agents to *Xf* (Das et al 2015; Hopkins 2005). Our strategy is to couple culture-dependent and –independent approaches to identify novel biocontrol agents (BCAs) and active natural molecules. Control of bacterial plant diseases with commercial BCAs has been an active area of research (Stockwell and Stack 2007; Stockwell et al 2010; Yuliar et al 2015). In addition, fungi and bacteria are receiving increasing attention from natural product chemists due to the diversity of structurally distinctive compounds they produce that have potential for use as antimicrobial compounds to cure plant diseases (Aldrich et al 2015; Ben Abdallah et al 2015). Our research team has made substantial progress in the past years and identify several potential BCAs and natural products that could be used as prophylactic and curative treatments for PD. Our goals are to evaluate in *in planta* bioassays those BCAs and natural products before field testing.

Objectives

1- Evaluate a single organism-based approach for PD management.
2- Evaluate natural products and 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 a single organism-based approach for PD management
The goal of this objective is to evaluate a cost-effective high throughput delivery method for three biological control agents (BCAs) including one fungus (*Cryptococcus* sp.) and two bacteria (*Achromobacter xylosoxidans* and *Pseudomonas fluorescens*) isolated from grapevine for effective management of PD.

PD-escaped and –symptomatic grapevines tissues (cane, sap, spurs) were previously sampled from several commercial vineyards in Riverside and Napa Counties (**Fig.1**) and were analyzed by culture-dependent (isolation on culture medium) and –independent (Illumina sequencing) approaches. A PD-escaped vine is defined as a grapevine located in a PD-hot spot (with high disease pressure) that is infected with *Xf* but only express no to little PD symptoms. We were able to identify *Achromobacter xylosoxidans* and *Pseudomonas fluorescens* as BCA candidates for *Xf* because they correlated negatively with *Xf* titer in grapevine with culture-independent approach (**Table 1**). Interestingly, we isolated *Achromobacter* sp. during our culture-dependent approach and found that that it also inhibited *Xf* growth *in vitro* and provided some increased immunity against PD in our established *in planta* bioassay when introduced in grapevine through vacuum infiltration (**Figs.2-3**). In addition, in a preliminary study conducted in 2016 we tested a strain of *P. fluorescens* isolated from grapevine that we obtained from B. Kirkpatrick bacterial collection at UC Davis. In this experiment, *P. fluorescens* was introduced *in planta* by needle inoculation one week prior to *Xf* inoculation and after 12 weeks of incubation we showed a reduction of PD symptoms (**Fig.4**). Both *Pseudomonas fluorescens* and *Achromobacter xylosoxidans* are known biological control agent and plant growth promoting rhizobacteria (Gruau et al 2015; Khmel et al 1998; Mayak et al 2004; Shen et al 2013). In parallel, we had showed that several endophytic fungi recovered by culture-dependent analyses inhibited *Xf in vitro* (**Fig.5**; Rolshausen et al 2013), but only *Cryptococcus* sp. mitigated consistently PD symptoms development and *Xf* titer in our *in planta* bioassay after being introduced in the plant by vacuum inoculation (**Fig.3**). *Cryptococcus* is a yeast and is also a known BCA of many plant pathogens (Schisler et al 2014; Ulises Bautista-Rosales et al 2014). In experiments conducted in 2016 we demonstrated that there was no reduction of PD symptoms when *Cryptococcus* was applied as soil drench or foliar spray suggesting that the organisms had to get inside the vine to become antagonistic to *Xf*. Results from the S. Lindow's lab supported those conclusions as they added break-thru® to foliar sprays of another potential BCA (*Burkholderia phytofirmans*) to mitigate PD symptoms development *in planta*. Break-thru® is a penetrating surfactant that allows the active material to get inside the plant. We had previously contacted the manufacturing company (Evonik Corporation) and have started to use break-thru® for our radicinin-based formulation in order to get the anti-*Xf* radicinin inside the plant (see objective 2). We are planning to expand our collaboration with Evonik Corporation in the future to develop and optimize BCA formulation in order to facilitate entry of our organisms inside grapevine and achieve PD control.

Figure 1: PD-symptomatic (red arrow) and PD-escaped (blue arrow) grapevines in a vineyard located close to a riparian area in the Napa valley, California.

Table 1 Bacterial OTUs correlating negatively with *Xylella fastidiosa*.

Operational Taxonomic Units (OTUs)	*P**	FDR Corrected*	r*
Pseudomonas fluorescens	**1.8E-18**	2E-16	-0.83
Achromobacter xylosoxidans	**8.9E-03**	2.8E-01	-0.32

*Pearson correlation analyses were performed between the numbers of *X. fastidiosa* sequencing reads and the relative abundance of other bacterial taxa. Standard (*P*) and FDR corrected probability values are presented for the most abundant OTU along with the correlation coefficient (r); *n* = 68.

Figure 2: Greenhouse bioassay used to evaluate efficacy of biocontrol fungi and fungal natural products for control of Pierce's Disease. The progression of PD in vines infected with *X. fastidiosa* is scored on a disease severity rating scale ranging from 0 (= healthy) to 5 (= dead or dying).

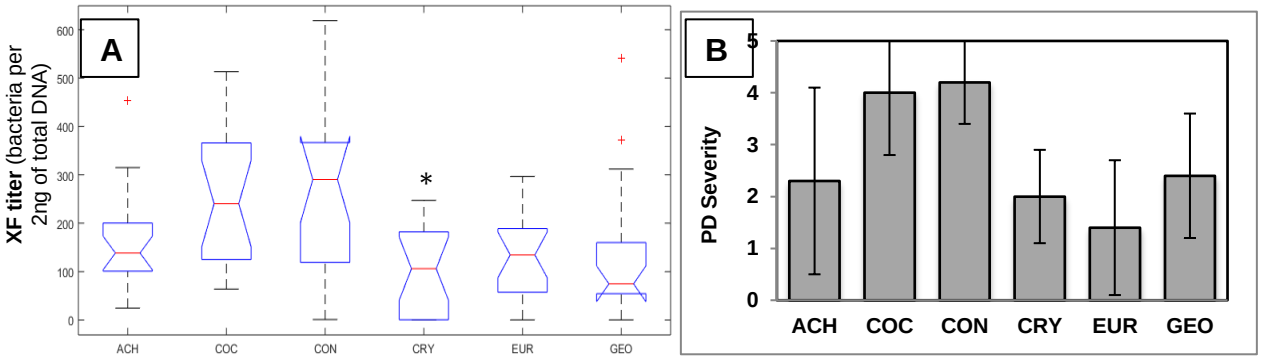

Figure 3: *Xf* titer and PD severity in grapevines (n=10) inoculated with 5 grapevine endophytes or 1X PBS alone (control) and challenged with *Xf* (ACH= *Achromobacter*; COC= *Cochliobolus*; CON= Control; CRY= *Cryptococcus*; EUR= *Eurotium*; GEO= *Geomyces*). **A**; Box plots illustrate the distribution of *Xf* titer in all 6 treatments. Asterisks * indicate significance at $P<0.05$. *Xf* titer was measured by qPCR. *Xf* titer was significantly decreased in vines that were pre-treated with *Cryptococcus* as compared to vines that were pre-treated with 1X PBS only. In addition, *Xf* titer was also decreased (just above statistical significance) in vines that were pre-treated with *Achromobacter* as compared to those inoculated with 1X PBS only. **B**; PD severity average as measured by our disease rating scale (0-5; **Fig.2**). Error bars represent standard deviation.

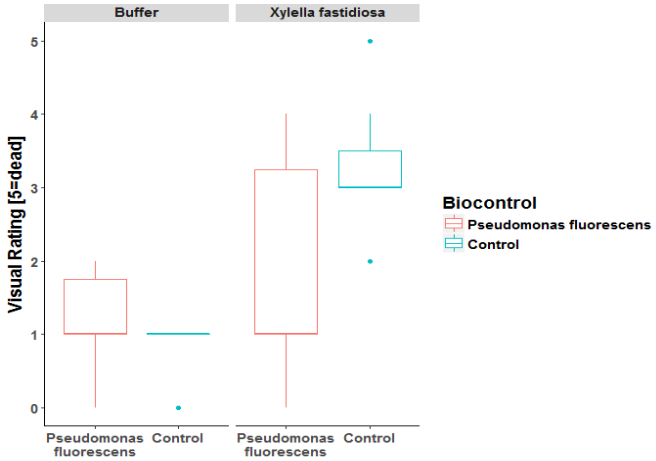

Figure 4: PD severity rating (**Fig.2**) in control grapevine vs. *Pseudomonas fluorescens* inoculated grapevine. *P. fluorescens* was introduced by needle inoculation one week prior to *X. fastidiosa* inoculation.

Biocontrol treatments efficacy are currently being evaluated on rooted grapevine cuttings planted in 1 gal pots in the greenhouse. We are evaluating three delivery methods of BCAs; (i) the foliar application delivery with surfactant break-thru®; (ii) vacuum infiltration method of grape cuttings; and (iii) the needle inoculation method of green shoots. For each application method, we test four treatments that include the two bacteria (*A. xylosoxidans* and *P. fluorescens*), one fungus (*Cryptococcus*) and control treatment (PBS buffer only). Twenty plants were used for each treatment so that a total of 80 plants are used for each experiment. *Achromobacter xylosoxidans* and *Pseudomonas fluorescens* were grown on nutrient agar, harvested in 1X PBS and adjusted to a concentration of 1000 cells/µl prior to inoculation. *Cryptococcus* was grown on PDA medium harvested in 1X PBS and adjusted to a concentration of 1000 spores/µl prior to inoculation.

For the vacuum infiltration treatments, the bacterial cell or spore suspensions were vacuum infiltrated into grapevine cuttings prior to rooting as described previously (Rolshausen et al 2013). Cuttings were then rooted and planted and when shoots reach 10-20 inches inoculated with Xf. For the foliar application treatments, plants were sprayed until runoff and inoculated with Xf two days later. For the needle inoculation experiment, BCAs were introduced in the plant one week before Xf inoculation. All plants were mechanically inoculated with Xf as previously described (Hill and Purcell 1995; Clifford et al 2013). PD symptoms will be rated after 10-12 weeks of incubation using a disease scale of 0 to 5 (Fig.2) as previously described (Guilhabert and Kirkpatrick 2005). Xf titer will also be evaluated using our Xf-detection qPCR method (Rolshausen et al 2013). All experiments will be repeated next year using Cabernet Sauvignon cultivar.

Objective 2- Evaluate fungal natural products and semisynthetic derivatives for their potential as curative treatments for vines already infected with PD.
Our goal is to find and develop a commercial product that growers can use as a curative treatment for PD-infected vines. In this objective we continued our bioprospecting to identify additional microbial natural products that have antagonistic activity against *Xf* and that are produced by the microbes we have isolated from the grapevine endosphere.

2.1. Identifying new *Xf* inhibitory natural products.
We previously isolated 8 endophytic fungi from grapevine showing anti-*Xf* properties in our *in vitro* assay (**Fig.5**) and have purified five active anti-*Xf* fractions produced by *Cochliobolus, Dreschlera, Geomyces, Ulocladium* and *Eurotium*. We have identified the chemical structure of two of these molecules; radicinin produced by *Cochliobolus* sp., and cytochalasin produced by *Dreschlera* (**Fig.6**; Grove 1964, Bottalico et al 1990). Because of the acute toxicity of cytochalasin to mammals, we decided not to explore further its application for PD management. Crude extracts of *Cryptococcus, Eurotium, Geomyces*, and *Ulocladium* also showed activity against *Xf* in our *in vitro* bioassay. Of particular interest, the active fraction from *Ulocladium* contained a relatively pure compound with an exact mass (*m/z* 260.9726) and distinctive isotope pattern consistent with a molecular formula of $C_{10}H_8Cl_2O_4$ (calculated $[M\text{-}H]^-$ 260.9727). 1D- and 2D- NMR spectroscopy on semi-purified fractions from *Ulocladium* sp. led us to identify several structural fragments that are consistent among the active fractions (**Fig.7**). Neither these fragments, nor the identified molecular formula match any known compounds in the AntiMarin

database. A time-course study showed that longer fermentation times (3 weeks or more) led to higher levels of this compound. We will be performing a larger-scale (10L) fermentation of *Ulocladium* sp. for a longer time to obtain additional compound for complete purification and characterization.

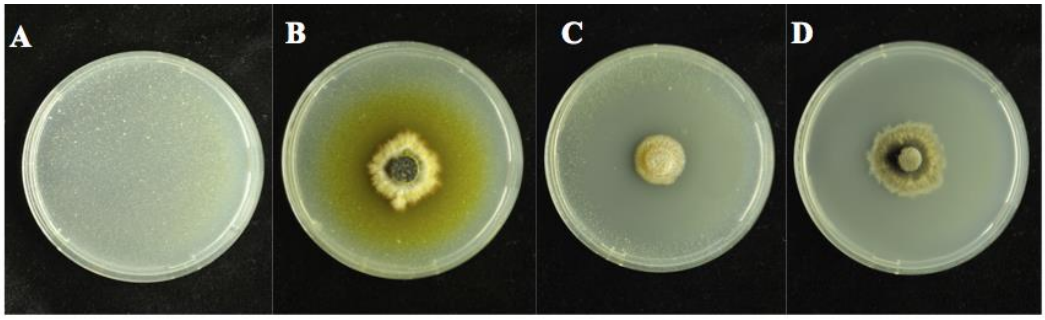

Figure 5: *In vitro* inhibition assay used to evaluate fungal activity towards *Xf*; *Xf* cells were plated in top agar and agar plugs containing fungi were placed on top. Inhibition was evaluated after 8 days of incubation at 28˚C. A) *Xf*-only control; B) No *Xf* inhibition; C) Mild *Xf* inhibition; D) Total *Xf* inhibition.

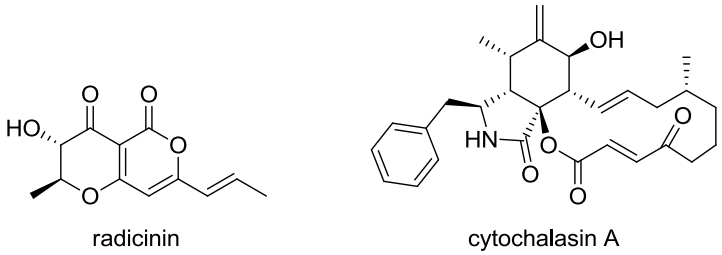

Figure 6: Chemical structures of radicinin and cytochalasin

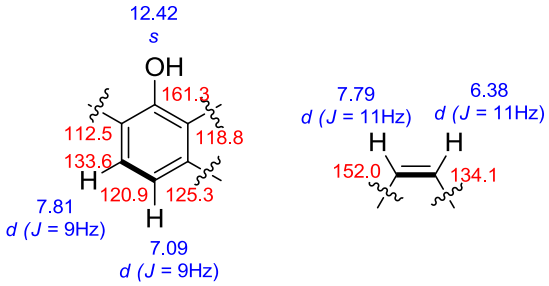

Figure 7: Structural fragments observed by NMR of active fractions from *Ulocladium* sp. Proton chemical shifts are shown in blue; Carbon-13 chemical shifts are shown in red, and their relative locations determined by analysis of HMBC and HSQC experiments. Proton-proton spin systems (in bold) were determined by analysis of a gCOSY experiment.

We will also repeat the cultivation and extraction of *Cryptococcus* sp. Thus far, *Cryptococcus* sp. has proven idiosyncratic in its production of the *Xf*-inhibitory compound. However, we currently have harvested active crude extract, which we can combine with any additional active extracts for purification by high-performance liquid chromatography (HPLC) and structure elucidation by two-dimensional NMR spectroscopy and MS.

2.2. Testing the anti-*Xf* natural product radicinin *in planta*.

Radicinin is known to possess antimicrobial activity and we have demonstrated that purified radicinin inhibits *Xf* growth in a dose-dependent manner (Aldrich et al 2015), but its limited water solubility (0.15 mg/mL) prevented testing radicinin's potential as a possible curative treatment for PD *in planta*. To overcome this problem, we

initially focused on developing water-soluble semisynthetic derivatives of radicinin by putting ionizable groups at the hydroxyl position of radicinin.

During the most recent project period, after a series of mostly-unsuccessful attempts at preparing radicinin derivatives, we changed our strategy and looked at using a combination of organic solvent and a surfactant to get radicinin into grapevines. First, we tested the solubility of radicinin in a variety of organic solvents that are compatible with agriculture and found that it was completely soluble in cyclohexanone. We then prepared an emulsion for spraying on plants consisting of radicinin in 10% cyclohexanone and 1% of Evonik TEGO SMO 80 emulsifier suitable plus 0.1% break-thru penetrating surfactant. Using this emulsion, we performed an exploratory study to establish an upper limit for the concentration of radicinin tolerated by grapevine. Specifically, we found that radicinin caused some leaf phytotoxicity when sprayed on grapevine at a concentration of 2 grams/liter (roughly 20 times the concentration of radicinin in *Cochliobolus* sp. culture broth). The water/cylohexanone/TEGO/Break-thru emulsion alone did not show any phytotoxicity, nor did radicinin at a concentration of 1 gram/liter (roughly 10 times the concentration in *Cochliobolus* sp. culture broth). With this information, we now plan to evaluate the curative potential of our radicinin-based formulation at 1 g/L on Xf-infected plants. Grape cvs. Cabernet Sauvignon were inoculated with Xf and will be sprayed with our radicinin-based formulation every two weeks. PD symptoms will be rated after 10 weeks of incubation using a disease scale of 0 to 5 (Fig.2) as previously described (Guilhabert and Kirkpatrick 2005). Xf titer will also be evaluated using our Xf-detection qPCR method (Rolshausen et al 2013). All experiments will be repeated next year using Cabernet Sauvignon cultivar.

Publications produced and pending, and presentations made that relate to the funded project.

- Aldrich, T.J., Rolshausen, P.E., Roper, M.C., Reader, J.M., Steinhaus, M.J., Rapicavoli, J., Vosburg, D.A., Maloney, K.M. 2015. Radicinin from Cochliobolus sp. inhibits *Xylella fastidiosa*, the causal agent of Pierce's Disease of grapevine. *Phytochemistry* 116:130-137.
- Deyett, E., Roper, M.C., Ruegger, P., Yang, J-I., Borneman, J., and Rolshausen, P.E. Microbial landscape of the grapevine endosphere in the context of Pierce's Disease. *Phytobiomes*. Under Review.
- Taking a stab at Pierce's Disease; Adventures in Genomics, Illumina movie (https://youtu.be/cDejq_47qxg).
- American Phytopathological Society annual meeting, Pasadena 2015. Invited speaker, live stream audience; http://www.apsnet.org/meetings/meetingarchives/2015annual/Pages/LiveStreaming.aspx (starts at min. 28).

Research relevance statement, indicating how this research will contribute towards finding solutions to Pierce's disease in California.

The overarching goal is to investigate both prophylactic and curative measures for PD that will ultimately contribute to a sustainable PD management strategy. We anticipate that the BCAs could be introduced into grapevine cuttings in nurseries as xylem infiltration as we have previously tested and demonstrated. We are also hopeful that those BCAs can be delivered into plants in a cost-effective high-throughput manner using penetrating surfactant. In addition, we are continuing to identify the chemical structure of natural products that inhibit *Xf in planta*. We have already developed a radicinin-based formulation to be tested *in planta*. We are working actively with the private sector to optimize the formulation so that the active anti-*Xf* product can be effectively delivered in the xylem tissue where *Xf* resides. We are in the last phase of the greenhouse evaluation of those treatments, and looking forward to scale up and further test our products under field conditions.

Layperson summary of project accomplishments.

This proposal focuses on testing grapevine endophytes as biocontrol agents against *Xf*. We have isolated one fungus that suppresses PD symptom development *in planta* and identified two bacteria that correlated negatively with *Xf* titer in naturally infected grapevines. We envision that these organisms could be used as commercial biocontrol agents for preventative control of PD. We also developed an anti-*Xf* product with a molecule isolated from a fungus living in grapevine and characterized two additional anti-*Xf* fungal molecules that also possess anti-*Xf* properties. These molecules have potential as curative treatments in commercial vineyards affected by PD. We

are currently testing in the greenhouse high-throughput and cost-effective delivery methods for these active molecules and biocontrol agents.

Status of funds.
Co-PIs have used their share of the funds. PI has $20,000 remaining on his funds.

Summary and status of intellectual property associated with the project.
The goal of this research is to identify biocontrol agents and natural products that are antagonistic to *Xf* that could be implemented as a preventive and/or curative management strategy. We have identified one fungus that mitigated PD development and *Xf* titer. In addition, we have identified two bacteria that could potential become promising biocontrol agents. Finally, we have isolated four compounds, and characterized the chemical structure of one of them, that are inhibitory to *Xf* that could become commercialize for PD management. 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.

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