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GREENHOUSE EVALUATION OF GRAPEVINE MICROBIAL ENDOPHYTES AND FUNGAL NATURAL PRODUCTS FOR CONTROL OF PIERCE'S DISEASE

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ABSTRACT.

The goal of this project was to characterize the microbial communities living in the endosphere of grapevine using culture-dependent and –independent (i.e. Illumina-based NGS) approaches and measure the impact of individual microbes of these resident communities on *X. fastidiosa* growth *in vitro* and PD infection *in planta*. We identified *Pseudomonas fluorescens* and *Achromobacter xyloxosidans* as negative correlates of *X. fastidiosa* (*Xf*). Hence, those bacteria were more abundant in vines that displayed a disease-escape phenotype in vineyards under high PD pressure. Those bacteria also proved to decrease PD symptoms in *in planta* bioassays. In addition, *in vitro* screening bioassays selected several grapevine-inhabiting fungi inhibitory to *Xf*. Among those *Cochliobolus* sp. inhibited *Xf* growth *in vitro* through the production of radicinin, and *Cryptococcus* sp. mitigated the development of PD symptoms *in planta*. This research has generated intellectual property, scientific publications, newsletter articles and public interest. We have assembled a collection of microbes and antimicrobial products that possess anti-*Xf* properties that are ready to be tested under field conditions.

LAYPERSON SUMMARY.

This proposal aimed at testing microbes inhabiting grapevine as potential biocontrol agents against *X. fastidiosa* (*Xf*). We also focused on the natural compounds that those microbes produce and determine if they could be deployed as bactericide against *Xf*. We isolated one fungus that two bacteria that suppressed PD symptom development in grapevine under greenhouse and/or field conditions. We envision that these organisms could be used in the future as commercial biocontrol agents for preventative control of PD. We also isolated and characterized an anti-*Xf* product from an additional fungus naturally occurring in grapevine. We envision that this bactericide could be used as a curative treatment in commercial vineyards affected by PD. We are now testing in both the greenhouse and the field cost-effective delivery methods for this bioactive molecule and biocontrol agents.

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 explored 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; Deyett et al 2017; Hopkins 2005; Lindow et al 2016). Our strategy was to couple culture-dependent and –independent approaches to identify novel biological control agents (BCAs) and active natural molecules. Control of bacterial plant diseases with commercial BCAs has been an active area of research (Loper et al. 2012; 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; Loper et al. 2012). Our research team has made substantial progress in the past years and identified three potential BCAs and one natural product that could be used as prophylactic and curative treatments for PD management.

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.

RESULTS AND DISCUSSION

1- Evaluate a single organism-based approach for PD management.

The goal of this objective is to evaluate individual fungal and bacterial grapevine endophytic strains for management of PD. PD-escaped and –symptomatic grapevines tissues (cane, sap, spurs) were previously sampled from several commercial vineyards in Riverside and Napa (**Fig.1**) Counties and were analyzed by culture-dependent and –independent (i.e., Illumina Next Generation 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.

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.

Using an Illumina-based culture-independent approach, we identified *Achromobacter xylosoxidans* and *Pseudomonas fluorescens* as the two most abundant bacteria inhabiting grapevine xylem that correlated negatively with *Xf* titer (**Table 1**; Deyett et al., 2017). In other words, those two bacteria were present in higher abundance in PD-escape than in PD-symptomatic grapevines, suggesting that those may be good BCAs candidates. We obtained cultures of *Achromobacter* sp. and *P. fluorescens* from grapevine wood re-isolations, and from Bruce Kirkpatrick's bacterial collection at UC Davis, respectively. In addition, using a culture-dependent approach we isolated several fungi that showed *Xf*-growth inhibition in *in vitro*-bioassay (**Fig.2**; Rolshausen et al 2013). In addition, we developed an i*n planta* bioassay to evaluate BCA candidates for their ability to reduce PD symptoms development (**Fig.3**), and a qPCR detection assay to measure *Xf* titer *in planta* (Rolshausen et al 2013).

Table 1 Bacterial OTUs correlating negatively with *Xylella fastidiosa* (Deyett et al. 2017)

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); $n = 68$.

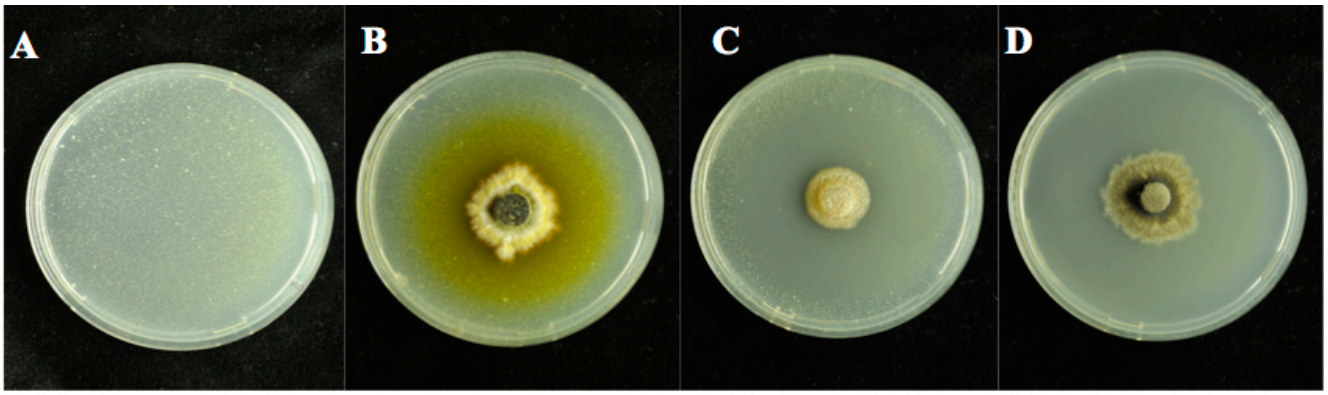

Figure 2: *In vitro* inhibition assay used to evaluate fungal activity towards *Xf*; *Xf* cells were plated in top agar and agar plugs containing fungi were placed on top. Inhibition was evaluated after 8 days of incubation at 28˚C. A) *Xf*-only control; B) No *Xf* inhibition; C) Mild *Xf* inhibition; D) Total *Xf* inhibition.

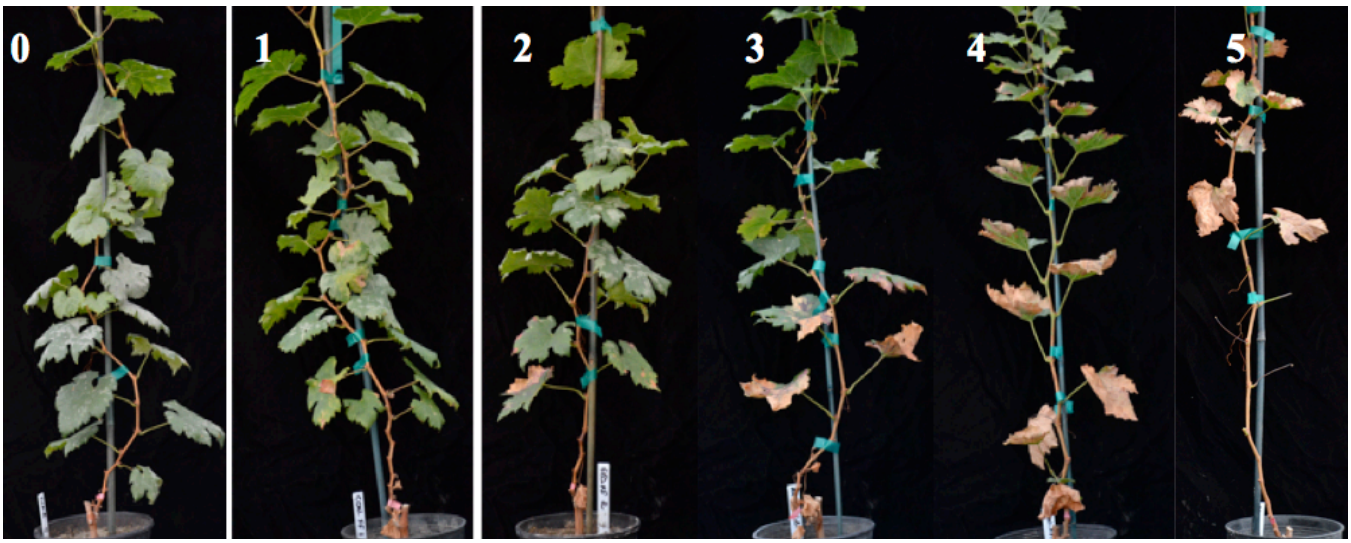

Figure 3: Greenhouse bioassay used to evaluate efficacy of biocontrol fungi and fungal natural products for control of Pierce's Disease. The progression of PD in vines infected with *X. fastidiosa* is scored on a disease severity rating scale ranging from 0 (= healthy) to 5 (= dead or dying).

Our results showed that *Cryptococcus* sp., *Achromobacter* sp. and *Pseudomonas fluorescens* were able to reduce PD symptoms development and *Xf* titer when they were introduced *in planta* (**Figs.4-5**) either through vacuum infiltration of grape cuttings before the rooting stage or needle inoculation of shoots (**Fig.6**). However, when those organisms were applied on the plant either through foliar spray or drench application, no reduction of PD severity was observed (data not shown), suggesting that they must be introduced in the plant vascular system where *Xf* resides, to be active. Nonetheless, this discovery is exciting because both *P. fluorescens* and *Achromobacter* are known as biological control agents and plant growth promoting bacteria in many plant systems (Abitha et al 2014; Gruau et al. 2015; Khmel et al. 1998; Mayak et al. 2004; Shen et al. 2013; Triki et al. 2012). In addition, *Cryptococcus* is a relatively abundant organism of grapevine wood (Deyett et al., 2017) and is also a known BCA of many plant pathogens (Schisler et al 2014; Ulises Bautista-Rosales et al 2014). We have proposed in our next research goals to optimize delivery of these BCAs *in planta* and evaluate them under field conditions.

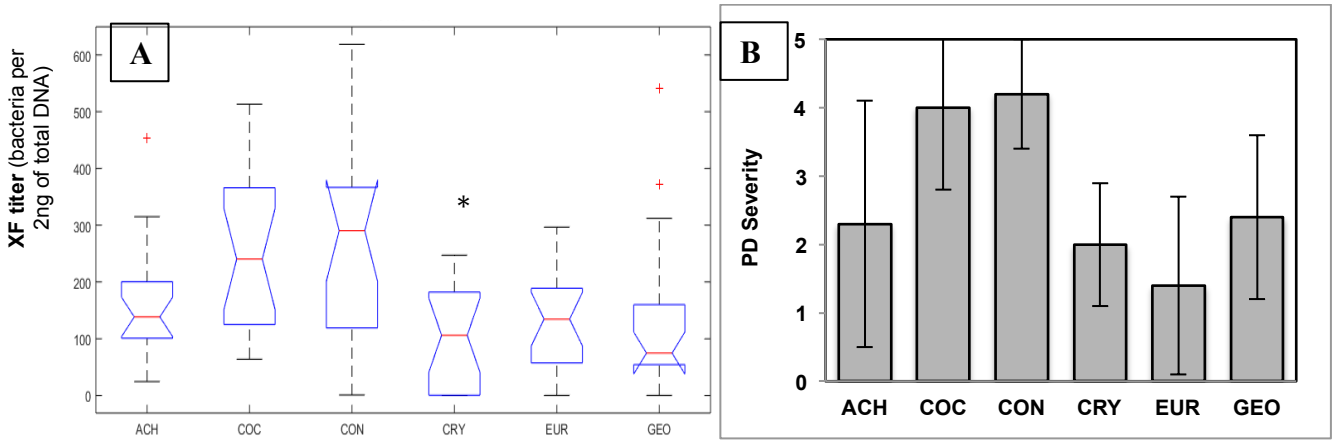

Figure 4: *Xf* titer and PD severity in grapevines (n=10) inoculated with 5 grapevine endophytes or 1X PBS alone (control) and challenged with *Xf* (ACH= *Achromobacter*; COC= *Cochliobolus*; CON= Control; CRY= *Cryptococcus*; EUR= *Eurotium*; GEO= *Geomyces*). A; Box plots illustrate the distribution of *Xf* titer in all 6 treatments. Asterisks (*) indicate significance at P<0.05. *Xf* titer was measured by qPCR. B; PD severity average as measured by our disease rating scale (0-5; **Fig.3**). Error bars represent standard deviation.

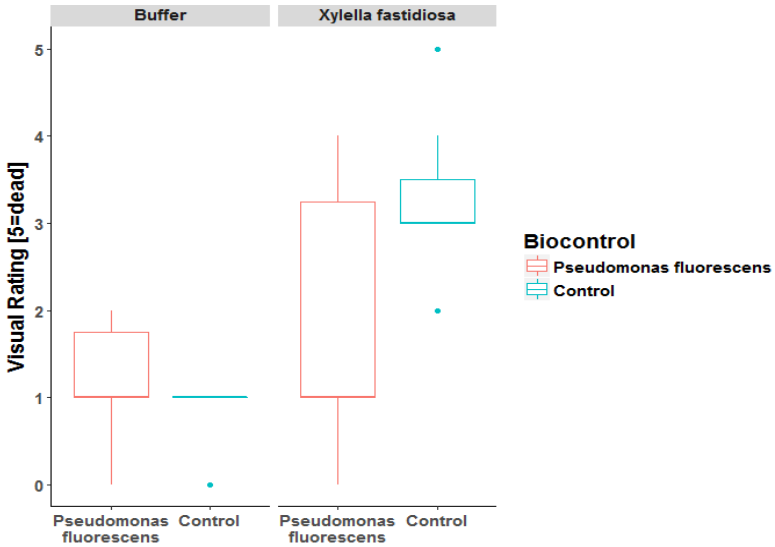

Figure 5: PD severity rating (**Fig.3**) in control grapevine vs. *Pseudomonas fluorescens* inoculated grapevine. *P. fluorescens* was introduced by needle inoculation one week prior to *X. fastidiosa* inoculation.

Figure 6: Methods used to re-introduced BCAs *in planta* for evaluation against PD in greenhouse bioassay (**Fig.3**). A- Vacuum infiltration; B- Needle inoculation.

2- Evaluate natural products and derivatives for their potential as curative treatments for vines already infected with PD.

The goal of this objective was to identify anti-*Xf* fungal natural products produced by endophytes isolated in a culture-dependent manner (**Obj.1**) that can be used as curative treatments for control of PD. We have purified three active natural products from endophytic fungi of grapevine showing anti-*Xf* properties in our *in vitro* assay. Those included radicinin produced by *Cochliobolus* sp., cytochalasin produced by *Dreschlera*, and alteichin (aka alterperylenol) produced by *Ulocladium* (**Fig. 7**, Grove 1964, Bottalico et al. 1990, Aldrich et al. 2015, Robeson et al. 1984, Okuno et al. 1983). Despite multiple attempts at optimizing production, alteichin was only obtained at miniscule yields (less than 1 milligram from 20 liters of culture), making it difficult to probe dose-response or mechanism of action. Cytochalasin has been studied extensively and is acutely toxic to mammals leading us to decide not to explore further its application for PD management. Radicinin showed the most promise, both for its abundance in fungal culture medium and low toxicity. We demonstrated that purified radicinin inhibited Xf growth in a dose-dependent manner by targeting protease activity (**Fig.8**, Aldrich et al. 2015).

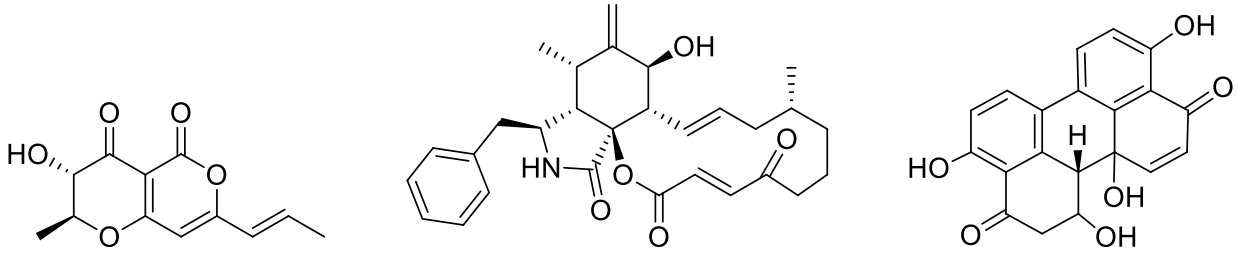

Figure 7: Chemical structures of anti-Xf natural products radicinin, cytochalasin and alteichin

After originally isolating radicinin from *Cochliobolus* sp. we have since observed that we can get higher yields of the compound from cultures of *Curvularia lunata*. So we obtained a culture of *C. lunata* from the American Type Culture Collection (ATCC #38850). We optimized culture conditions by growing the culture in Potato Dextrose Broth with shaking at 30°C for 21 days. The culture broth was extracted by shaking with XAD-7 solid-phase extraction resin, organic compounds eluted from the resin with methanol, and solvent was removed under vacuum to give a crude extract. This residue was then recrystallized from methanol to obtain pure radicinin. To maximize recovery, the remaining radicinin was purified from the filtrate by flash chromatography. In addition, we developed a radicinin formulation. Radicinin's limited water solubility (0.15 mg/mL) poses a challenge for testing radicinin's potential as a possible curative treatment for PD *in planta*, but we were able to formulate a water-soluble solution of radicinin using a combination of organic solvent commonly used in agriculture (10% cyclohexanone), emulsifier (1% TEGO SMO 80) and wetting surfactant (0.1% BREAK- THRU®), which we used in an exploratory study to establish an upper limit for the concentration of radicinin tolerated by grapevine (2 grams/liter). Unfortunately, foliar sprays of this formulation did not result in a reduction of PD severity, likely due to low penetration of the active compound in the plant xylem. We have proposed in our next research goals to evaluate injection of a radicinin-based formulation on *Xf*-host colonization and PD symptoms development.

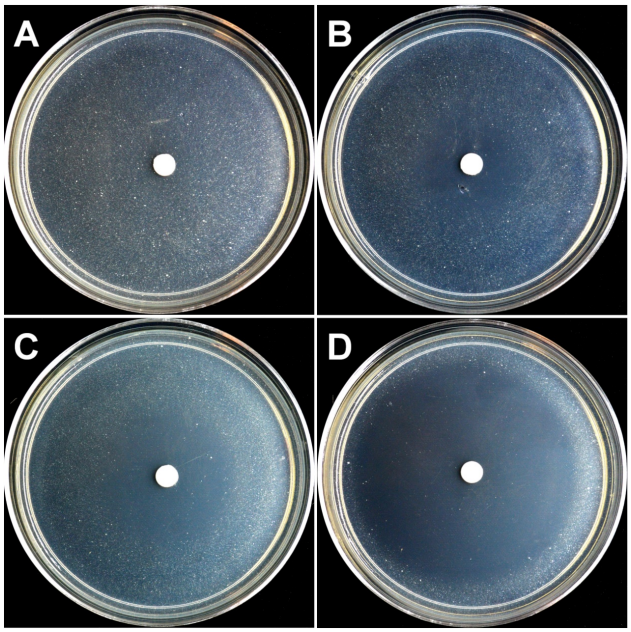

Figure 8: Radicinin purified from the *Cochliobolus* sp. crude extract displays dose-dependent inhibition of *Xf*. (A) 0.05 mg, (B) 0.10 mg, (C) 0.25 mg, (D) 0.50 mg. (Aldrich et al., 2015)

CONCLUSIONS

We aimed to investigate prophylactic and curative measures for management of PD as part of a sustainable PD management program. Our strategy is to utilize both the microbes associated with grapevines and their anti-*Xf* natural molecules. The commercialization of biological control agents and/or novel chemistries will provide a solution for the grape industry to manage PD and if successful could also be expanded beyond grapevine. To date, we have discovered three potential biological control agents to *Xf* (*Pseudomonas, Achromobacter*, and *Cryptococcus*) and developed formulation of one active anti-*Xf* fungal natural product with practical application (radicinin). The next phase will be to optimize the delivery method of those anti-Xf bioproducts and evaluate their performance under natural vineyard settings.

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