CONTROL OF PIERCE'S DISEASE WITH FUNGAL ENDOPHYTES OF GRAPEVINES ANTAGONISTIC TO *XYLELLA FASTIDIOSA*

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ABSTRACT

The goal of this research is to identify xylem dwelling fungi that are antagonistic to *Xylella fastidiosa* (*Xf*) that could be implemented as a preventive or curative treatment for Pierce's disease. We hypothesize that some of the fungal endophytes present in PD-escaped grapevines possess anti-*Xf* properties, likely due to the production of secondary metabolites. We have identified and sampled from vineyards located in Napa and Riverside Counties that are under high disease pressure and identified both diseased and PD-escaped grapevines. We isolated fungal endophytes living in the xylem sap and in one-year-old canes and wood spurs of these vines. We identified them by PCR and sequence analysis of the ribosomal DNA. *Aureobasidium* and *Cladosporium* were predominant in both the xylem sap and wood tissue in all vineyards sampled. Several other fungi had a low incidence and were found only in certain varieties. Notably, three fungal strains have inhibitory effects on *Xf* growth *in vitro*. Furthermore, crude extracts of one of these antagonistic fungi showed similar inhibitory effects. In future work, we will test these strains and fungal products *in planta*.

LAYPERSON SUMMARY

Several management strategies for Pierce's disease (PD) are currently being deployed but as of today, successful management largely involves vector control through the use of insecticides. Here we propose to test an alternative control strategy to complement those currently in place or being developed. Our goal is to identify fungi inhabiting grapevine that are antagonistic to *Xylella fastidiosa* (*Xf*). We hypothesized that in natural field settings grapevine escape PD because the organisms residing in the vine do not allow the establishment of *Xf*. In 2009 and 2010, we have sampled from vineyards in Napa and Riverside Counties that are under high disease pressure and identified fungi living in the sap, canes and spurs of diseased and escaped grapevines. We have selected three fungi that inhibit *Xf* growth in culture. We also extracted compounds secreted by these fungi and identified one fungus able to make natural products inhibitory to the bacterium. In the future our goals are to; 1) extend our sampling in order to screen more fungi with potential for *Xf* growth inhibition; 2) pre-introduce the selected fungal candidates in PD inoculated grapevine cuttings to see if it results in prophylactic control; 3) identify the chemical nature of the natural products produced by the fungi that are antagonistic to *Xf* and determine if we can use them as a curative treatment on PD-infected grapevines.

INTRODUCTION

Current Pierce's disease (PD) management strategies largely involve vector management through the use of insecticides (Byrne and Toscano, 2009). This has contained the spread of the disease (Jetter and Morse, 2009). However, for sustained control of PD, strategies that either target the bacterium or impart resistance to the plant host are required. There are several ongoing research avenues investigating the use of transgenic grapevines and rootstocks that show resistance to PD (Aguero et al., 2005; Kirkpatrick, 2009; Lindow, 2009; Gilchrist and Lincoln, 2009). There is also a traditional breeding approach focused on introducing PD resistance into *Vitis vinifera* grapevines (Walker and Tenscher, 2009). Integrated control strategies are also being investigated in natural vineyard settings. These include the use of natural parasitoids to the glassy-winged sharpshooter (GWSS) (Cooksey, 2009) and inoculation of grapevine with mild *Xylella fastidiosa* (*Xf)* strains that may provide cross protection prior to infection with a virulent three strain of *Xf* (Hopkins, 2009). However, there are no effective curative measures that can clear an infected grapevine of *Xf* besides severe pruning, assuming that the bacteria have not colonized the trunk of the grapevine resulting in a chronic infection.

Notably, control of PD with fungi or fungal metabolites is a largely unexplored research area, although fungi are known to produce an array of secondary compounds that have antimicrobial properties (Getha et al., 2009; Mathivanan et al., 2008). Indeed, using fungi as biocontrol agents against plant disease is an active area of research. Some examples include the use of *Trichoderma* species to control avocado white root rot, the use of *Penicillium oxalicum* to control powdery mildew of strawberries, and the use of fungal endophytes to control frosty pod of cacao (Cal et al. 2008; Mejia et al, 2008; Rosa and

Herrera, 2009). In addition, bio-pesticides that are fungal spore-based are commercially available and registered on grapevine in California.

This proposal focuses on identifying endophytic fungi in grapevine and evaluating their potential as biocontrol agents against *Xf*. Our objectives are to characterize the microbiological 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. Once identified, we will assess the ability of these endophytes and their natural products (ie. secondary metabolites) for inhibitory activity against *Xf in vitro*. Finally we will determine in greenhouse tests if 1) endophytic fungi have potential use as prophylactic bio-control agents for control by inoculating grapevine cuttings with endophytic, *Xf*-antagonistic fungi has a prophylactic effect against PD; and 2) if injection of fungal natural products have curative properties in PD-infected grapevines cuttings. If successful, we envision that these control strategies can be implemented at the nursery level (for endophytes) or directly in the field (for natural products).

OBJECTIVES

1. Identify fungal endophytes that are present in xylem sap and xylem tissues of PD-escaped grapevines but not in PD-symptomatic grapevines.
2. Evaluate the antagonistic properties of the fungal candidates to *Xf in vitro* and conduct a preliminary characterization of the chemical nature of the inhibitory compound(s).
3. Evaluate biological control activity of the fungal candidates *in planta*.

RESULTS AND DISCUSSION

Field Sampling and identification of fungi. In August 2009, we sampled one-year-old canes from grapevines varieties Chardonnay and Cabernet Sauvignon at the research farm on the UC Riverside campus. Although apparently healthy, these grapevines were submitted to the constant disease pressure present in Riverside County, especially given that this vineyard was next to the UCR Citrus Germplasm Depository that supports a large population of the GWSS. Canes were pressure-bombed and 100µl of the sap was plated on general fungal medium, Potato Dextrose Agar (PDA), amended with tetracycline to inhibit bacterial growth. After two weeks of growth at room temperature, the fungi growing were transferred to fresh PDA medium in order to obtain pure cultures. Fungal DNA was extracted from these pure cultures with a Qiagen DNA extraction kit. Following this, the ribosomal DNA was PCR-amplified (600 base pairs) and sequenced (forward and reverse). Fungal taxa were identified after comparing the r-DNA sequence to homologous sequences posted in the GenBank database. We identified five taxa from the sap of these vines, namely *Aureobasidium, Cladosporium, Cryptococcus, Cochliobolus and Chaetomium*.

In August 2010, we sampled from four varieties in two vineyards in Napa County and one vineyard in Riverside. Grapevine varieties included Chardonnay, Merlot, Riesling and Cabernet Sauvignon. We collected one-year-old canes including the wood spur from blocks that had both diseased and PD-escaped grapevines (**Figure 1**). Samples were brought back to the lab and canes were pressure-bombed to extract the xylem sap. Following extraction, 100µl of the xylem sap was plated on general fungal medium, Potato Dextrose Agar (PDA), amended with tetracycline to inhibit bacterial growth. In addition, wood chips were excised from the one-year-old cane and spur and were also plated on PDA-tetracycline medium. Fungi were cultured and identified as described above. The list of endophytic fungi present in escaped and diseased grapevines is presented in **Table 1**. *Cladosporium* and *Aureobasidium* were present in all grapevine varieties and were also found in the xylem sap. Other fungi listed were only found in one grapevine variety of the four sampled and primarily from one-year-old canes and spurs. Additional gene sequencing and morphological identification is currently being conducted to determine the species name of the fungal taxa isolated from the escaped and PD-diseased grapevines.

***In vitro* inhibition assays**. Culturable fungal candidates were evaluated in an *in vitro* inhibition assay for antagonism against *Xf*. In brief, *Xf* liquid cultures were adjusted to OD600nm=0.1 (approx. 10^7 CFU/ml). 300 µl of the *Xf* cell suspension was added to 3 ml of PD3 medium containing 0.8% agar and briefly vortexed. This mixture was overlayed onto a petri plate containing PD3 medium. A #4 size cork borer was flame sterilized and used to cut out a circle of agar containing fungal mycelium from a petri plate containing a fungal culture. This circle was placed onto the plates previously inoculated with *Xf*. Plates were incubated at 28ºC for 10 days and then observed for an inhibition zone around the fungal colony. Measurements were taken of the inhibition zone and recorded (**Figure 2**). Fungal species with inhibition zones were considered inhibitory to *Xf*. To date, we have identified three fungal taxa that are antagonistic to *Xf in vitro*. We are currently testing the other fungi for inhibition of *Xf* using this assay.

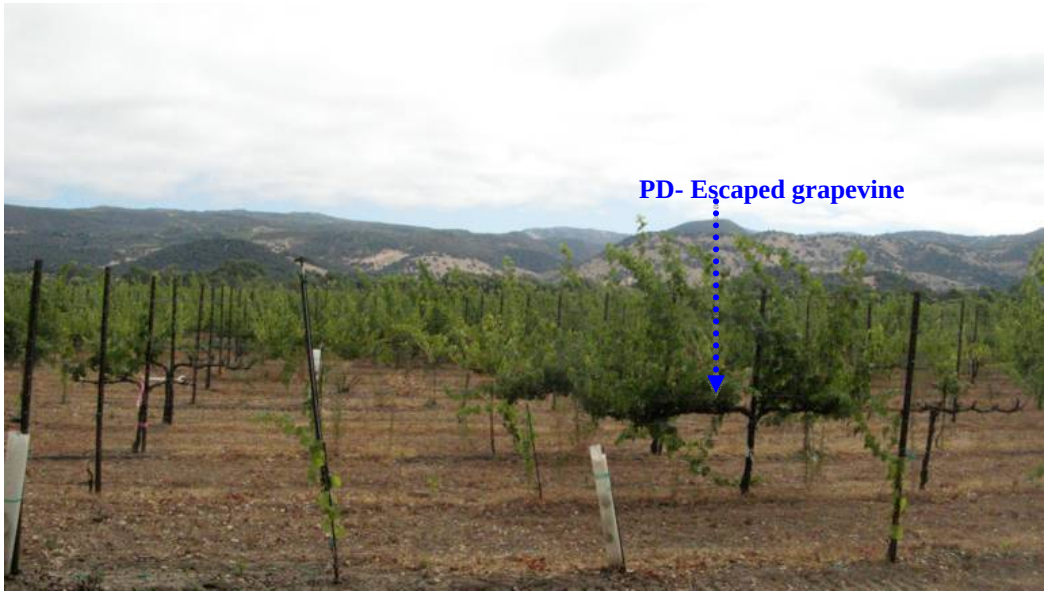

Figure 1. PD-escaped- and diseased grapevines in a Riesling block in Napa County.

Table 1: Identification and percent recovery of fungal taxa from PD-escaped and PD-infected grapevines. Results are based on sampling from three vineyards in Napa and Riverside County, and include four grapevine varieties (Merlot, Cabernet Sauvignon, Chardonnay, Riesling). Fungi were isolated from xylem sap and one-year-old cane and spur wood.

Escaped Grapevine (n=11)		Diseased Grapevine (n=11)	
Fungal Taxa	% Recovery	Fungal Taxa	% Recovery
		Fusarium	18
		Ulocladium	27
		Pezizomycete	9
		Didymella	9
		Unknown taxon 4	9
Cladosporium	82	*Cladosporium*	63
Aureobasidium	82	*Aureobasidium*	91
Alternaria	27	*Alternaria*	27
Cryptococus	9	*Cryptococus*	18
Geomyces	9	*Geomyces*	9
Penicillium	9	*Penicillium*	9
Ustilago	18		
Drechslera	9		
Discostroma	9		
Unknown taxa 1	9		
Unknown taxa 2	9		
Unknown taxa 3	9		

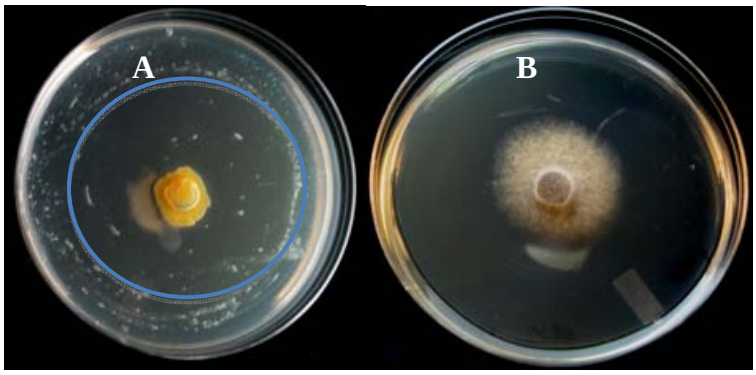

Figure 2: *In vitro* inhibition assay. Two fungal taxa were co-cultured with *Xf* on PD3 medium. Results show a halo of inhibition around the fungal growth (A) indicated by the blue circle and total inhibition of *Xf* growth (B) in comparison to the control (**Figure.3A**).

Isolation of fungal natural products inhibitory to *Xf*. Crude extracts of the three inhibitory fungi were prepared as follows. Agar plugs of 0.5 cm diameter of each fungus were used to inoculate 250 mL liquid media, and the fungi cultivated at room temperature with shaking. After seven days, each culture was extracted with three portions of 125 mL ethyl acetate, the extracts dried over sodium sulfate, and the solvent removed *in vacuo*.

***In vitro* inhibition assay using crude natural product extracts**. *Xf* cultures were prepared as described above. Crude extracts from the three different inhibitory fungi were re-suspended in sterile ethyl acetate to a concentration of 2 mg/ml. Volumes corresponding to a total extract mass of 1 mg, 0.1mg, and 0.01mg were pipetted onto sterile paper discs and allowed to dry in a laminar flow hood. Once dry, the paper discs containing the crude extracts were placed onto the *Xf* cultures and incubated at 28ºC for seven days. Following this, plates were observed for a halo of inhibition around the paper disc. To date, we have identified one fungal taxon producing natural compounds that are antagonistic to *Xf in vitro* (**Figure 3**).

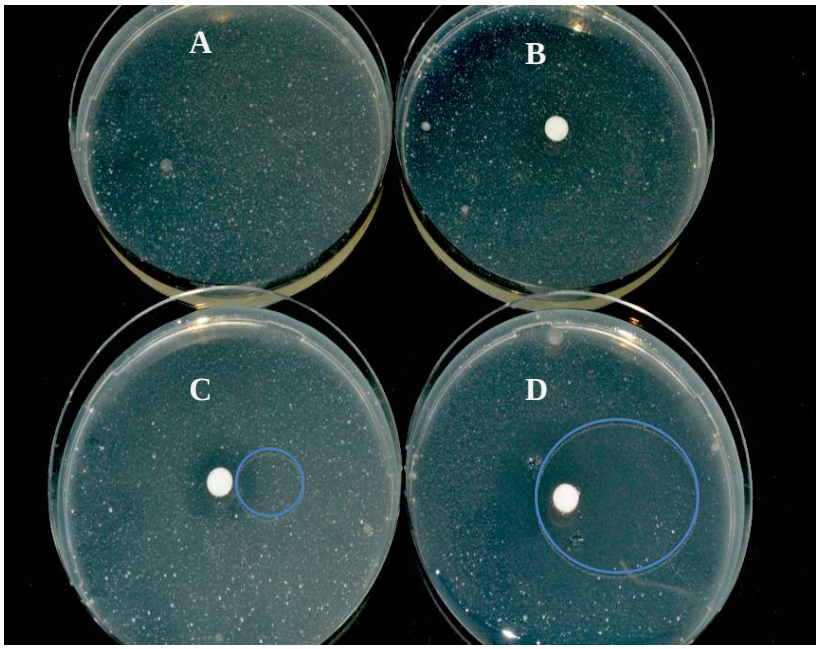

Figure 3: *In vitro* inhibition assay for natural fungal products. Crude fungal natural product extracts were re-suspended in ethyl acetate and pipetted onto paper disc to yield a range of extract masses. Solid PD3 medium was inoculated with *Xf* and overlayed with the paper discs. **A** (*Xf* only) and **B** (ethyl acetate only) show no halo of inhibition, whereas, **C** (0.1 mg crude extract) and **D** (1 mg crude extract) show a halo of inhibition that increases as the concentration of crude extract increases.

CONCLUSIONS

The goal of this research is to identify fungal strains or natural fungal products that have an antagonistic effect towards *Xf*. Thus far, we have isolated three promising fungal candidates that inhibit *Xf in vitro*. In addition, one of these fungi is producing measurable amounts of a secreted natural product that is inhibitory to *Xf*. We are currently characterizing and testing the other fungi that we have isolated from PD-escaped vines as well as the natural product extracts produced by these fungi. In future work, we will inoculate these fungal strains into grapevine cuttings to evaluate their efficacy as a prophylactic control treatment for PD that would limit the establishment of the bacterium in grapevines in natural vineyard settings. Additionally, we are further characterizing the natural products by anti-*Xf* fungi and will evaluate their efficacy as an application product that may have curative effects for grapevines already infected with PD.

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