Title of Report

Interim Progress Report for CDFA Agreement Number 10-0280

Title of Project

Control of Pierce's Disease with Fungal Endophytes of Grapevines Antagonistic to *Xylella fastidiosa*

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Time Period Covered by the Report

07/01/2010 to present

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 largely 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 by inoculating grapevine cuttings with endophytic, *Xf*-antagonistic fungi and 2) if treatments PD-infected grapevine with fungal natural products have curative properties. 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. Identify fungal endophytes that are present in xylem sap and xylem tissues of PD-escaped grapevines but not in PD- symptomatic grapevines.

Objective 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).

Objective 3. Evaluate biological control activity of the fungal candidates *in planta*.

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

Objective 1: Identify fungal endophytes that are present in xylem sap and xylem tissues of PD-escaped grapevines but not in PD- symptomatic grapevines.

The goal is to identify the fungal endophytic populations inhabiting grapevines infected with PD and apparently healthy grapevines adjacent to PD-infected vines (PD-escaped) (**Fig. 1**) with classical isolation techniques and DNA-based methods. Plant tissues/fluids (xylem sap, green shoots, petioles, and wood spurs) were collected at bud-break and before harvest from vineyards grown in Riverside and Napa Counties (**Table 1**) and brought back to the laboratory. Culturable fungi were isolated on fungal medium (Potato Dextrose Agar, PDA), and were identified after comparing the PCR-amplified rDNA sequence to homologous sequences posted in the GenBank database.

Results in **Table 2** show showed that based on our samplings to date, *Cladosporium* sp. and *Aureobasidium* sp. are the most widespread culturable fungi inhabiting grapevine xylem. Both of these species have a high incidence in both diseased and PD-escaped grapevines (xylem sap, green shoots and wood spurs). Furthermore, these fungi are repeatedly identified in the xylem sap of grapevine. We also found other fungal species occurring in both diseased and PD-escaped grapevines, albeit, at a lower frequency. These include *Alternaria* sp., *Cryptococcus* sp., *Penicillium* sp., *Nigrospora* sp., *Biscogniauxia* sp. and a *Geomyces* sp. Some fungi were only present in PD-escaped or diseased grapevines. The fungal species found only in diseased vines include *Epicoccum* sp., *Phomopsis* sp., *Fusarium* sp., *Cryptosporiopsis* sp., *Ulocladium* sp., *Pezizomycete* sp, and *Didymella* sp. Most interestingly, we found several species only inhabiting PD-escaped grapevines. These include *Peyronellae* sp., *Drechslera* sp., *Discostroma* sp., *Cochliobolus* sp., *Chaetomium* sp., *Aspergillus* sp., *Pyronema* sp., *Neofusicoccum* sp., *Phaeosphaeria* sp., *Oidodendron* sp., and *Diplodia* sp. Identification of fungi from sampling in February/March of 2012 from vineyards 2, 3, and 4 is currently underway.

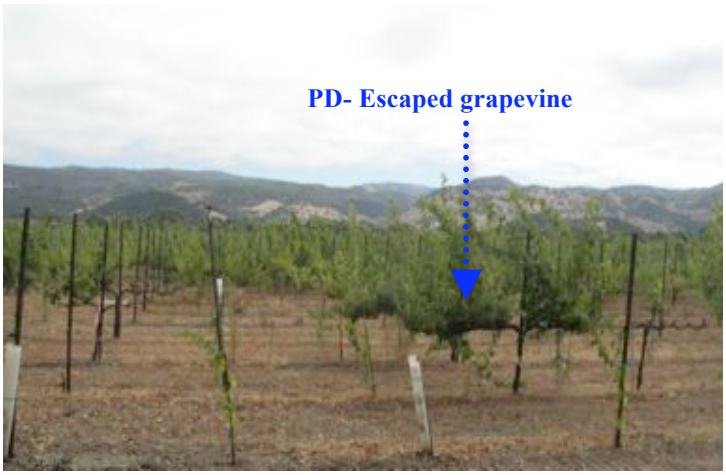

Figure 1: PD-escaped- grapevines in a Riesling block infected with Pierce's Disease in Napa County.

Table 1: Location, variety and timing of the sampling of vineyards.

Location	Vineyard #	Variety	Timing of Sampling
Riverside County	Vineyard 1	Cabernet Sauvignon Chardonnay	August 2009
	Vineyard 2	Syrah	September 2010 March 2011 October 2011 March 2012[a]
Napa County	Vineyard 3	Riesling Chardonnay Merlot	August 2010 April 2011 October 2011 February 2012[a]
	Vineyard 4	Chardonnay	August 2010 April 2011 October 2011 February 2012[a]

[a:] sampling was completed and fungi are currently being identified

Table 2: Identification and percent recovery of fungal taxa from PD-escaped and PD-infected grapevines. Results are based on sampling from 5 grapevine varieties (Merlot, Cabernet Sauvignon, Chardonnay, Riesling, Syrah), in 4 vineyards located in Napa and Riverside County. Fungi were isolated from xylem sap, green shoots and wood spur.

Fungal Taxa	Percent Recovery	
	Escaped Grapevines (n=37)	Diseased Grapevines (n=30)
Cladosporium sp.	63	57
Aureobasidium sp.	59	60
Alternaria sp.	11	30
Cryptococcus sp.	14	7
Penicillium sp.	3	3
Geomyces sp.	3	7
Biscogniauxia sp.	3	3
Nigrospora sp.	3	3
Peyronellae sp.	5	
Drechslera sp.	3	
Discostroma sp.	3	
Cochliobolus sp.	3	
Chaetomium sp.	5	
Aspergillus sp.	3	
Phaeosphaeria sp.	3	
Pyronema sp.	3	
Oidiodendron sp.	3	
Diplodia sp.	3	
Neofusicoccum sp.	3	
Epicoccum sp.		7
Phomopsis sp.		3
Fusarium sp.		7
Cryptosporiopsis sp.		3
Ulocladium sp.		13
Pezizomycete sp.		7
Didymella sp.		3

Characterization of the fungal population using oligonucleotide-based fingerprinting of rRNA genes (Valinsky et al., 2004) is underway to characterize the total (culturable and non-culturable) fungal population inhabiting grapevines. We have extracted the total DNA from diseased and PD-escaped grapevines using Qiagen Plant DNA extraction kit, and were able to PCR amplify the total ribosomal DNA. We have cloned the PCR products into the cloning vector PCR2.1-TOPO (Invitrogen Life Technologies) and are currently sequencing the PCR products.

Objective 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).

The goal of this objective is to identify fungal species and fungal natural products produced by these species that can be used as treatments for control of PD. Fungal cultures recovered from xylem sap, shoot, petioles and spur isolations (Obj. 1) were evaluated in an *in vitro* inhibition assay for antagonism against *Xf*. In brief, *Xf* liquid cultures were adjusted to OD_{600nm}=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 sterile circle of agar was drawn from the margin of an actively growing pure fungal culture and was placed onto the plates previously inoculated with *Xf.* Plates were incubated at 28ºC for 7 days and then observed for an inhibition zone around the fungal colony (**Fig. 2**). Fungal species with a halo of inhibition were considered

antagonistic to *Xf*. All the fungal specimens showing inhibition will be identified to the species level using multi-gene sequencing and morphological identification.

In addition, crude extracts collected from the fungal cultures showing inhibition towards *Xf* was collected for evaluation using the growth inhibition assay as described above. Fungal crude extracts were extracted as follows; 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 7 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 were 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. The efficacy of fungal crude extracts was initially pre-screened in a high throughput method using three paper discs per plate (data not shown) and when inhibition of *Xf* growth was observed, the experiment was repeated using one disc per plate (**Fig. 3**).

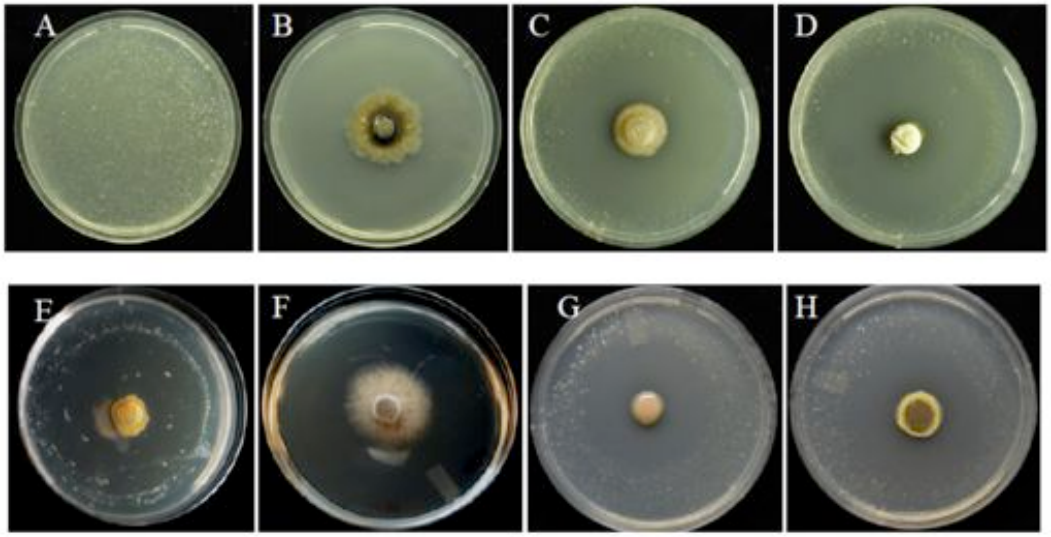

Figure 2: *In vitro* inhibition assay used to evaluate fungi. A) *Xf*-only Control; *Xf* cells were plated in top agar and agar plugs containing fungi were placed on top. Following 8 days of incubation, several fungi strongly inhibited *Xf* growth as indicated by the large zone of inhibition around the fungal colony (Panels C,D,E,G, and H). Two fungi completely inhibited the growth of *Xf* (Panels B, F).

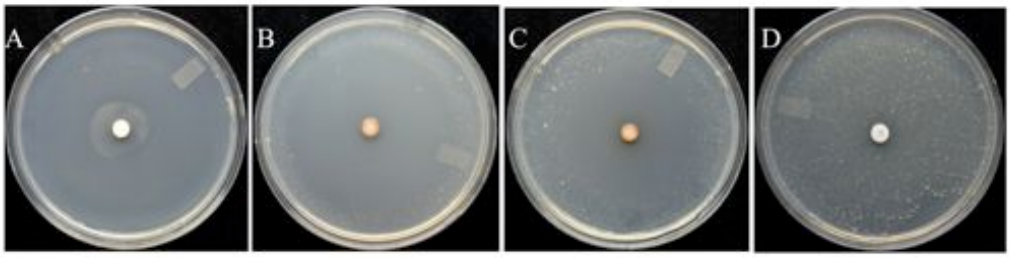

Figure 3: *In vitro* inhibition assay used to evaluate crude ethyl acetate extracts from culture supernatants of fungi. These extracts strongly inhibit *Xf* growth at concentrations of 1 mg/ml as indicated by either a complete lack of growth (Panel A) or a large inhibition zone around the paper disc 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.

From the field sampling we have identified several fungal taxa that provided good inhibition of *Xf* growth *in vitro*, either directly when co-cultured with *Xf* or indirectly when *Xf* was grown in the presence of the fungal natural products. We are currently testing the potential of these fungi as biocontrol agents *in planta* (see Obj. 3). We are currently fractionating the crude extracts from these fungi in order to purify and identify the inhibitory molecules. Thus far, we have purified one individual molecule that is active against *Xf* growth *in vitro* and characterized its chemical structure. 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.

Objective 3. Evaluate biological control activity of the fungal candidates *in planta*.

The goal of this objective is to provide increased tolerance to PD by inoculating grapes with natural fungal endophytes that possessed anti-*Xf* properties. We have selected four fungal candidates that showed inhibitory effect of *Xf* in *in vitro* assays (Obj. 2). Fungal spores were grown on PDA medium were harvested in sterile water and the concentration was adjusted to 10^5 to 10^6 to spores/ml. Grape cuttings var. 'Merlot' (with 2 buds) were vacuum infiltrated (**Fig. 4**) with the fungal spore suspension, planted and placed in the greenhouse. Control plants were infiltrated with sterile water only. In June of 2011, 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 was left un-inoculated with *Xf* to determine if the concentration of fungal spore treatment used is detrimental itself to the grape cuttings. Plant symptoms were rated from 0 to 5 weekly (0= no symptoms; 5=Plant dead or dying) according to Guilhabert and Kirkpatrick (2005). (**Fig.5**).

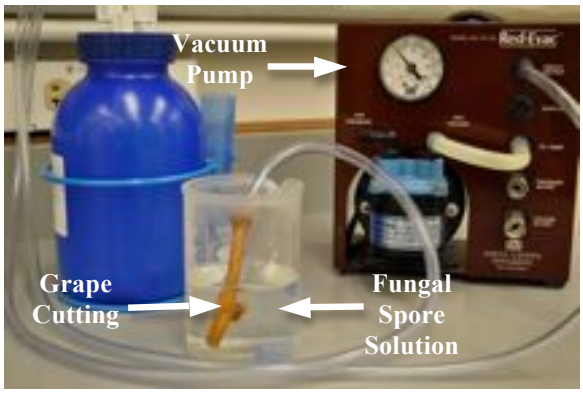

Figure 4: Technique used to vacuum infiltrate grape cuttings with spores of fungal endophytes that showed inhibitory effects in the *Xf in vitro* inhibition assay.

Our results (**Fig. 6**) showed that the progression of the disease symptoms over a 18 week-period were less when grape cuttings were previously vacuum infiltrated with two fungal endophytes (fungus 1 and 3) that showed inhibition to *Xf* in the previous *in vitro* inhibition assay (Obj. 2). The two other fungal endophytes (fungus 2 and 4) show little to no reduction in disease progression. No fungi were detrimental to the plant.

After 18 weeks incubation in the greenhouse, petioles were harvested, surface-sterilized and ground in 1X PBS buffer. The grindate were serially diluted and plated on PD3 medium and allowed to incubate at 28ºC for 10 days. Following incubation, colonies were enumerated and normalized to petiole weight to give a measure of CFU (colony forming units)/g tissue. We measured a lower *Xf* titer in plants inoculated with fungi 2,3,4 than in control plants (**Fig. 7**).

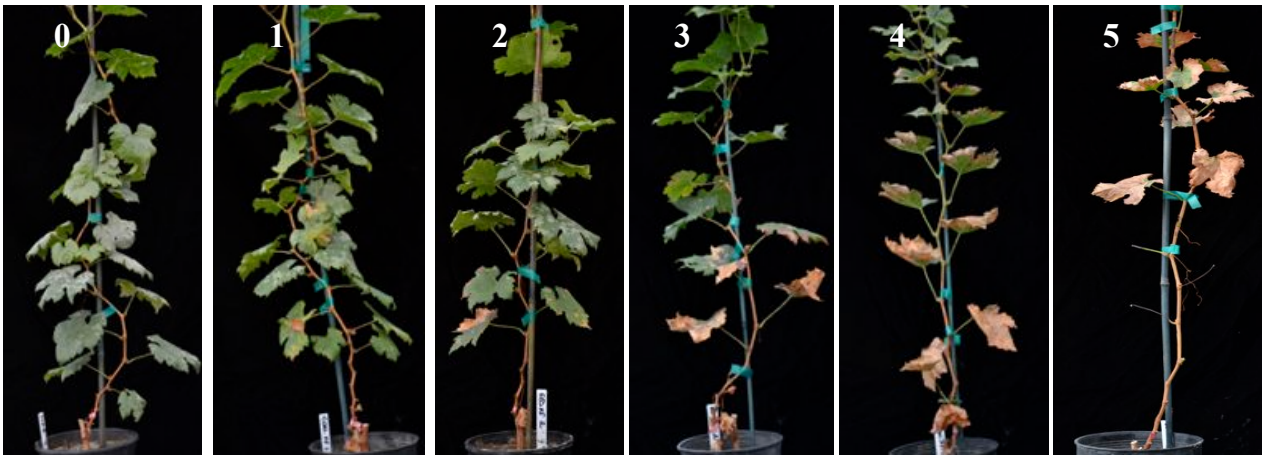

Figure 5: Pierce's Disease symptoms severity rating in grapevine cv. 'Merlot'; 0 = no symptoms (Mock inoculation); 1 through 5= grapes infected with the wild type strain Temecula showing an increase in the disease severity.

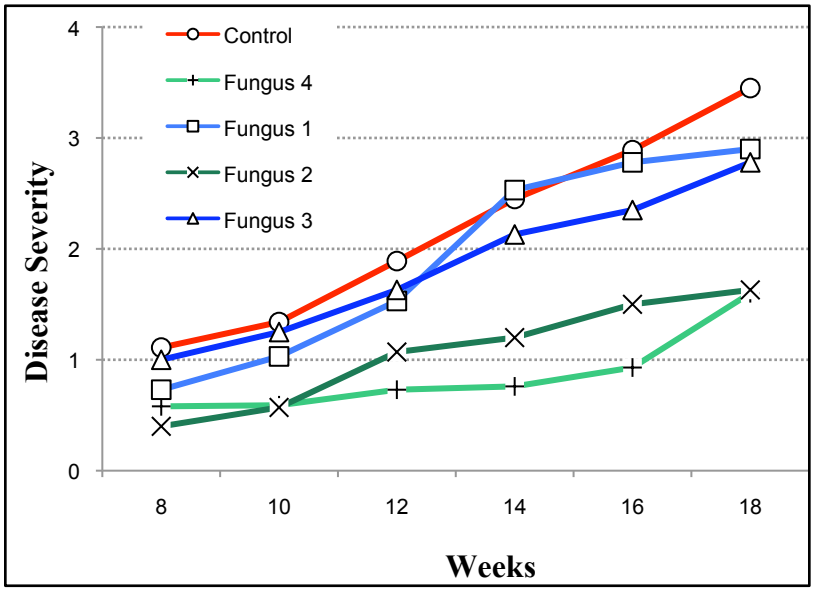

Figure 6: Pierce's Disease severity progression between eight and fourteen weeks post-inoculation with *X. fastidiosa* (*Xf*) 'Temecula' strain on grape cuttings cv. 'Merlot' previously vacuum infiltrated with four different fungal endophytes of grapevine showing inhibition to *Xf* in *in vitro* assays.

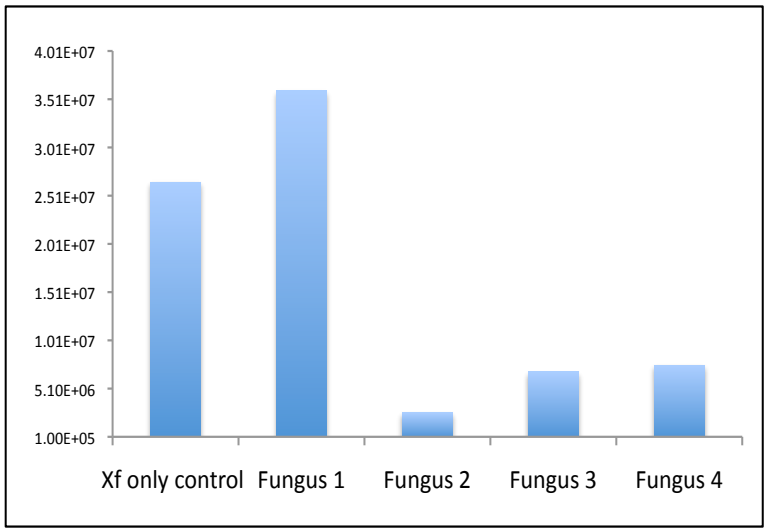

Figure 7: *Xf* population counts in grapevines inoculated with the potential biocontrol fungi. Plants inoculated with Fungi 2, 3 and 4 harbored a lower titer of *Xf* as compared to plants inoculated with *Xf* only which correlates with a decrease in PD severity.

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, 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.

Research Relevance Statement

We aim to investigate 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 have already shown that this strategy is effective in the greenhouse. The remaining step is to confirm that the endophytic fungi provide PD control in a field setting. 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 in which to deliver a sustain supply of the anti-*Xf* compounds effectively into the xylem of an infected vine. We have already discovered one active anti-*Xf* compound that we will test this year 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. In the event that 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 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 was 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, 2010 and 2011 we have sampled from vineyards in Napa and Riverside Counties that are 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. Four fungi were re-introduced in grape cuttings that were inoculated with *Xf*, and two of them show a reduction in PD-disease progression. 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) repeat the experiment with introduction of fungi in grape cuttings with additional potential biocontrols and evaluate these beneficial fungi in the field experiment under natural disease pressure; 2) elucidate the chemical structure of additional fungal natural products antagonistic to *Xf* and test them as a curative treatment on PD-infected grapevines in the greenhouse and 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 February 2012, the remaining funds were of $25,507, which will cover the Principal Investigator (Philippe Rolshausen) salary and benefits ($23,792) until May 2012. The remaining of the funds ($1,715) will be used for the sequencing costs that are necessary to identify the fungal endophytes living in grapevine tissues (Objective 1), and for supplies.

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 several fungi 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.

Literature Cited

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