CDFA RENEWAL PROGRESS REPORT 2011

I- Project Title: Control of Pierce's Disease with Fungal Endophytes of Grapevines Antagonistic to *Xylella fastidiosa*

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III- List of objectives and description of activities conducted to accomplish 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.

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 glassy-winged sharpshooter. 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 2 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 rDNA sequence to homologous sequences posted in the GenBank database.

In August 2010, we sampled from four varieties in two vineyards in Napa County and one vineyard in Riverside County. 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 transferred to obtain pure cultures and identified as described above.

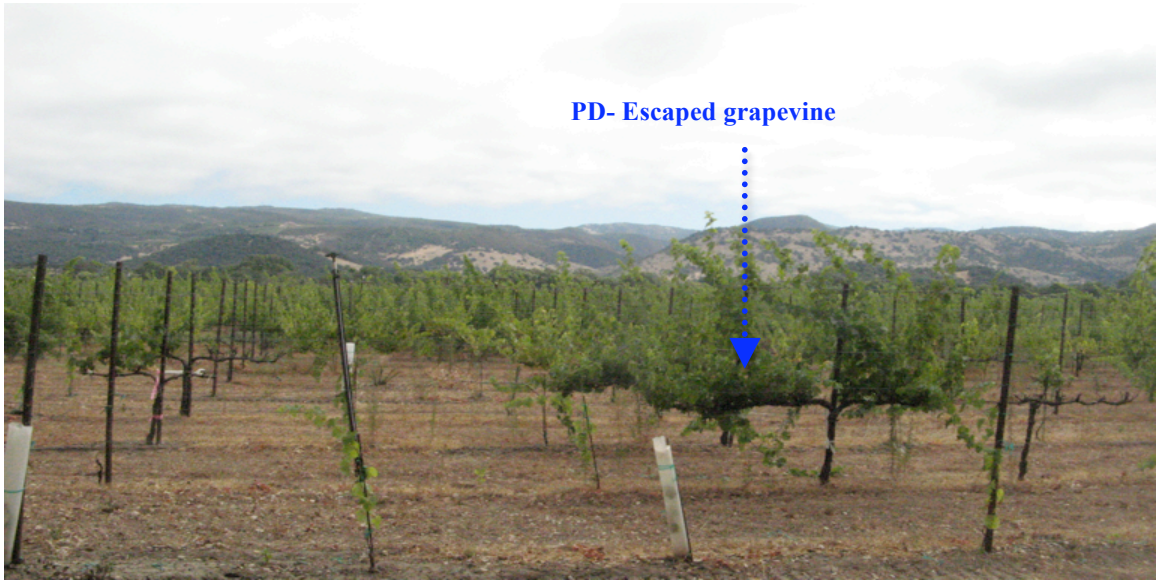

Figure 1: PD-escaped- and diseased grapevines in a Riesling block in Napa County.

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).

Fungal cultures recovered from sap, cane and spur isolations were evaluated in an *in vitro* inhibition assay for antagonism against *Xylella fastidiosa* (*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 #4 size cork borer was flame sterilized and used to cut out a circle of agar from the margin of an actively growing pure 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 (Figure 2). Fungal species with a halo of inhibition were considered antagonistic to *Xf*.

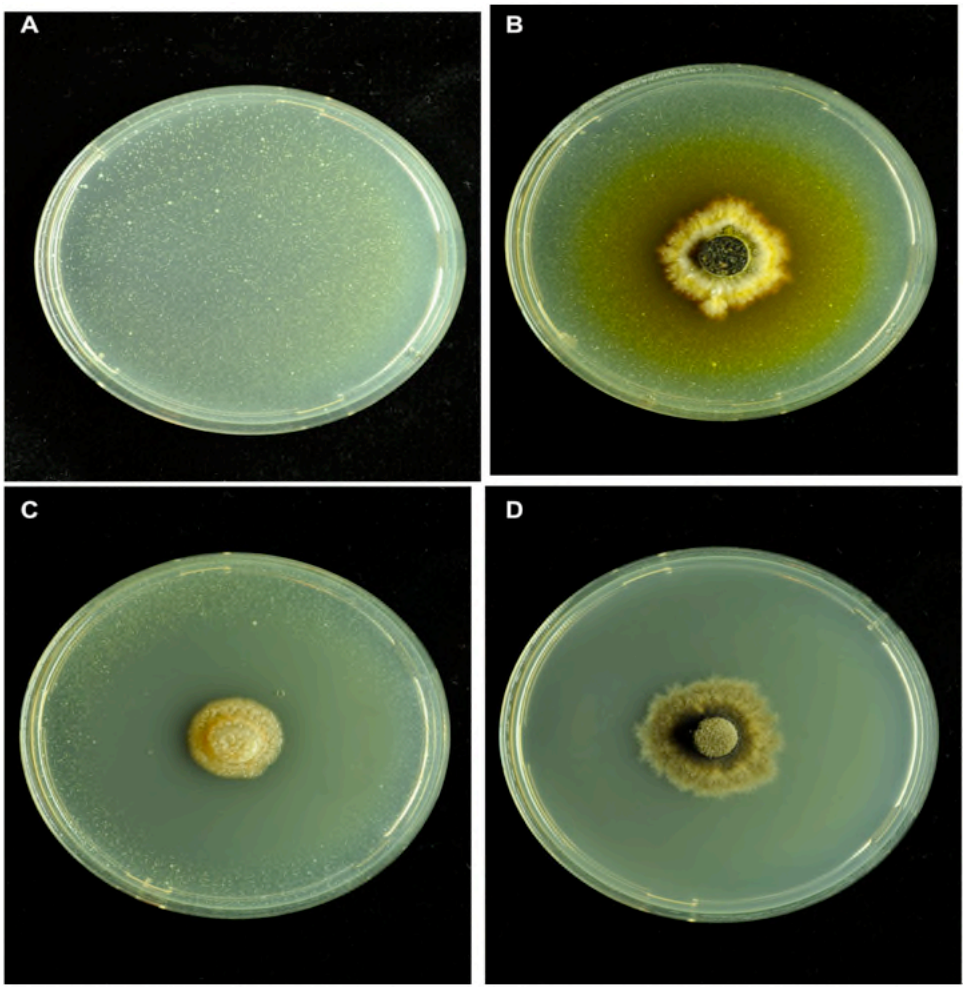

Figure 2: *In vitro* inhibition assay. Three fungal taxa were co-cultured with *Xylella fastidiosa (Xf)* on PD3 medium. Results show; (A) control; (B) no inhibition of *Xf* around fungal growth; (C) partial inhibition of *Xf* as shown with the halo around the fungal growth (D) total inhibition of *Xf.*

In addition, crude extracts collected from these fungal cultures were evaluated in an *in vitro* inhibition assay for *Xf*. *Xf* cultures were grown as previously 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 with Whatman paper and further extracted with three portions of 125 mL ethyl acetate, the extracts dried over sodium sulfate, and the solvent removed *in vacuo*. Fungal crude extracts were re-suspended in sterile ethyl acetate to an extract mass of 1mg, pipetted onto sterile paper discs that were 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 ethyl acetate only (Figure 3).

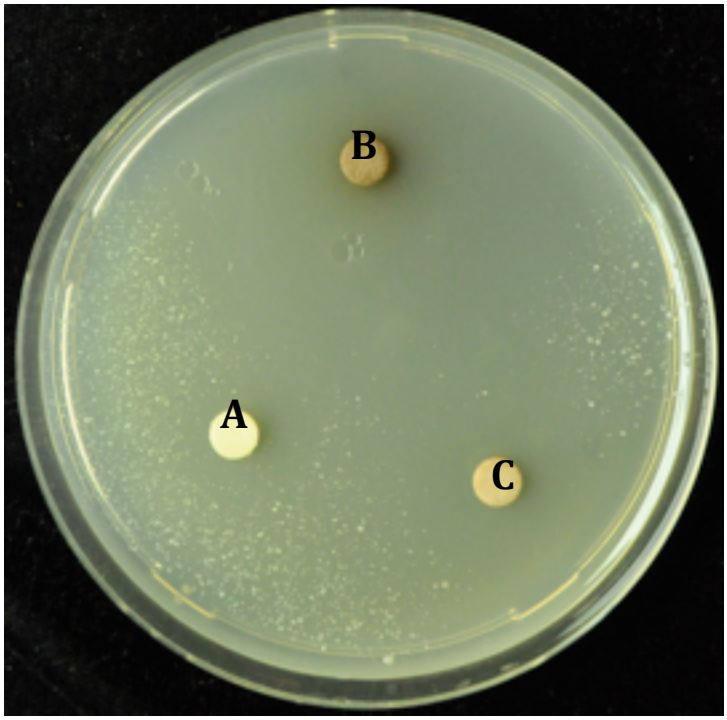

Figure 3: *In vitro* inhibition assay for natural fungal products. Crude extracts of three different fungal cultures were re-suspended in ethyl acetate and pipetted onto paper disc with volumes corresponding to extract mass of 1mg. Solid PD3 medium was inoculated with *Xf* and overlayed with the paper discs. Results show; **A** no inhibition of *Xf*; **B** good inhibition of *Xf* as shown by the halo around the disc; **C** intermediate inhibition of *Xf*.

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

We selected fungal candidates that displayed two features; 1) they showed inhibitory effect of *Xf* in *in vitro* assays; 2) they were heavily sporulating in culture. Spore formation is an important criteria because we need to be able to re-introduce these fungal endophytes into grape cuttings by vacuum filtration (Figure 4). Because of their small size and shape, fungal spores are more likely to infiltrate and colonize the plant xylem vessels than fungal hyphae. Fungal spores were harvested in sterile water and the concentration was adjusted to 10^5 to 10^6 to spores/µl.

Grapes cuttings var. Merlot of 2 buds were vacuum infiltrated (Figure 4) with the fungal spore suspension, planted and placed in the greenhouse. Control plants were infiltrated with sterile water only. In April, shoots arising from the planted cuttings will be inoculated with *X. fastidiosa* (Temecula strain) by mechanical needle inoculation. A sub-sample of plants will not be inoculated to determine if the concentration of fungal spores used are detrimental to the grape cuttings. Planted cuttings will be evaluated for PD symptoms in September of 2011.

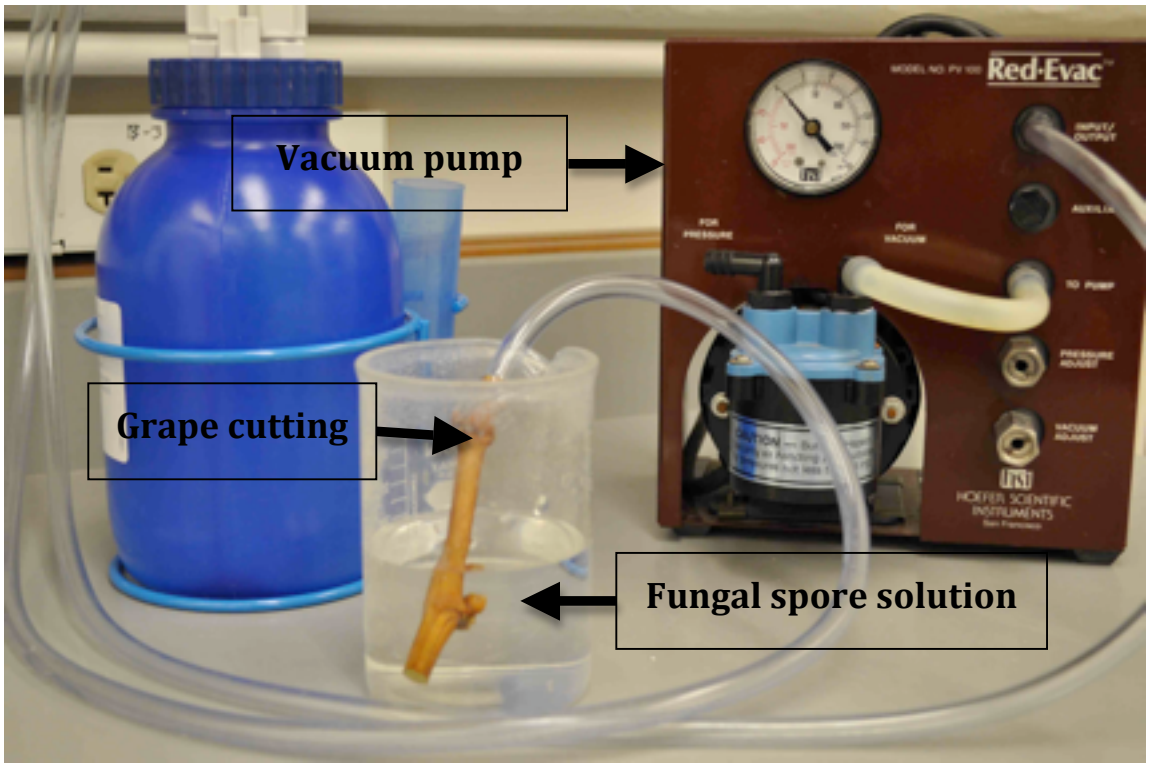

Figure 4: Technique used to vacuum infiltrate grape cuttings with spores of fungal endophytes that showed inhibitory effects in the *Xf in vitro* inhibition assay.

IV- Summary of major research 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.

From our 2009 sampling in Riverside we identified 5 endophytic fungi namely *Aureobasidium, Cladosporium, Cryptococcus, Cochliobolus and Chaetomium*. From our 2010 sampling we identified several endophytic fungi in the sap, canes, and spurs of escaped and diseased grapevines (Table 1). Our results indicate that *Cladosporium* and *Aureobasidium* are the most widespread fungi and have a high incidence in both diseased and escaped grapevines. However, some fungi were only present in escaped or diseased grapevines. Additional gene sequencing and morphological identification is currently being conducted to identify to the species level these fungal taxa isolated from the escaped and PD-diseased grapevines.

Table 1: Identification and percent recovery of fungal taxa from PD-escaped and PD-infected grapevines. Results are based on sampling from 3 vineyards in Napa and Riverside County, and include 4 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
		Cryptosporiopsis	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		
Oidiodendron	9		

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).

From the sampling of 2009 and 2010 we have identified seven fungal taxa that inhibited *Xf* growth *in vitro* with various degrees of inhibition. We have obtained the crude extracts from the culture medium of these fungi and are currently testing the efficacy of these extracts by measuring the concentration at which we can detect a level of inhibition in the growth of *Xf*. Once identified, we will separate the various fractions of the fungal crude extracts and evaluate the efficacy of these fractions in the *Xf in vitro* inhibition assay and characterize the chemical nature of the natural compounds in these fractions.

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

From the seven fungal taxa that showed antagonistic effects towards *Xf* (Objective 2), we have selected 3 to be inoculated *in planta* because they were heavily sporulating *in vitro*. We have currently not collected any data for this objective as we will inoculate the biocontrol-infiltrated cuttings with *Xf* in April of 2011 and rate PD severity in the fall of 2011.

V- Publications or reports resulting from this project.

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.

VI- Presentations on research

Rolshausen, P.E., Maloney, K., Aldrich, T., Kirkpatrick, B., and Roper, M.C. Control of Pierce's Disease with fungal endophytes of grapevines antagonistic to *Xylella fastidiosa*. 2010 Pierce's Disease Research Symposium, San Diego, CA.

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.

VII- Research relevance statement.

These research findings will lead to a practical management strategy for PD for the California wine and table grape industry that targets the bacterium itself. The outcome of this research could lead to control of PD with two different strategies. The first control strategy is to use the biocontrol fungal candidates prophylactically by inoculating grapevine cuttings at the nursery level, thereby providing an increased tolerance to PD in natural vineyard settings because of the antagonistic properties of the fungal strains to *Xf*. The second control strategy is to use the fungal natural products that these fungi produce as curative treatments for PD. Fungal natural products antagonistic to *Xf* could be commercialized and applied as a treatment directly on PD infected grapevines in the field.

VIII- Lay summary of current year's results.

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, grapevines escape PD because the microorganisms 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 for both PD-infected and PD-escaped grapevines. We have selected seven fungi that inhibit *Xf* growth in culture. We are currently extracting compounds secreted by these fungi and testing their inhibitory efficacy against *Xf*. At this point we identified one fungus capable of producing natural products that are strongly inhibitory to the bacterium. We have also re-introduced three fungal candidates in grapevines cuttings that will be inoculated with *Xf* in the spring of 2011 to determine if they can provide prophylactic control against PD. In the future our goals are to; 1) expand our sampling in order to screen more fungi with potential for *Xf* growth inhibition; 2) pre-introduce selected fungal candidates in PD inoculated grapevine cuttings to see if this will

result in prophylactic control of PD; 3) continue to 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.

IX- Status of funds.

The funding for the first year of this project was obtained in August 2010. As a result, we have only used a portion of the funds allocated for 2010-2011. However, we anticipate that the remaining funds will be used by the end of the 2010-2011 budget year. Specifically, we have currently spent $22,952 on the Principal Investigator's (Dr. P. Rolshausen) salary and benefits as well as supplies and travel. We project to spend the remainder of the funding allocated for the 2011-2011 budget year (year 1) on the Principal Investigator (Dr. P. Rolshausen) salary and benefits, one month of the Co-Principal Investigator's summer salary (Dr. C. Roper) as well as supplies and travel. We have also requested a second year of funding as part of this project proposal. If granted, we will spend these dollars on Principal Investigator (Dr. P. Rolshausen) salary and benefits, one month of the Co-Principal Investigator's summer salary (Dr. C. Roper) as well as supplies and travel as outlined in the Budget Justification of the originally funded proposal.

X- Summary and status of intellectual property produced during this research project.

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. Thus far, we have isolated seven promising fungal candidates that inhibit *Xf in vitro* and we have re-introduced three 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. In addition, we are currently characterizing and testing crude natural product extracts obtained from the inhibitory fungi that we identified in our screen. We have currently isolated anti-*Xf* fungal crude natural product extracts from one strongly inhibiting fungal strain and were able to demonstrate that these extracts also possess anti-*Xf* properties. We have begun the initial steps of fractionation/purification that will lead to the isolation of the precise molecule(s) responsible for inhibiting *Xf* in our *in vitro* studies. In future work we will evaluate the efficacy of the isolate molecule(s) as an application product that may have curative effects for grapevines already infected with PD.

The results of this research has been disclosed to the UC Riverside Office of Technology Commercialization and a case number as been allocated (UC Case No. 2011-401-1) and are currently being reviewed for patentability.