PROGRESS REPORT FOR CDFA CONTRACT NUMBER 10-0280

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

II- Principal Investigators:

Philippe Rolshausen, Ph.D
Dept. of Plant Pathology and Microbiology
University of California
Riverside, 92521
Phone: (951) 827-6988
Fax: (951) 827-4294
philrols@ucr.edu

Caroline Roper, Ph.D
Dept. of Plant Pathology and Microbiology
University of California
Riverside, 92521
Phone: (951) 827-3510
Fax: (951) 827-4294
mcroper@ucr.edu

Cooperators:

Bruce Kirkpatrick, Ph.D
Dept. of Plant Pathology
University of California
Davis, 95616
(530) 752-5674
bckirkpatrick@ucdavis.edu

Katherine Maloney, Ph.D
Dept. of Chemistry
Harvey Mudd College
Claremont, CA 91711
(909) 607-9019
maloney@hmc.edu

James Borneman, Ph.D
Dept. of Plant Pathology and Microbiology
University of California
Riverside, 92521
(951) 827-3584
borneman@ucr.edu

Donald Cooksey, Ph.D
Dept. of Plant Pathology and Microbiology
University of California
Riverside, 92521
(951) 314-8904
cooksey@ucrac1.ucr.edu

III- Time period: 07/01/2010 to Present.

IV- Objectives, Materials & Methods and Results.

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 of this objective is to identify the fungal endophytic populations inhabiting grapevines infected with Pierce's Disease and apparently healthy grapevines adjacent to PD-infected vines (PD-escaped) (Fig. 1) with classical isolation techniques and DNA-based methods. Plant tissues (sap, green shoots, 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. Plant sap was collected at bud-break after placing a falcon tube below a bleeding wound and before harvest by pressure-bomb of woody canes. A total of 100 µl of the sap was plated onto a general fungal medium, Potato Dextrose Agar (PDA), amended with tetracycline to inhibit bacterial growth. Fungi were recovered from green shoots and wood spurs using classical isolation techniques on PDA medium with tetracycline (Rolshausen et al., 2010). The remaining plant tissues were kept frozen for characterization of the fungal population by oligonucleotide-based fingerprinting of rRNA genes (Valinsky et al., 2004). After 2 weeks of growth at room temperature, the culturable fungi 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.

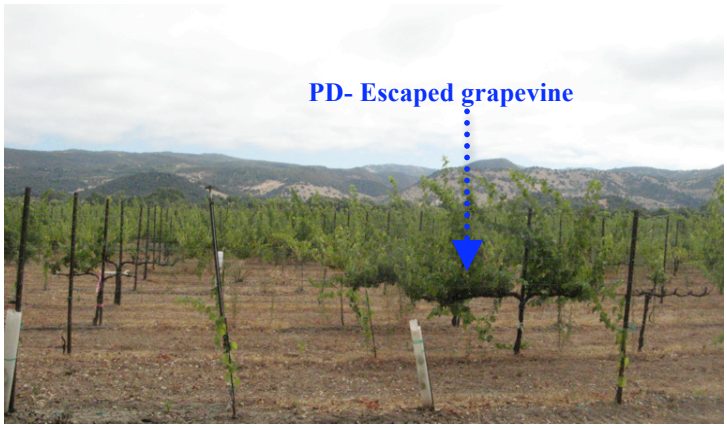

Figure 1: PD-escaped- grapevines in a Riesling block infected with Pierce's Disease in Napa County.

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 Mars 2011
Napa County	Vineyard 3	Riesling Chardonnay Merlot	August 2010 April 2011
	Vineyard 4	Chardonnay	August 2010 April 2011

Identification of fungi using classical isolation techniques showed that *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 escaped grapevines. We also found other fungal species occurring in both diseased and escaped grapevines, albeit, at a lower frequency. These include *Alternaria* sp., *Cryptococcus* sp., *Penicillium* sp., and a *Geomyces* sp. Some fungi were only present in escaped or diseased grapevines. The fungal species found only in diseased vines include *Epicoccum* sp., *Phomopsis* sp., *Fusarium* sp., *Biscogniauxia* 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., *Phaeosphaeria* sp., *Oidodendron* sp., and *Diplodia* sp. (Table 1)

Table 1: Identification and percent recovery of fungal taxa from PD-escaped and PD-infected grapevines. Results are based on sampling from 4 vineyards in Napa and Riverside County, and include 5 grapevine varieties (Merlot, Cabernet Sauvignon, Chardonnay, Riesling, Syrah). Fungi were isolated from xylem sap and green shoots and wood spur.

Fungal Taxa	Percent Recovery	
	Escaped Grapevines (n=26)	Diseased Grapevines (n=19)
Cladosporium sp.	77	53
Aureobasidium sp.	81	74
Alternaria sp.	12	16
Cryptococcus sp.	12	11
Penicillium sp.	4	5
Geomyces sp.	4	5
Peyronellae sp.	8	
Drechslera sp.	4	
Discostroma sp.	4	
Cochliobolus sp.	4	
Chaetomium sp.	8	
Phaeosphaeria sp.	4	
Oidiodendron sp.	4	
Diplodia sp.	4	
Epicoccum sp.		5
Phomopsis sp.		5
Fusarium sp.		11
Biscogniauxia sp.		5
Cryptosporiopsis sp.		5
Ulocladium sp.		16
Pezizomycete sp.		11
Didymella sp.		5

Additional sampling will occur in August/September of 2011 and March/April of 2012 at the same locations to complement these results. All the fungal specimens recovered will be identified to the species level using multi-gene sequencing and morphological identification. Characterization of the fungal population using oligonucleotide-based fingerprinting of rRNA genes (Valinsky et al., 2004) is underway in order to identify non-culturable fungi inhabiting grapevines. We have extracted the total DNA from diseased and escaped grapevines using Qiagen Plant DNA extraction kit and are currently conducting the DNA based population analysis.

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 sap, shoot and spur isolations (Obj. 1) 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*.

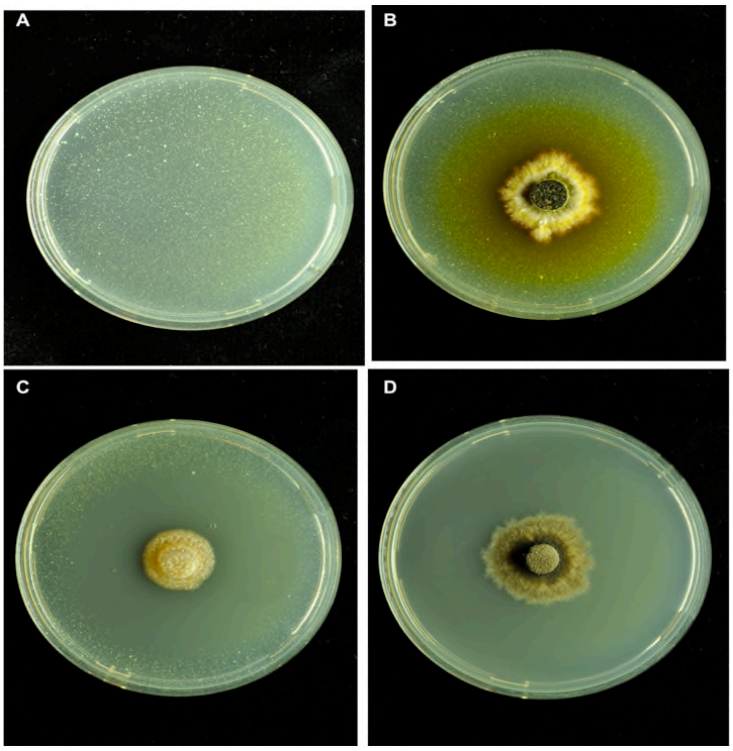

Figure 2: *In vitro* inhibition assay. Three fungal taxa were co-cultured with *Xylella fastidiosa (Xf)* on PD3 medium. Results show; (A) control; (B) no inhibition of *Xf* around fungal growth; (C) partial inhibition of *Xf* as shown with the halo around the fungal growth (D) total inhibition of *Xf.*

In addition, crude extracts collected from the fungal cultures showing inhibition towards *Xf* was collected for evaluation using the growth inhibition assay as described above. *Xf* cultures were grown as previously described. 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 methanol 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 methanol only. The efficacy of fungal crude extracts was initially pre-screened in a high throughput method using three paper discs per plate (Figure 3). When inhibition of *Xf* growth was observed, the experiment was repeated using only 1 disc per plate (Figure 4) and the diameter of the halo of inhibition was recorded.

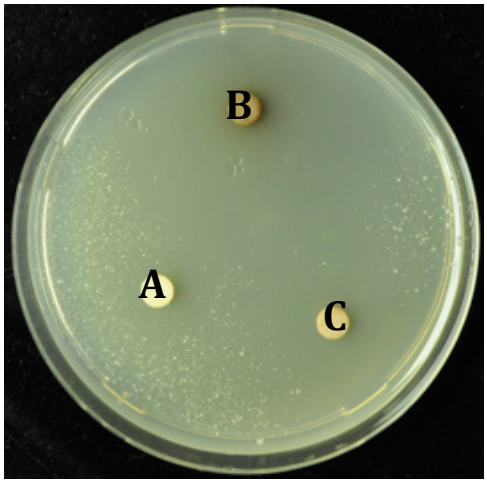

Figure 3: High throughput *in vitro* inhibition assay for natural fungal products. Crude extracts of three different fungal cultures were re-suspended in methanol and pipetted onto paper disc with volumes corresponding to extract mass of 1mg. *Xf* was suspended in 0.8% top agar and poured onto solid PD3 medium and allowed to solidify. Following this, the plates containing *Xf* were overlayed with three paper discs per plate. 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*.

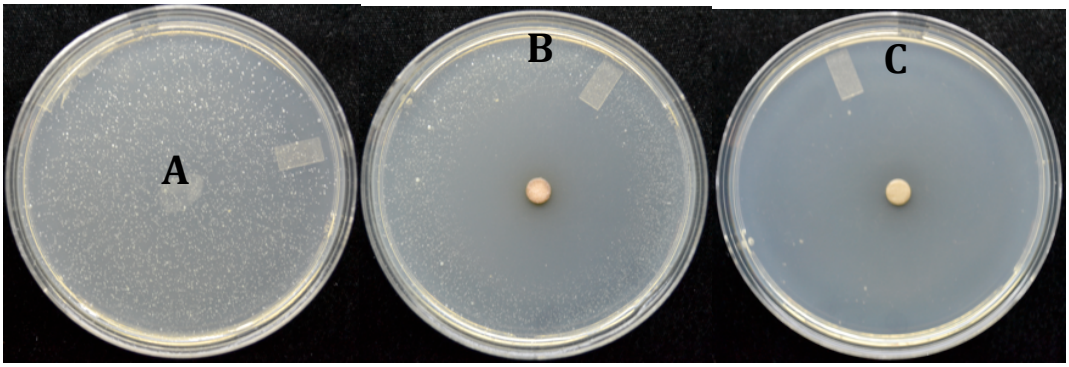

Figure 4: *In vitro* inhibition assay for fungal crude extracts. Crude extracts of two different fungal cultures were re-suspended in methanol and pipetted onto paper disc with volumes corresponding to extract mass of 1mg *Xf* was suspended in 0.8% top agar and poured onto solid PD3 medium and allowed to solidify. Following this, the plates containing *Xf* were overlayed with three paper discs per plate.. Results show; **A** Control *Xf*; **B** intermediate inhibition of *Xf* as shown by the halo around the disc; **C** good inhibition of *Xf*, as shown by the absence of *Xf* growth around the disc.

From the field sampling we have identified nine fungal taxa that inhibited *Xf* growth *in vitro* with various degrees of inhibition. We are currently testing the potential of three of these fungi as a biocontrol agent *in planta* (see obj. 3). In addition, we have found six additionally fungal crude extracts showed inhibition of *Xf* growth *in vitro.* We are currently fractionating the crude inhibitory fungal fractions in order to purify this extract to identify the inhibitory molecule. Thus far we have purified two molecules that are active against *Xf* growth *in vitro* and identified them to the chemical structural level. 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 is to provide increased tolerance to PD by inoculating grapes with natural fungal endophytes that showed anti-*Xf* properties. We have selected three fungal candidates that displayed two features; 1) they showed inhibitory effect of *Xf* in *in vitro* assays (obj. 2); 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. 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 5) 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 were inoculated with *X. fastidiosa* (Temecula strain) by mechanical needle inoculation (Hill and Purcell, 1997). A sub-sample of plants was left un-inoculated with *Xf* to determine if the concentration of fungal spores used is detrimental to the grape cuttings.

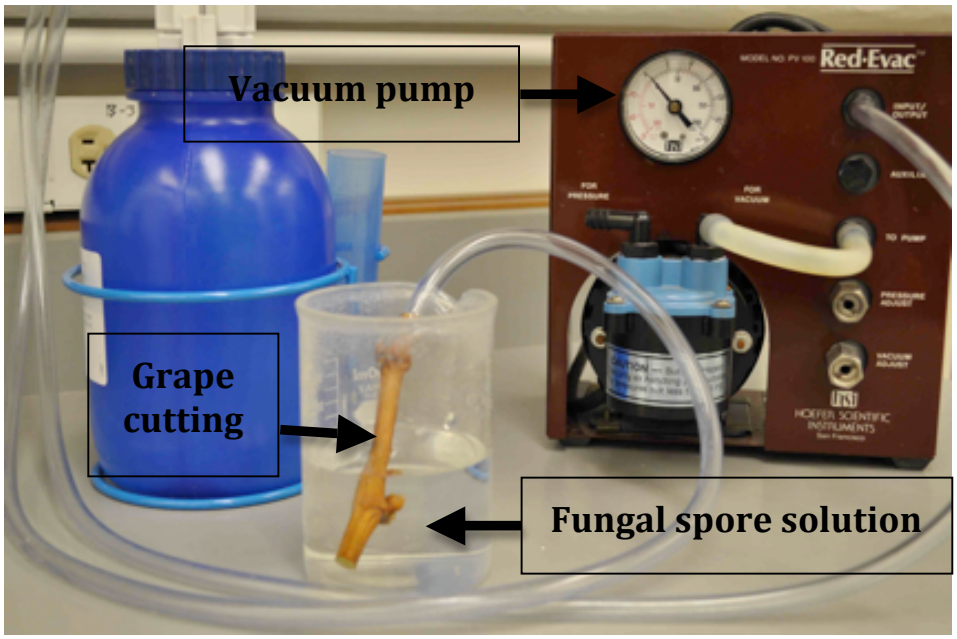

Figure 4: Technique used to vacuum infiltrate grape cuttings with spores of fungal endophytes that showed inhibitory effects in the *Xf in vitro* inhibition assay.

Planted cuttings will be evaluated for PD symptoms in September of 2011 (approximately 12 weeks following inoculation) as described by Roper et al. (2007). Presence or absence of fungal endophytes in inoculated cuttings will also be evaluated by classical isolation techniques. This experiment will be repeated in 2012 with additional potential biocontrols.

V- Intellectual property.

The goal of this research is to identify xylem dwelling fungi that are antagonistic to *Xf* that could be implemented as preventive or curative treatment for Pierce's disease. We hypothesize that some of the fungal endophytes found in PD-escaped grapevines possess anti-*Xf* properties, likely due to the production of secondary metabolites. Thus far, we have isolated nine 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 biocontrol treatments for PD. In addition, we have isolated six fungal crude extracts with anti-*Xf* properties and purified two active molecules from these crude extracts.

The results of this research have 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. Thus, the names of the biocontrol fungi and active molecules cannot be disclosed in this report.

VI- References and Publications resulting from this project.

Publications:

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.

References:

Hill , B. and Purcell, A. 1997. Populations of *Xylella fastidiosa* in plants required for transmission by an efficient vector. *Phytopathology*, 87, 1197-1201.

Roper, M.C. Greve, L.C., Warren, J.G., Labavitch, J.M, and Kirkpatrick, B. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. *Molecular Plant Microbe Interactions*, 20. 411-419.

Rolshausen, P.E., Úrbez-Torres, J.R., Rooney-Latham, S., Eskalen, A., Smith, R.J., and Gubler, W.D. 2010. Evaluation of pruning wound susceptibility and protection against fungi associated with grapevine trunk diseases. Am. J. Enol. Vit. 61:113-119.

Valinsky, L., Scupham, A.J., Vedova, G.D., Liu, Z., Figueroa, A., Jampachaisri, K., Yin, B., Bent, E., Press, J., Jiang, T., Borneman, J., 2004. Oligonucleotide fingerprinting of rRNA genes, In: Kowalchuk, G.A., de Bruijn, J.J., Head, I.M., Akkermans, A.D.L., van Elsas, J.D. (Eds.), Molecular Microbial Ecology Manual, 2nd. Ed. Kluwer Academic Publishers, New York NY, pp. 569–585.

VII- Research relevance statement.

The goal of this research project is to identify a practical management strategy of 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 by two different strategies. The first control strategy is a prophylatic strategy using tbiocontrol fungal candidates that could be inoculated into grapevine cuttings at the nursery level. This practice could, thereby, provide an increased tolerance to PD in natural vineyard settings due to the antagonistic properties of the fungal strains to *Xf*. To date we have identified three fungal specimens that have biocontrol potential and are currently testing them in greenhouse experiments. The second control strategy is to use the fungal natural products that these fungi produce as curative treatments for vines already infected with PD. Fungal natural products antagonistic to *Xf* could be commercialized and applied as a treatment directly on PD infected grapevines in the field. To date we have purified and identified two active molecules that have inhibitory effect towards *Xf* and are currently identifying additional ones.