RENEWAL PROGRESS REPORT, MARCH, 2011

I. Project title

BIOLOGICAL CONTROL OF PIERCE'S DISEASE OF GRAPEVINE WITH BENIGN STRAINS OF *XYLELLA FASTIDIOSA*

II. Principal investigators and cooperators

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III. List of objectives and description of activities conducted to accomplish each objective

Objective 1. To evaluate strain EB92-1 of *X. fastidiosa* for the biological control of Pierce's disease of grapevine in new plantings in the vineyard in California. All plants for the vineyard tests were planted in April 2008 in greenhouses at UC Davis. The cultivars were Orange Muscat (propagated by the grower, Imre Cziraki, and starting budbreak when planted April 6), Cabernet Sauvignon/110R (dormant rooted vines from Vintage Nursery, planted April 30), Reisling/3309 (dormant rooted vines from Vintage Nursery, planted April 30), Chardonnay/3309 (dormant rooted vines from Vintage Nursery, planted April 30), Barbera/110R (dormant rooted vines from Sunridge Nursery, planted April 30, and Viognier/110R (growing potted vines from Vintage, planted April 30.

The biocontrol strain, EB92-1, was recovered from storage in glycerol at -70 C. Five and 6-day cultures of second transfer of the bacterium from storage on PD3 solid medium were hand-carried by Don Hopkins on a flight to California. For biocontrol treatment of the grape plants, a slightly cloudy solution of EB92-1, approximately 0.25 OD at 600 nm ($10^7 – 10^8$ CFU/ml) was prepared in 75 ml of SCP buffer (disodium succinate, 1.0 g/L; trisodium citrate, 1.0 g/L; K_2HPO_4, 1.5 g/L; KH_2PO_4, 1.0 g/L; pH 7.0) in Bruce

Kirkpatrick's laboratory at UC Davis. A pin pricking technique was used to inoculate the biocontrol into the xylem vessels of the treated grapevine. A drop (0.02 ml) of the biocontrol suspension was placed onto each of two lower internodes of the plants. The stem was pierced 3-5 times through the drop with a syringe needle. The inoculum was pulled into the plant by the negative pressure of the pierced xylem vessels. Approximately 5×10^5 to 5×10^6 bacteria were inoculated into each node.

For transplanting into the Bella Vista Vineyard in Temecula, 50 Orange Muscat were inoculated with the biocontrol strain (EB92-1) on June 26, and 50 were left untreated as controls. Fifty Cabernet Sauvignon/110R were treated and 50 were untreated controls. These plants were transported to Temecula and transplanted into plots in the Bella Vista Vineyard on July 21-22, 2008. For Preston Vineyards in Sonoma, 50 Barbera/110R and Viognier/110R from were inoculated with EB92-1 and 50 vines of each were left as untreated controls. These plants were transported to Sonoma and transplanted the last week of July, 2008. For transplanting into the Beringer Vineyard in Napa, 50 Reisling/3309 and 50 Chardonnay/3309 were treated with EB92-1 on June 25 and 50 vines of each were left untreated as controls. The vines were transplanted in Beringer Vineyard in early April 2009.

Objective 2. To evaluate strain EB92-1 of *X. fastidiosa* for the protection of older established grapevines against Pierce's disease in California vineyards. Since PD is rapidly developing in the mature Chardonnay block at Beringer Vineyard in Napa, it was chosen for an evaluation of EB92-1 for the prevention of PD development in mature, producing grapevines. Randomly, forty vines were inoculated with EB92-1 and 40 vines were chosen as controls. On September 8, 2010, the vines were inoculated with strain EB92-1 in the main trunk, approximately equidistant from the graft and the trellis wire. Vines were injected by boring a small hole into the trunk with an electric drill. Two ml of the bacterial suspension will be injected into each hole using a nail-injector syringe.

Objective 3. To develop a PCR based assay that can quickly differentiate the PD biocontrol strain EB 92-1 from pathogenic, wild type Xylella strains. We will continue to evaluate the tandom repeat numbers (TRN) of additional PD Xf reference strains as well as samples that we collect from the biocontrol plots. For the field samples we will initially test approximately 6 to 8 individual colonies from any one sample to determine the degree of potential polymorphisms in colonies isolated from a symptomatic vine. DNA extraction is very straightforward as a single colony will be removed from a medium plate and suspended in 40ul of sdH2O. The sample will be boiled for 3 minutes and 5ul of the supernatant DNA will be used as the PCR template. This method was used to prepare DNA template for the gel shown in Figure X. PCR conditions and primers to be used are described by Chen, et al., 2008. We will initially evaluate all PCR field samples using agarose gel electrophoresis to get a clear visual representation of the size and number of the PCR products in each sample.

We will also evaluate the efficacy of determining the size of the TRN PCR products using quantitative real-time PCR (QRT-PCR) following the methodology described by Bextine and Child, 2007. These researchers used QRT-PCR to differentiate gyrase B genes from 3 Xf strains that only differed in a few nucleotides. It is likely that

TRNs that differ in size by many base pairs should be readily differentiated using this method. If successful, QRT-PCR has the advantage that samples will not have to be evaluated by gel electrophoresis. Determining the identity of an Xf strain in a vine would simply involve isolating Xf colonies on PD3, picking a representative number of colonies from the plate and directly analyzing their TRN number by QRT-PCR which generates unique temperature melting profiles for each size TRN.

Objective 4. To evaluate rapid, efficient methods of treatment with strain EB92-1 of *X. fastidiosa* for the biocontrol of PD in *V. vinifera* in the vineyard. On May 29, 2007, Merlot/101-1 plants were injected with EB92-1 in the greenhouse. Treatments were (1) EB92-1 in scion only, (2) EB92-1 in rootstock only, (3) EB92-1 in both rootstock and scion, and (4) Nontreated. On June 21, vines were transplanted into the vineyard in 3 replications of 3 plants per treatment. In another experiment, Chardonnay cuttings from the MREC vineyard were grafted onto Salt Creek rootstock rooted cutting from the vineyard. The grafted plants were transplanted into the vineyard on August 14. The treatments included 1) Chardonnay cuttings from mature vines that had been treated 3 years ago with EB92-1 on Salt Creek, 2) Chardonnay cuttings from mature nontreated vines on Salt Creek, and 3) Chardonnay cuttings from mature nontreated vines on Salt Creek, with the scion injected with EB92-1 in the vineyard on August 29.

New experiments are being initiated to evaluate infiltration of EB92-1 biocontrol strain into propagation material for control of PD. Two to three node green cuttings with leaf attached at top node will be allowed to take up a suspension of
EB92-1, will be rooted in a mist bed, and planted in the vineyard or used as a rootstock for a green graft. Treatments will include 1) EB92-1 treated rooted cuttings of Chardonnay, 2) untreated rooted cuttings of Chardonnay, 3) EB92-1 treated Salt Creek as a rootstock for Chardonnay, and 4) untreated Salt Creek as a rootstock for Chardonnay.

IV. Summary of major research accomplishments and results for each objective

Objective 1. To evaluate strain EB92-1 of *X. fastidiosa* for the biological control of Pierce's disease of grapevine in new plantings in the vineyard in California.
In September 2010, all the young plants in the Bella Vista vineyard appeared to have severe water and nutritional stress. PD-like symptoms were extensive in the plants that were still alive, treated and untreated. Many plants died without ever having any PD symptoms, probably due to the lack of water and poor nutrition. It is difficult to discern whether the PD-like symptoms are due to water stress or whether water stress increases PD. In the Orange Muscat test, 35-40% of the vines had died after 2 years from something other than PD, probably lack of water. Twenty-two percent of the Cabernet Sauvignon also had died, probably from water stress. In both the Cabernet Sauvignon and Orange Muscat, many of the vines were severely stunted and barely reached the trellis wire after 3 seasons and more than 2 years. Therefore, the Orange Muscat test is definitely lost. The Cabernet Sauvignon test will probably have to be abandoned also, but better irrigation and fertilization could salvage it. We will re-evaluate it in 2011. To replace the lost tests in southern California, a replacement test is being established in the spring of 2011.

In September 2010 in Preston Vineyards in Sonoma, there were still no symptoms in the Barbera block, either in the new test vines or the older vines. This test will not be evaluated in 2011, because of the lack of disease. The Viognier block has significant PD incidence in the mature vines and these new test vines began to develop PD symptoms in 2010 (Table 1). Symptoms were not very severe, but there were more symptomatic vines in the untreated vines than in the EB92-1 vines. This trend indicated that EB92-1 was reducing the incidence of PD in the Viognier. With the amount of symptoms in the mature Viognier vines, PD should continue to develop in the young test vines.

Table 1. Biocontrol of PD in 2-year-old grapevines in Northern California vineyards 9/8/10.

	EB92-1 treated vines:		**Untreated vines:**	
Cultivar	**#PD vines/total**	**Disease rating[1]**	**#PD vines/total**	**Disease rating[1]**
Beringer Vineyard, Napa				
Chardonnay	3/45 (7%)	0.1	4/48 (8%)	0.1
Reisling	4/47 (9%)	0.1	6/51 (12%)	0.1
Preston Vineyard, Sonoma				
Viognier	8/48 (17%)	0.2	13/48 (27%)	0.3
Total	15/140 (11%)	0.1	23/147 (16%)	0.2

[1]Disease rating was an average per vine on a scale of: 0 = no symptoms; 1 = any symptom of PD, such as marginal necrosis (MN) on a basal leaf; 2 = definite, moderate symptoms on <50% of vine; 3 = severe symptoms on >50% of vine; 4 = dead plant.

With better irrigation, both the Chardonnay and Reisling test vines in the Beringer Vineyard were vigorous and grew well in this second growing season. PD began to develop in the test vines of both varieties (Table 1). The trends were for less PD in the EB92-1 treatments and more PD in Reisling than Chardonnay. However, these are very early results as the plants are only in their second season and less than 2 years old. With extensive PD in the mature Chardonnay and Reisling vines, disease should continue to develop in the young test vines.

Objective 2. To evaluate strain EB92-1 of *X. fastidiosa* for the protection of older established grapevines against Pierce's disease in California vineyards.

A plot was established in Napa in 2010; no results are available.

Objective 3. To develop a PCR based assay that can quickly differentiate the PD biocontrol strain EB 92-1 from pathogenic, wild type Xylella strains.

EB 92-1 colonies have consistently produced the same size PCR fragment that differed in size from the PD wild type strains. Although our sample size has been comparatively small the initial results provide rationale for further evaluating this as a tool for differentiating EB- 92-1 from wild type Xf strains which will strengthen our ability to conclude whether EB 92-1 provides PD protection under California conditions.

Objective 4. To evaluate rapid, efficient methods of treatment with strain EB92-1 of *X. fastidiosa* for the biocontrol of PD in *V. vinifera* in the vineyard.
In 2010 comparisons of treating scion versus rootstock, the EB92-1 treated vines had less PD than the untreated (Table 2). PD biocontrol was obtained whether EB92-1 was injected into the scion, the rootstock, or both. The treatments appeared to be equally effective.

Table 2. Effect of methods of treatment of grape plants with *Xylella fastidiosa* strain EB92-1 on biological control of Pierce's disease.

	% PD incidence in August 2010 in:[1]
Treatment	**Merlot/101-14**
Scion injection	13
Rootstock injection	11
Scion & Rootstock injection	14
Scion field injection	-
Untreated	38

[1]%PD is the number of plants with symptoms divided by total number of plants x 100.

In 2010, plants that consisted of scion wood from mother vines infected with EB92-1 had less PD than untreated vines (Table 3). In contrast to 2009, there was less PD in plants developed from scion wood from EB92-1 infected mother plants than in plants that were injected directly with EB92-1. While it may be too early to draw conclusions, this indicates that there could be transfer of the biological control from the mother plant through scion wood. Further development of the symptoms will be observed. This evaluation of scion from treated mother vines is especially significant, because scion wood from infected mother vines could be an efficient treatment method that does not require a lot of additional hand labor over normal production practices.

Table 3. Transmission of biocontrol in scion from infected Chardonnay mother plant grafted onto Salt Creek rootstock.	
	% PD incidence in August 2010:
Treatment	
Scion from clean Chardonnay	40
Scion from clean Chardonnay injected with EB92-1 in the field	27
Scion from EB92-1 Chardonnay mother plant	9

V. Publications or reports resulting from the project

Hopkins, D. L. 2007. Biological control of Pierce's disease of grapevine with benign strains of *Xylella fastidiosa* subsp. *piercei*. IN: Proceedings of the Pierce's Disease Research Symposium, December 12-14, 2007, San Diego, CA, California Department of Food and Agriculture.

Hopkins, D.L. and Thompson, C.M. 2008. Biological control of Pierce's disease in the vineyard with a benign strain of *Xylella fastidiosa.* J. Plant Pathol. 90S:115.

Hopkins, D. L., B. Kirkpatrick, B. Hill, R. Smith, and D. Johnson 2008. Biological control of Pierce's disease of grapevine with benign strains of *Xylella fastidiosa* subsp. *piercei*. Pages 164-166, IN: Proceedings of the Pierce's Disease Research Symposium, December 15-17, 2008, San Diego, CA, California Department of Food and Agriculture.

Hopkins, D. L., B. Kirkpatrick, B. Hill, R. Smith, and D. Johnson 2009. Biological control of Pierce's disease of grapevine with benign strains of *Xylella fastidiosa*. Pages 129-132, IN: Proceedings of the Pierce's Disease Research Symposium, December 9-11, 2009, Sacramento, CA, California Department of Food and Agriculture.

Hopkins, D.L. 2010. Management strategies for Pierce's disease: An increasing threat to grape production in the southern US. Phytopathology 100(6):S201.

Hopkins, D. L. 2010. Biological control with *Xylella fastidiosa* strain EB92-1 for the prevention of Pierce's disease development in mature, producing grapevines. Phytopathology 100(6):S52.

Hopkins, D. L., B. Kirkpatrick, B. Hill, R. Smith, and D. Johnson 2010. Biological control of Pierce's disease of grapevine with benign strains of *Xylella fastidiosa*. Pages 187-190, IN: Proceedings of the Pierce's Disease Research Symposium, December 15-17, 2010, San Diego, CA, California Department of Food and Agriculture.

VI. Presentations on research

Poster at Pierce's Disease Research Symposium in San Diego, December 12-14, 2007

Oral presentation at Pierce's Disease Research Symposium in San Diego, December 15-17, 2008

Oral presentation at Texas PD Symposium on "Biological control of Pierce's disease with a benign strain of *Xylella fastidiosa*" at Fredericksburg Texas in April, 2009

Oral presentation on "Biological control of Pierce's disease with *Xylella fastidiosa* strain EB92-1 in Vineyards with Different Rates of Disease Development" at the Pierce's Disease Research Symposium in Sacramento, December 9-11, 2009

Oral presentation at Pierce's Disease Research Symposium in San Diego, December 15-17, 2010

VII. Research relevance statement

The successful completion of the proposed research could lead to an effective control of Pierce's disease that is environmentally friendly. The strains utilized in this study are naturally occurring and are not genetically modified in any way. Thus, we would avoid the concerns associated with introducing genetically modified organisms or plants. This should lead to faster implementation than could be attained with genetically engineered plants or biocontrol organisms. This project should yield results within the next 3-4 years and if the control is successful, there should be a biological control for Pierce's disease available for commercial use in vineyards in California.

VIII. Lay summary of current year's results

In trial plantings of Orange Muscat and Cabernet Sauvignon in Bella Vista Vineyard in Temecula, almost half of the Orange Muscat, treated or untreated vines have died from something other than PD, probably water stress. Many of the surviving vines were severely stunted and barely reached the trellis wire after more than 2 years and 3 growing seasons. This makes it impossible to obtain good data so the trial has been abandoned. Approximately a fourth of the Cabernet Sauvignon also have died and this test will be abandoned unless plant survival and vigor improve. In Preston Vineyards in Sonoma, EB92-1 was controlling PD in Viognier when compared to untreated vines. In Beringer Vineyard in Napa, PD began to develop in the tests on Chardonnay and Reisling and trends were for reduced PD in the EB92-1 treated compared to the untreated. In both the Sonoma and Napa trials, symptoms are just beginning to develop so it is early to draw definitive conclusions on the control trends. We are evaluating the use of mother vines infected with the biocontrol strain EB92-1 as propagation material for scion wood. In 2010, vines developed using scion wood from mother vines of Chardonnay infected with EB92-1 had less PD than vines developed with uninfected scion wood. Development of plants with scion wood from infected mother vines could eliminate the need to inject every vine by pin pricking.

IX. Status of funds

Good, adequate support through June 30, 2012

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

None, UF owns patent on strain, EB92-1