

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

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INTRODUCTION

Pierce's disease (PD) of grapevine is an endemic, chronic problem in the southeastern USA where it is the primary factor limiting the development of a grape industry based on the high-quality European grapes (*Vitis vinifera* L.) (Hopkins and Purcell, 2002). PD is also endemic in California and has become more of a threat to the California grape industry with the introduction of the glassy-winged sharpshooter. While vector control has been effective for PD control in some situations, the only long-term, feasible control for Pierce's disease has been resistance. Almost 20 years of research on the biological control of Pierce's disease of grapevine by cross protection with weakly virulent strains of *X. fastidiosa* has demonstrated that this is a potential means of controlling this disease (Hopkins, 2005). One strain of *X. fastidiosa* that was able to control PD in *V. vinifera* for 14 years in Central Florida has been identified. We are testing this strain in commercial vineyards in several states and, if these tests are successful, the strain will be ready for commercial use. In most trials with the biocontrol strain, the bacteria were injected into the grapevines either in the greenhouse or in the vineyard after transplanting. This is a labor-intensive procedure. Treatment methods that would make the technology less labor-intensive, less costly, and more consistent are being evaluated. The overall goal of this project is to develop a biological control system for Pierce's disease (PD) of grapevine that would control the disease in California and other areas where PD and the glassy-winged sharpshooter (GWSS) are endemic.

OBJECTIVES

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.
2. To evaluate strain EB92-1 of *X. fastidiosa* for the protection of older established grapevines against Pierce's disease in California vineyards.
3. To develop a PCR based assay that can quickly differentiate the PD biocontrol strain EB 92-1 from pathogenic, wild type Xylella strains.
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.

PROGRESS AND RESULTS

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.**

Southern California tests. For transplanting into the Bella Vista Vineyard in Temecula, 50 Orange Muscat were inoculated with the biocontrol strain (EB92-1) on June 26, 2008 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.

In late fall 2008, PD-like symptoms were observed in most of the vines at Bella Vista, treated or untreated (Observation by Barry Hill). However, it was very hot and dry in 2008 and some of these symptoms may have been due to the weather. In the summer of 2009, PD symptoms were still extensive in the Bella Vista Vineyard, but were observed in only about half of the vines that had symptoms in 2008. All of the vines, treated and untreated, were under severe water stress and this may have caused some of the PD-like symptoms. Differences in the incidence of leaf scorch between the treated and untreated vines were not significant. The Orange Muscat planting was interspersed with mature vines that were nearly 100% infected with Pierce’s disease. This entire planting, except our experimental vines were removed during the winter of 2009, leaving only our young plants scattered in the vacant vineyard.

In September 2010, all the young plants in the Bella Vista vineyard appeared to have severe water and nutritional stress. Many plants died without ever having any visible PD symptoms, probably due to the lack of water and poor nutrition. 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. Therefore, the trials were abandoned.

To replace the lost tests in southern California, a replacement test was established in 2011 at UC Riverside. For transplanting into the UC Riverside vineyard, 100 Merlot/1103 plants and 100 Pinot Noir/1103 plants were obtained from Sunridge Nursery in March 2011 and maintained in UC Davis greenhouse. Fifty Merlot and 50 Pinot Noir were inoculated with EB92-1 in July 2011 and fifty plants of each cultivar were kept as untreated controls. These plants were maintained in the greenhouse for 6 weeks and then moved outside to harden them off. These plants were transported to Riverside in mid-October and transplanted into the plot at UCR. In 2012, there was very little PD development and no differences between treatments (Table 1). This plot will continue to be monitored even though the project has ended.

| Cultivar | Untreated vines: | | EB92-1 treated vines: | |
|--|------------------------|-----------------------------|------------------------|-----------------------------|
| | Incidence ¹ | Disease rating ² | Incidence ¹ | Disease rating ² |
| Merlot | 0/26 (0%) | 0 | 3/45 (7%) | 0.07 |
| Pinot Noir | 3/43 (7%) | 0.09 | 1/49 (2%) | 0.02 |
| ¹ PD incidence is the number of PD symptomatic vines over total vines in treatment. | | | | |
| ² 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. | | | | |

Sonoma tests. 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 as replants for missing vines in a mature vineyard the last week of July, 2008. On August 26, 2009, these vines were mapped for symptoms. All of the Barbera vines appeared to be healthy with no PD symptoms. The block of Barbera did not appear to have any PD symptoms, even in the older vines and this test was abandoned because of the lack of disease.

In the Viognier test, there were a few vines that had minor yellow and/or necrotic leaf margins on the basal leaves in 2009, but there were no definitive symptoms. Minor PD symptoms began to develop in a very few vines in the Viognier test in 2010. However, there were fewer vines with PD symptoms in 2011 than in 2010. After 3 years, the PD incidence in the test vines was very low, but there was a large increase in incidence in both the untreated and treated vines in 2012 (Table 2). There were no significant differences between treatments.

Table 2. Effect of EB92-1 on the incidence of PD in young “Voignier” grapevines in Preston Vineyards in Sonoma California.¹

| Year | Untreated vines | | EB92-1 treated vines | |
|------|------------------------|-----------------------------|------------------------|-----------------------------|
| | Incidence ¹ | Disease rating ² | Incidence ¹ | Disease rating ² |
| 2010 | 13/48 (27%) | 0.3 | 8/48 (17 %) | 0.2 |
| 2011 | 6/48 (12%) | 0.2 | 5/48 (10%) | 0.2 |
| 2012 | 32/48 (67%) | 1.0 | 29/48 (60%) | 0.8 |

¹PD incidence is the number of PD symptomatic vines over total vines in treatment.

²Disease 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.

More important than the incidence of PD symptoms may be the incidence of severe PD symptoms, which we define in this study as incidence of vines with a disease rating of 2-4 (Table 3). These severely infected vines have 50%, or more, of the leaves with symptoms and will usually die. Vines with fewer symptomatic leaves may not show any symptoms the following year. For example, six Voignier vines had a disease rating of 1 in 2011 and 4 of these 6 vines had no PD symptoms in 2012. This could be the result of vine recovery or misdiagnosis of plants with only a few symptomatic leaves. The disease rating incorporates both the incidence and severity of the PD symptoms. Incidence of severe PD was much lower than total PD (compare Table 2 with Table 3). Untreated vines had 25% incidence of severe symptoms; whereas, EB92-1 vines had 17% incidence of severe symptoms (Table 3). Interestingly, this trial is next to a riparian area and there were 3 times as many vines with severe PD symptoms in the 5 rows next to the river as in the 5 rows further from the river. Statistical analysis of the disease ratings showed that there was no difference between the treated and untreated. This plot will continue to be monitored even though the project has ended.

Table 3. Effect of EB92-1 on the incidence of severe PD (Disease rating of 2 - 4) in young “Voignier” grapevines in Preston Vineyards in Sonoma California in 2012.

| Treatment | Incidence of severe PD ¹ | | |
|----------------------|-------------------------------------|------------|-------------|
| | 5 rows near river | Rows 6-10 | All rows |
| Untreated vines | 9/20 (45%) | 3/28 (11%) | 12/48 (25%) |
| EB92-1 treated vines | 6/20 (30%) | 2/28 (7%) | 8/48 (17%) |

¹Incidence of severe PD is the number of vines with a rating of 2 or greater over total vines in a treatment.

Napa tests. For transplanting into the Beringer Vineyard in Napa, 50 Reisling/3309 and 50 Chardonnay/3309 were treated with EB92-1 on June 25, 2008 and 50 vines of each were left untreated as controls. The vines were transplanted as replants for missing vines in Beringer Vineyard in early April 2009. In 2011, there still was essentially no disease in either the Chardonnay or Reisling. Only 1 Chardonnay vine and 2 Reisling vines were considered to have the beginning of PD symptoms, but these were still questionable. In 2012, there still is only one Chardonnay vine with minor PD symptoms (Table 4). However, there was a considerable increase in the number of Reisling vines with PD symptoms. These were mostly vines with a disease rating of 1 and could have no symptoms next year (see Sonoma test section above). There were no significant differences in disease ratings between the untreated and treated plots. This plot will continue to be monitored even though the project has ended.

Table 4. Effect of EB92-1 on the incidence of PD in young grapevines Beringer Vineyard in Napa, CA, October, 2012.

| Cultivar | Untreated vines: | | EB92-1 treated vines: | |
|--------------------------------|------------------------|-----------------------------|------------------------|-----------------------------|
| | Incidence ¹ | Disease rating ² | Incidence ¹ | Disease rating ² |
| <i>Beringer Vineyard, Napa</i> | | | | |
| Chardonnay | 0/40 (0%) | 0 | 1/42 (2%) | 0.02 |
| Reisling | 9/42 (21%) | 0.24 | 12/43 (28%) | 0.40 |

¹PD incidence is the number of PD symptomatic vines over total vines in treatment.

²Disease 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.

Conclusions. Due to the loss of trials in Temecula and the lack of PD development in trials in Sonoma and Napa, no definitive information on the biological control of PD with EB92-1 in California was obtained through 2011. To replace the lost tests in southern California, a test was established in 2011 at UC Riverside. With the PD pressure in southern California, this test should yield conclusive results over the next 2-3 years. In the 2012 season, PD increased significantly in the Sonoma and Napa test. If this continues, data should be obtained on the effectiveness of the biocontrol in these vineyards in 2013 and 2014. With the successful trials in other states, this project could yield results within the next 2-3 years that would provide a commercial biological control for Pierce's disease for vineyards in California.

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 treated 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. In the first year, none of the mature vines, treated or untreated, had developed any PD symptoms. In October 2012, there was one severely diseased vine of 43 in the untreated and one vine of 49 with mild symptoms in the EB92-1 treated plot. This plot will continue to be monitored even though the project has ended.

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.

The tandom repeat numbers (TRN) of PD Xf reference strains as well as samples that we collect from the biocontrol plots were determined. For the field samples, approximately 6 to 8 individual colonies from any one sample were tested to determine the degree of potential polymorphisms in colonies isolated from a symptomatic vine. DNA extraction is very straightforward as a single colony is removed from a medium plate and suspended in 40ul of sdH₂O. The sample is boiled for 3 minutes and 5ul of the supernatant DNA used as the PCR template. PCR conditions and primers to be used are described by Chen, et al., 2008. We evaluated 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.

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.

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.

Effectiveness of using scion from grapevines infected with EB92-1. Plants of Chardonnay/Salt Creek were obtained by grafting green cuttings from Chardonnay plants from the vineyard onto rooted cuttings of Salt Creek. The grafting was done between May and July in 2007. Grafted plants were transplanted into the vineyard on August 14, 2007. Treatments included (1) Cuttings from Chardonnay not infected with EB92-1 on Salt Creek, (2) Cuttings from EB92-1 inoculated Chardonnay on Salt Creek, and (3) Cuttings from Chardonnay not infected with EB92-1 on Salt Creek, but injected in the vineyard with EB92-1 on August 29.

| Table 5. Transmission of biocontrol in scion from infected Chardonnay mother plant grafted onto Salt Creek rootstock. | |
|--|------------------------|
| | % PD incidence: |
| Treatment | 8/2/2012 |
| Scion from clean Chardonnay | 90 |
| Scion from clean Chardonnay injected with EB92-1 in the field | 27 |
| Scion from EB92-1 Chardonnay mother plant | 64 |

In 2012, the incidence of PD symptoms in the scion from clean Chardonnay was high (Table 5). As expected, field injection of these clean scion plants with EB92-1 reduced the incidence of PD from 90% to 27%. The scion wood from mother vines of Chardonnay infected with EB92-1 developed less PD than did the uninfected scion but more than the field injected scion. Thus, transfer of the biological control from the mother plant through scion wood did occur but was less effective than direct injection.

Effectiveness of treating scion and/or rootstock with EB92-1. 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 2009, PD began to occur in a few of the Merlot vines. Symptoms have continued to progress in the untreated, with 43% of the vines having some symptoms (mostly minor) in 2011 (Table 6). All 3 treatments with EB92-1 were reducing symptoms. EB92-1 was controlling PD equally well after injection into the rootstock, scion, or rootstock and scion.

| Table 6. Effect of methods of treatment of grape plants with <i>Xylella fastidiosa</i> strain EB92-1 on biological control of Pierce's disease. | | |
|--|----------------------|------------------|
| | Merlot/101-14 | |
| Treatment | Aug 2010 | June 2011 |
| Scion injection | 13 | 22 |
| Rootstock injection | 11 | 13 |
| Scion & Rootstock injection | 14 | 17 |
| Untreated | 38 | 43 |

PUBLICATIONS

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.

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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.

Hopkins, D. L. 2011. Biological control of Pierce's disease of grapevine with benign strains of *Xylella fastidiosa* subsp. *fastidiosa*. *Pierce's Disease Research Symposium Proceedings*. 2011:128-131.

Hopkins, D. L. 2012. Long-term control of Pierce's disease in various grape genotypes with a benign strain of *Xylella fastidiosa*. *Phytopathology* 102:S4.55 (Abstract).

PRESENTATIONS

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

Oral presentation "Biological control of Pierce's disease of grapevine with EB92-1, a benign strain of *Xylella fastidiosa*" at the International Symposium on Biocontrol of grapevine diseases on May 26, 2011 in Toulouse, France.

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. A commercial company has licensed the patent and is preparing to register this strain with the EPA for commercial control of PD and a commercial formulation of EB92-1 could be on the market in 3-4 years to control PD of grapevine in California and other areas where PD is a problem.

LAYPERSON SUMMARY OF PROJECT ACCOMPLISHMENTS

In trial plantings of Orange Muscat and Cabernet Sauvignon in Bella Vista Vineyard in Temecula, almost half of the Orange Muscat and a fourth of the Cabernet Sauvignon treated or untreated vines 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 made it impossible to obtain good data so the trial was abandoned. In 2011, the lost Temecula trials were replaced with trials on Merlot and Pinot Noir in the UC Riverside Vineyard. In 2012, PD began to develop, with no differences between treatments. After 3 years of no increase in PD in the Sonoma and Napa trials, PD began to spread in 2012. In the Viognier trial in Preston Vineyards in Sonoma, more than 50% of the vines had PD symptoms. However, many of these plants had very minor symptoms and could be symptomless in 2013; however, 12 untreated vines and 8 vines treated with EB92-1 developed severe symptoms of PD. Grapevines with severe PD symptoms do not recover and eventually die. In this 4th year of the test in Beringer Vineyards in Napa, there still was only 1 vine with PD symptoms out of 82 total Chardonnay vines; however, PD did spread rapidly in the Reisling trial. Twenty-five percent of the Reisling vines had some symptom of PD, most symptoms were mild. In the Riverside, Sonoma, and

Napa trials, symptoms are just beginning to develop so it is too early to draw definitive conclusions on the control trends. We will continue to monitor these field trials, even though the project has ended. Data on the effectiveness of EB92-1 for the biocontrol of PD in California should be obtained over the next two seasons. A trial to evaluate the effectiveness of the biocontrol strain in protecting mature, producing grapevines against infection with PD was established in Beringer Vineyard in 2010. Mature Chardonnay vines were inoculated with biocontrol strain EB92-1 by boring a small hole into the trunk with an electric drill and injecting 2 ml of bacterial suspension into the hole. In 2012, one treated and 1 untreated vine had developed PD. In tests in Florida to determine the most efficient and effective way to apply the biocontrol strain, direct injection of EB92-1 into the vines still appeared to be the most effective method.

IX. Status of funds

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

REFERENCES

- Hopkins, D. L. 2005. Biological control of Pierce's disease in the vineyard with strains of *Xylella fastidiosa* benign to grapevine. *Plant Dis.* 89:1348-1352.
- Hopkins, D. L. and Purcell, A.H. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. *Plant Dis.* 86:1056-1066.