IDENTIFICATION OF A NEW VIRULENCE FACTOR REQUIRED FOR PIERCE'S DISEASE AND ITS UTILITY IN DEVELOPMENT OF A BIOLOGICAL CONTROL

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ABSTRACT.

Xylella fastidiosa is a serious phytopathogen that infects a number of important crops including citrus, almonds, and coffee. The *X. fastidiosa* Temecula strain infects grapevines and induces Pierce's disease. We examined a *X. fastidiosa* gene, PD1311, which is a putative acyl CoA synthetase; acyl CoA synthetases are an enzyme class involved in many different processes including secondary metabolite production. We deleted the PD1311 gene, characterized numerous behavioral responses, and found the strain to be avirulent. When we co-inoculated the PD1311 deleted strain with wild-type *X. fastidiosa*, disease symptoms were reduced. Therefore the PD1311 strain has potential as a biocontrol for management of Pierce's disease.

LAYPERSON SUMMARY.

We discovered that deleting the *X. fastidiosa* Temecula gene, PD1311, results in a strain that is avirulent. We performed research to determine how PD1311 plays such a central role in disease development. Most importantly, we have evidence that the strain deleted for PD1311 may function as a biocontrol. When inoculated with wild-type *X. fastidiosa*, disease development becomes reduced. Options for managing Pierce's disease are limited, which makes a possible new biocontrol critically important.

INTRODUCTION.

X. fastidiosa (*Xf*) is a Gram-negative, xylem-limited bacterium that induces Pierce's disease (PD) in grapevines (Chatterjee et al. 2008). *Xf* is transmitted to plants by insect vectors and once in the xylem, *Xf* is postulated to migrate, aggregate, and form biofilms that clog the vessels leading to PD. While clogging vessels appears to be key to disease, we wanted to explore whether virulence factors facilitate symptoms, and, in particular, genes with potential roles in secondary metabolites. We examined an *Xf* gene, PD1311, that is a putative ACS (acyl- and aryl-CoA synthetase) (Chang et al. 1997, Gulick 2009). The most studied bacterial ACS is the *Escherichia coli* FadD*,* which catalyzes exogenous long-chain fatty acyl-CoA from fatty acid, coenzyme A,

and ATP (Black 1992). ACS metabolite intermediates are involved in beta-oxidation, phospholipid biosynthesis, cell signaling, protein transportation, and protein acylation (Korchak et al. 1994, Glick et al. 1987, Gordon et al. 1991, Barber et al. 1997). In addition, ACSs are involved in the synthesis of virulence factors. A *Streptomycetes coelicolor* putative ACS is critical for antibiotic production (Banchio & Gramajo 2002). A *Sinorhizobium meliloti* ACS is involved in motility and *nod* gene expression; mutation of the gene limits infection (Soto et al. 2002). In *Xanthomonas campestris*, an ACS FadD homolog, RpfB, is critical for producing quorum-sensing DSF (diffusible signaling factor) and pathogenicity (Barber et al. 1997).

We report herein that an *Xf* strain deleted of the PD1311 gene (ΔPD1311) is avirulent when inoculated in grapevines. In addition, the ΔPD1311 strain reduces disease development by the wild-type Temecula strain when they are co-inoculated. Therefore we propose that it has potential as a biocontrol for PD. The weakly virulent *Xf* elderberry strain, EB92-1, has been studied as a PD biocontrol (Hopkins 2005, Hopkins 2012). Additional PD control strategies that are, or have been, studied involve naturally resistant rootstocks (Cousins and Goolsby 2011) and scion varieties and transgenic varieties (Dandekar 2014, Gilchrist et al. 2014, Gilchrist & Lincoln 2014, Kirkpatrick 2014, Lindow 2014, Powell & Labavitch 2014). Given the limited options for PD treatment and control, exploring the ΔPD1311 strain opens a new potential avenue for limiting disease.

OBJECTIVES.

The overall goal of this project was to understand how the PD1311 protein influences virulence, and test if the PD1311 mutant strain functions as a biocontrol for PD. This project was initially funded with a one year grant for 2013-2014 (written as objective 1-2 below), and it was amended for an additional year for 2014-2015 (written as objective 3-4 below). Given the overlapping nature of the objectives for each year, we will describe our progress in terms of objectives 1-2.

Objective 1. Characterize the *X. fastidiosa* ΔPD1311 mutant.
- a. Complete *in vitro* behavioral assays critical for disease.
- b. Determine the role(s) of PD1311 in producing virulence factor(s).

Objective 2. Determine the effectiveness of ΔPD1311 Temecula strain as a biological control of Pierce's disease.
- a. Determine conditions for biological control.
- b. Examine spread of ΔPD1311and wild-type strains simultaneously in grapevines.

Objective 3. Determine the biocontrol potential of the ΔPD1311 strain.

Objective 4. Examine motility and how the PD1311 protein impacts virulence.

RESULTS AND DISCUSSION.

Objective 1. Characterize the *X. fastidiosa* ΔPD1311 mutant.

1a. Complete *in vitro* behavioral assays critical for disease.

We deleted the PD1311 gene and complemented the ΔPD1311strain as previously described (Matsumoto et al. 2009, Shi et al. 2009). The ΔPD1311strain grew in grapevine xylem sap, although slower than the wild-type strain (Fig. 1A). We then examined the ability of the mutant to move, aggregate, and form biofilm, which are key steps in PD development (Chatterjee et al. 2008). We examined movement by the *in vitro* fringe assay where fringe around the bacterial colony directly correlates with type IV pilus twitching motility (Meng et al. 2005, Li et al. 2007). The ΔPD1311 strain produces a fringe on PW agar plates (Davis et al. 1981) but not on sap, suggesting that the mutant requires a component in rich media not provided in nutrient poor sap (Fig. 1B). We found that the ΔPD1311 strain had decreased aggregation compared to the wild-type and complemented strains (Fig. 1C), and the ΔPD1311strain produces less biofilm than wild-type *Xf* (Fig. 1D). Previous studies show that decreased

motility and biofilm production correlate with decreased pathogenicity (Cursino et al. 2009, Shi et al. 2009, Cursino et al. 2011).

Real-time RT-PCR data suggested the ΔPD1311strain has down-regulated expression of i) type IV pili gene, PD1926, which we have found to regulate motility (data not shown), ii) type I pili gene, *fimA*, which is important for cell adhesion (Li et al. 2007), and iii) afimbrial adhesion gene, *hxfB* (hemagglutinin *Xylella fastidiosa* B) (Feil et al. 2007) (Table 1). These results are consistent with the reduced motility, aggregation, and biofilm formation phenotype observed with this mutant. Surprisingly, adhesion gene, *xadA* (*Xanthomonas* adhesin-like protein A) (Feil et al. 2007), was upregulated in the mutant strain. This result requires further exploration. Overall our findings suggest a role of PD1311 in regulation of multiple virulence factors at the transcriptional level.

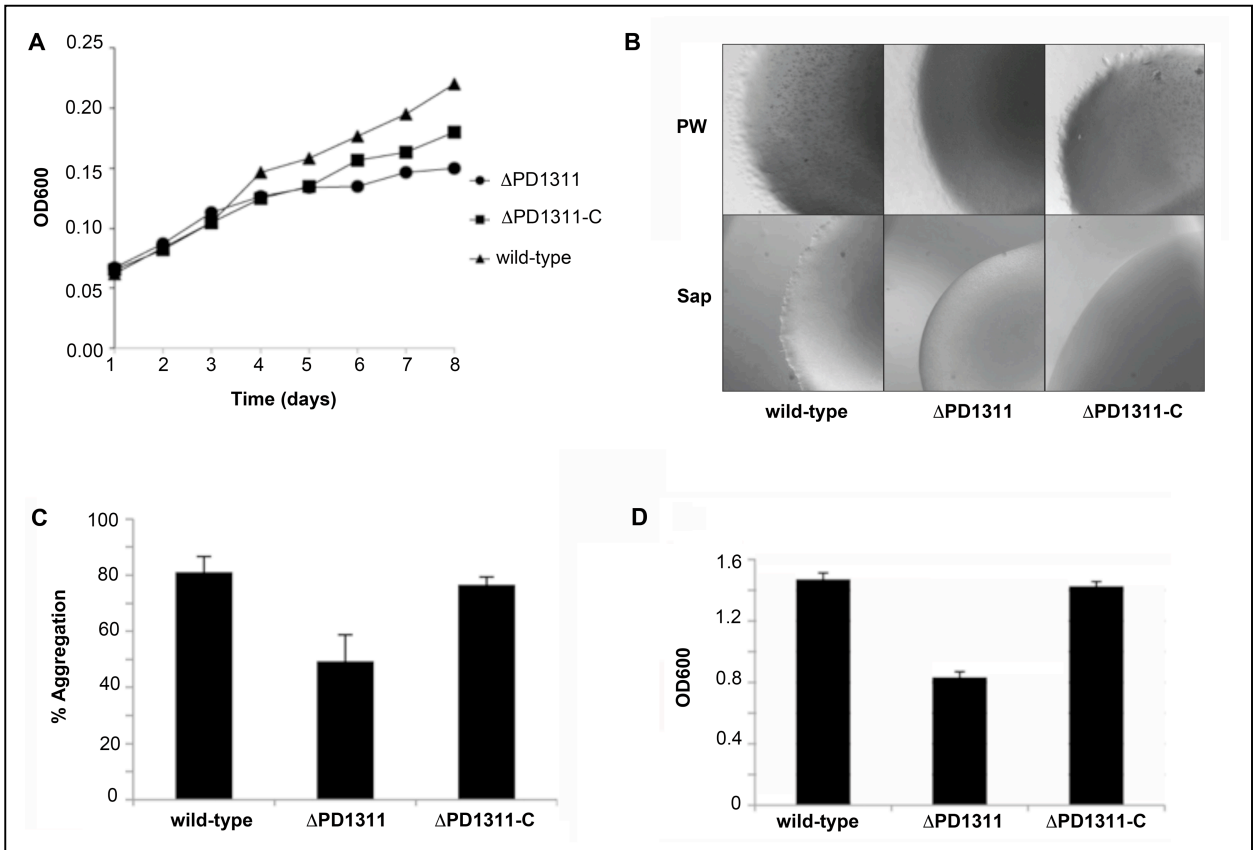


Fig. 1. Previous phenotypic findings with the ΔPD1311 strain. A) Wild-type *Xf* (triangle), mutant (ΔPD1311 - circle), and complemented mutant (ΔPD1311-C – square) strains were grown for eight days in 100% *Vitis vinifera* cv. Chardonnay xylem sap and growth was determined by OD_{600} readings. **B**) Colony fringes of strains were assayed on PW and sap agar plates (80% Chardonnay sap, 20% water, and 12g/L agar). Colonies were assessed after five days of growth (Meng et al. 2005, Li et al. 2007). Colonies photographed at 90X magnification. Experiment was repeated three (PW plates) or two (sap plates) times. **C**) Aggregation of wild-type, mutant and complemented mutant strains grown in test tubes for five days in 3 ml of PD2 (Burdman et al. 2000, Davis et al. 1980, Shi et al. 2007). The experiment was repeated three times. **D**) Quantification of biofilm formation in 96 well plates (Zaini et al. 2009). Experiment was repeated three times with 24 replicates each.

Table 1. Expression of key genes in ΔPD1311 strain compared to wild-type strain.

PD1926	*fimA*	*hxfB*	*xadA*
0.03± 0.03	0.23± 0.11	0.08± 0.02	15.15± 3.77

Wild-type or ΔPD1311 mutant cells were grown in PD2 broth for three days and collected for RNA extraction. The *petC* and *nuoA* genes were used as reference genes. Data represents expression in mutant compared to expression in wild-type cells. Three biological samples were included for each strain and the experiment was conducted once.

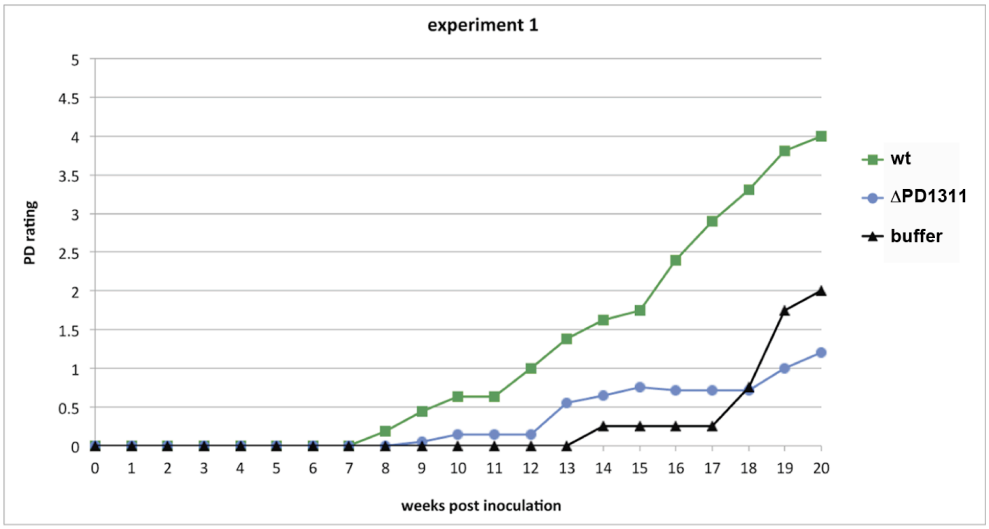
1b. Determine the role(s) of PD1311 in producing virulence factor(s).

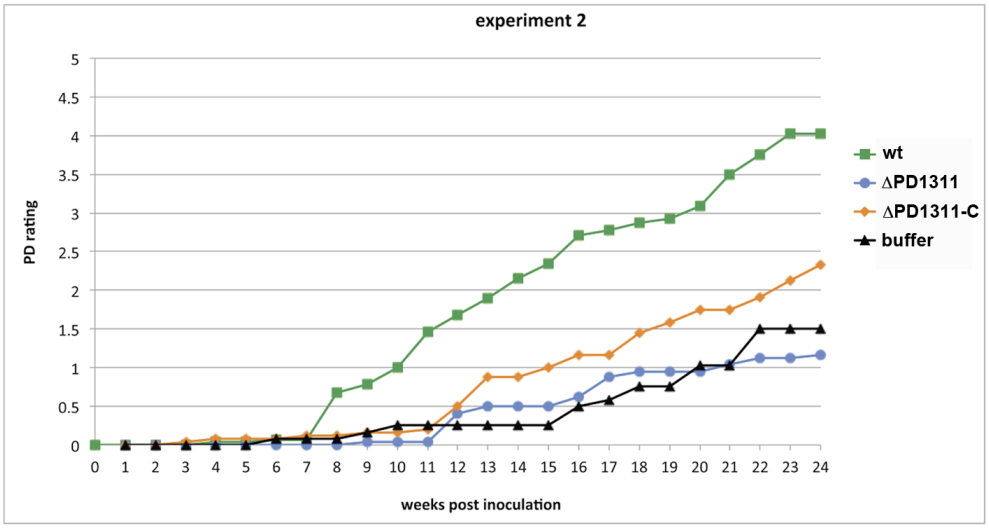
Given that PD1311 may be an ACS protein and ACS proteins are implicated in the production of quorum sensing molecules (Barber et al. 1997; Chatterjee et al. 2008) we asked if it was involved in DSF production. We streaked wild-type *Xf* and the ΔPD1311 strain onto PW agar plates for 8 days to allow production of DSF. The *Xanthamonas campestris campestris* (*Xcc*) indicator strain 8523 (kindly provided by Prof. Steven Lindow, U. California, Berkeley) was streaked perpendicular to either the wild-type or the ΔPD1311 strain for 24 hours (Newman et al. 2004). A suspension was made of the *Xcc* strain 8523 cells and fluorescence was visualized using a confocal microscope, and we found no changes in fluorescence by the ΔPD1311 strain (data not shown) indicating that PD1311 is not involved in DSF production.

Objective 2. Determine the effectiveness of ΔPD1311 Temecula strain as a biological control of PD.

To determine the impact of the ΔPD1311 strain on PD development *in planta*, we inoculated *V. vinifera* cv. Cabernet Franc vines per standard procedures (Cursino et al. 2011) and recorded disease development of PD using the five-scale assessment (Guilhabert & Kirkpatrick 2005). Our first trial gave promising results and led to repeat experiments the following year (Fig. 2). Trial two once again showed that PD1311 produces little disease. Trial three also suggested little disease but the wild-type strain did not show the expected PD phenotype at week 16, making conclusions difficult. Trial three was begun late in the season, which might explain the unexpected data for controls.

We believe that the ΔPD1311 strain may act as a biocontrol, as we previously found that it reduced biofilm production by the wild-type *Xf* strain. For this study, we grew wild-type *Xf* cells constitutively expressing green fluorescent protein (wt-GFP) with either the ΔPD1311strain or wild-type cells. We previously used this strain (kindly provided by Prof. Steven Lindow, U. California, Berkeley) and found it to produce biofilm similar to wild-type *Xf* (data not shown). As stated above, wild-type cells produce more biofilm than the ΔPD1311 strain so mixtures of wt-GFP/ΔPD1311 should have equal or greater fluorescence than mixtures of wt-GFP/wt, if the strains did not impact each other. In a previous grant period we found that the wt-GFP/ΔPD1311 mixture had less fluorescence than the wt-GFP/wt mixture, indicating that the ΔPD1311 strain impacts biofilm produced by wt *Xf* (Fig. 3).





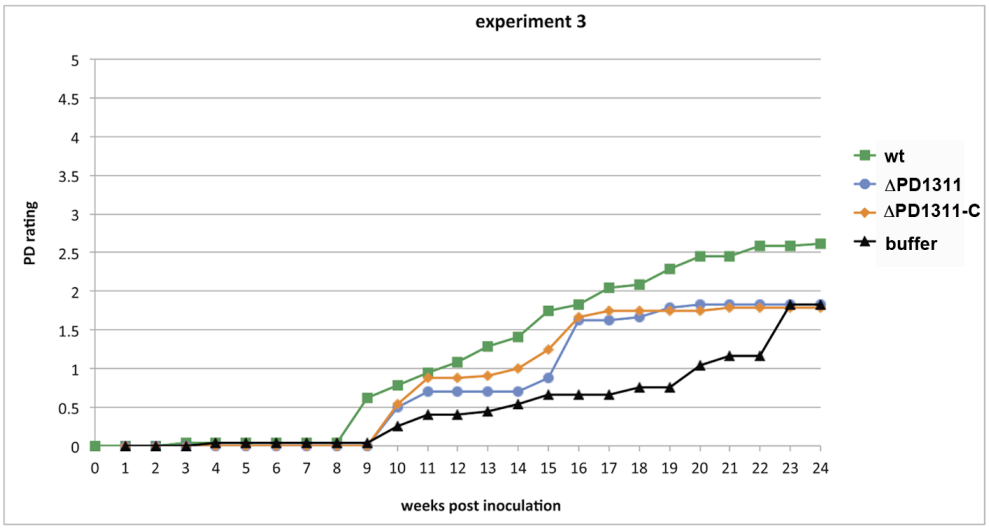


Fig. 2. Development of Pierce's disease. Grapevines were inoculated with wild-type *Xf* (square), ΔPD1311 strain (circle), ΔPD1311 complement (triangle), and buffer. Symptoms were monitored and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005, Cursino et al. 2009). Trial one did not include the complement strain.

2a. Determine conditions for biological control.

Given our findings that the ΔPD1311strain induces low virulence and impacts biofilm production by wild-type cells, we began greenhouse studies to determine if the ΔPD1311 strain can be a viable biocontrol for PD. We had three different inoculation conditions: i) co-inoculated wild-type and ΔPD1311strains, ii) inoculation with the ΔPD1311strain followed two weeks later by wild-type *Xf* [following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins 2005)], and iii) inoculation of wild-type *Xf* followed two weeks later by the ΔPD1311 strain (Fig. 4). The ΔPD1311 strain reduced the disease development of wild-type *Xf* whether co-inoculated or inoculated prior or after the wild-type strain. The mechanism by which this occurs is currently being investigated in our current 2015-2016 grant.

2b. Examine spread of ΔPD1311and wild-type strains simultaneously in grapevines.

Our initial trial *in planta* indicated that the ΔPD1311strain can be detected in plants (Table 2), suggesting that limited PD symptoms from ΔPD1311incoluation was not due to an inability of the ΔPD1311to survive in the grapevines. However, we have found that the ΔPD1311 is extremely sensitive to steps used to disinfect plant material required to limit contamination on plates, which has made determining the spread of the mutant difficult. We have recently refined the isolation protocol, which should allow us to assess the presence of ΔPD1311 *Xf* from our current third year of biocontrol experiments.

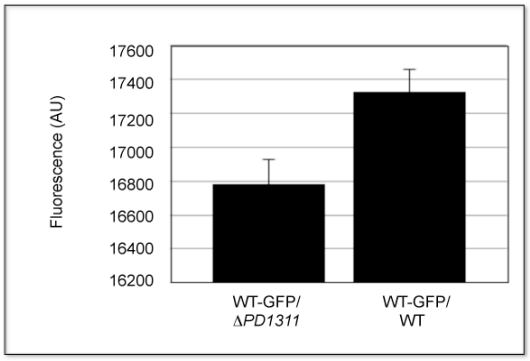


Fig. 3. The ΔPD1311 strain impacts biofilm formation by wild-type cells. Quantification of biofilm in 96 well plates with agitation with equal amounts of wild-type *Xf* constitutively expressing green fluorescent protein (WT-GFP) and either wild-type *Xf* (WT) or the ΔPD1311 strain (Δ*PD1311*). Experiment was performed with 24 replicates. Fluorescence in arbitrary units (AU).

CONCLUSIONS.

Xf motility, aggregation, and biofilm formation are key steps in PD development (Chatterjee et al. 2008). Concerning objective 1a, we have shown that PD1311 plays a role in motility, aggregation, and biofilm formation. For objective 1b, our studies suggest that PD1311 is not involved in DSF production. For objective 2a, we have found that the ΔPD1311strain impacts biofilm production by wild-type *Xf*. Additionally, the mutant strain inhibited wild-type virulence *in planta*. Objective 2b is in process under our 2015-2016 grant. Overall, this work will help further understanding of disease development and prevention.

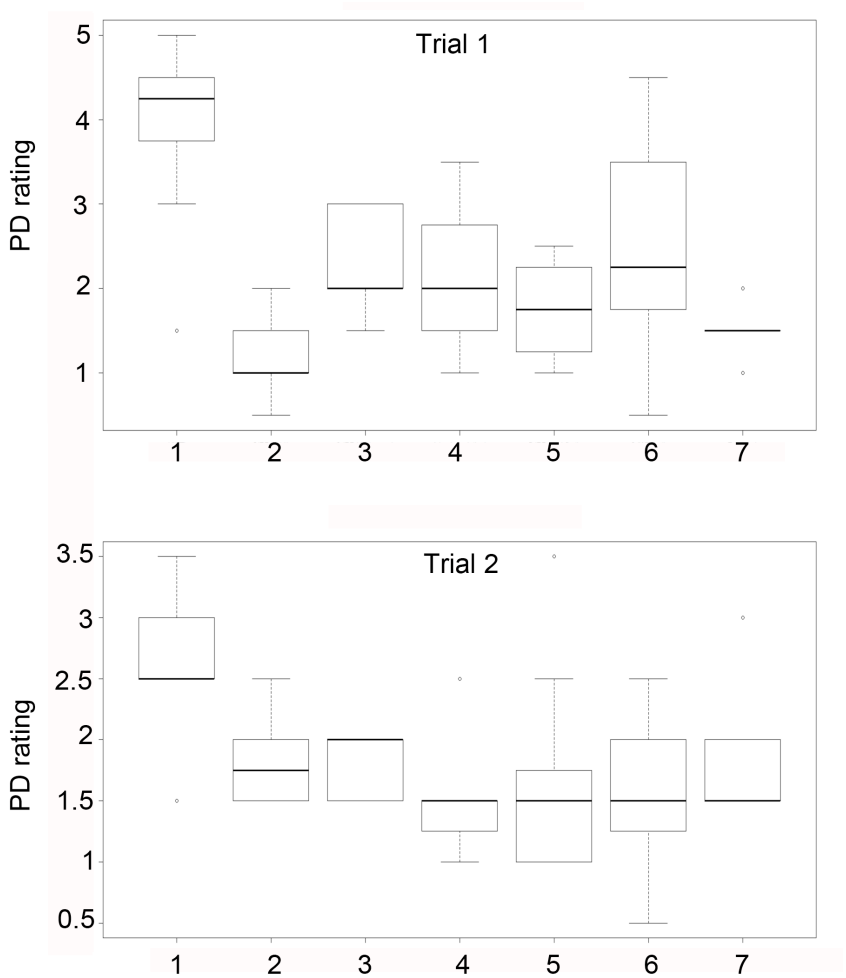


Fig. 4. ΔPD1311 strain reduces PD. Grapevines were inoculated with: 1) wild-type *Xf* , 2) ΔPD1311strain, 3) ΔPD1311 complement strain, 4) co-inoculation ΔPD1311 and wild-type *Xf*, 5) pre-treat with ΔPD1311 strain two weeks before wild-type cells, 6) post-treat with ΔPD1311 strain two weeks after wild-type cells, and 7) buffer. Bold lines represent the median values and circles representing outliers of each data group. Symptoms have been monitored on 12 plants for each treatment for 24 weeks and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005).

Table 2. ΔPD1311 strain detected *in planta*.

	Plant 1		Plant 2		Plant 3		Plant 4	
	up	down	up	down	up	down	up	down
WT	-	-	-	-	-	+	-	-
ΔPD1311	-	+	-	-	+	+	-	-

Five microliters of 10^^{9} CFU/mL of wild-type (WT) or mutant (ΔPD1311) *Xf* were inoculated into young grapevines in the 6-7th node counting from the top. The petioles directly above (up) and below (down) the inoculation point were sampled 10 days post-inoculation for PCR detection using *Xf* specific primers. + or – represents the presence or absence of the characteristic band.

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