CHARACTERIZATION OF *XYLELLA FASTIDIOSA* GENES REQUIRED FOR PATHOGENICITY

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ABSTRACT
Xylella fastidiosa (Xf) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease of grapevine (Wells et al., 1981). *Xf* is closely related to *Xanthomonas oryzae* pv. *oryzae* (*Xoo)*. Recent findings indicate that the sulfated Type 1 secreted protein Ax21 is required for density-dependent gene expression and consequentially pathogenicity of *Xoo*. Two two-component regulatory systems (TCSs) are required for Ax21 mediated immunity. Orthologs for both of the TCSs and Ax21 have been found in *Xf*. In this study, we will investigate the role of Ax21 and the two TCSs that regulate Ax21 in *Xf*.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) is a plant pathogenic bacterium and the causal agent of disease in a variety of economically important crops, including Pierce's disease of grapevine. *Xf* causes disease by colonizing the xylem vessels, blocking the flow of water in the grapevine. In many plant pathogenic bacteria, biofilm formation plays a key role in virulence. A biofilm is a population of microorganisms attached to a solid or liquid interface. The production of biofilm is regulated by quorum sensing system, in which bacteria communicate with one another via small molecular weight compounds. In *Xanthomonas oryzae* pv. *oryzae (Xoo)*, a bacterial species related to *Xf*, it has been shown that Ax21, a sulfated peptide, is a quorum sensing compound that is required for biofilm formation and virulence. Furthermore, two two-component regulatory systems (TCSs) have been identified that are required for Ax21 activity in *Xoo*. In this research, we will investigate the biological function of Ax21 and the two TCSs orthologs that were identified in the *Xf* genome.

INTRODUCTION
Xylella fastidiosa (Xf) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease (PD) of grapevine (Wells et al., 1981). *Xf* is found embedded in the plant in clumps, which leads to the xylem vessel blockage. The formation of biofilms allows for bacteria to inhabit an area different from the surrounding environment, potentially protecting itself from a hostile environment. Furthermore, biofilm formation is an important factor in the virulence of bacterial pathogens. Biofilm formation is a result of density-dependent gene expression (Morris and Monier, 2003). Density-dependent biofilm formation is triggered by the process of quorum sensing (QS). In QS, bacteria are able to communicate with each other via small signal compounds, generically called "auto-inducers" and in *Xanthomonas* and *Xf* the molecules are referred to as diffusible signal factors (DSF). The auto-inducer is a means by which bacteria recognize bacterial population size, and mediate the expression of specific genes when bacterial populations reach a threshold concentration. (Fuqua and Winans, 1994; Fuqua et al., 1996).

In *Xanthomonas oryzae* pv. *oryzae (Xoo)*, Ax21 is a sulfated, Type 1 secreted protein that is a quorum sensing compound. Ax21 was recently shown to be a requirement for induction of density-dependent gene expression, including biofilm formation (Lee et al., 2006; Lee et al., 2009). In *Xoo*, two two-component regulatory systems (TCSs) required for Ax21-mediated activity have been found and orthologs of the TCSs and Ax21 were identified in the *Xf* genome (Simpson et al., 2000). In order for an active Ax21 gene product to be produced, two TCSs are required: RaxR/H and PhoP/Q (Burdman et al., 2004; Lee et al., 2008). The goal of this research is to investigate the role of homologs of Ax21 and the associated two component regulatory genes in *Xf*.

OBJECTIVES
1. Determine the functional role of the Ax21 homolog in *Xf*.
2. Determine the functional role of the PhoP/PhoQ two-component regulatory system in *Xf*.
3. Identify GacA-regulated genes in *Xoo* through microarray analysis and compare with *Xf GacA*-regulated genes.

RESULTS AND DISCUSSION

In the few months that we have worked on this project we made deletion knockout strains of Ax21, *PhoP* and *PhoQ* in *Xf*. For the Ax21 knockout strain, we conducted a variety of assays including pathogenicity on grapevines, biofilm formation, cell-cell aggregation and growth rate. We will repeat the pathogenicity assays again next year and also inoculate grapevines with *Xf∆PhoP* and *Xf∆PhoQ* mutants.

Xf has an ortholog of ax21 gene (Lee, et al. 2009). To test if *Xf* has Ax21 activity, we carried out an Ax21 activity assay using our previous described method (Lee, et al., 2006). Rice leaves from TP309, susceptible to Xoo PXO99, and TP309-XA21, resistant to PXO99, were cut at the tip and pretreated with supernatants from wild type (*Xf*) and Ax21 knockout (*Xf*Δax21) of *Xf*. Supernatants from *Xoo* PXO99 and PXO99Δax21 were used as positive and negative controls, respectively. Five hours later the pretreated leaves were inoculated with the *raxST* knockout strain (PXO99Δ*raxST*), which lacks Ax21 activity. Ax21 activity was evaluated by measuring lesion lengths three weeks after inoculation. If *Xf* had Ax21 activity, leaves of TP309-XA21 pretreated by supernatants from *Xf* would show resistance to PXO99Δ*raxST* strain, but not leaves pretreated by supernatants from *Xf∆*ax21. However, both leaves pretreated by supernatants from *Xf* and *Xf∆*ax21 were susceptible to PXO99Δ*raxST*. This result suggests that *Xf* Ax21 is unable to trigger Ax21-mediated immunity in our rice plant bioassay (**Figure 1**). A lack of secretion and/or sulfation system in *Xf* may be the cause of the lack of Ax21 activity because *Xf* does not have orthologs of *raxA*, which is required for secretion of Ax21, and *raxST*, which is required for sulfation of Ax21. Further research will be conducted to better understand the role of Ax21 in *Xf* pathogenicity and cell-cell communication.

Based on cell growth, cell-cell aggregation and biofilm production assays, we found some differences between the wild-type *Xf* and *Xf∆*ax21. Based on cell growth assays, *Xf∆*ax21 grows to a lower population density than wild type *Xf* (**Figure 2**). There was no significant difference (95% CI) in biofilm formation or cell aggregation between *Xf* Fetzer (wt) and *Xf* Δ*ax21* (**Figures 3, 4**). Pathogenicity assays on Thompson seedless grapevines were conducted in the greenhouses this summer. We found no significant differences in colonies isolated from the point of inoculation or 25cm above the point of inoculation (**Figure 7**). Furthermore, we found similar levels of disease severity in *Xf* Δ*ax21* and wt *Xf* 18 weeks post-inoculation (**Figure 8**).

Both *Xf*Δ*phoP* and *Xf*Δ*phoQ* were found to have significantly (95% CI) less biofilm formation than wt *Xf* after ten days static incubation using the crystal violet assay (**Figure 5**). Furthermore, we also found that *Xf*Δ*phoP* and *Xf*Δ*phoQ* had significantly (95% CI) less cell-aggregation (**Figure 6**). We also found that there was no significant difference in biofilm formation or cell-cell aggregation between *Xf*Δ*phoP* and *Xf*Δ*phoQ*. This result would be expected, since PhoP and PhoQ collectively make up a two-component regulatory system (TCS). A mutant deficient in one gene should exhibit the same phenotype as a mutant deficient in the second gene of the TCS. Pathogenicity assays on Thompson seedless grapevines were conducted in the greenhouses this summer. We found no colonies isolated from the point of inoculation or 25cm above the point of inoculation (**Figure 7**). Furthermore, we found significantly reduced levels of disease severity compared to grapevines inoculated with wt *Xf* 18 weeks post-inoculation (**Figure 8**).

Although we isolated no live colonies from grapevines inoculated with *Xf* Δ*phoP*, and *Xf* Δ*phoQ*, it appeared that the vines exhibited mild PD-related symptoms. These mild symptoms are most likely due to a variety of non-PD issues that were stressing the grapevines including nutrient deficiency, insect damage and scorching from the greenhouse lamps. Therefore, we think the observed PD symptoms were difficult to differentiate from the other problems we encountered in the greenhouse. We plan to do PCR on the inoculated grapevines to track the movement of the mutants within the grapevine as well as to double check that all grapevines were successfully inoculated.

CONCLUSIONS

We have made good progress on determining the functional role of Ax21 and the PhoP/Q TCS in *Xf*, although further comparison of wild-type *Xf* and *Xf∆*ax21 needs to be done. We are currently awaiting final results from microarray analysis of *Xf*Δ*ax21, Xf*Δ*phoP*, and *Xf* Δ*phoQ*. We are currently conducting microarray analysis and once complete, we will continue to look into genes regulated by PhoP/Q. Furthermore, we are confirming whether or not a metabolically active Ax21 peptide is secreted by *Xf*. We will begin work on Objective 3 this fall.

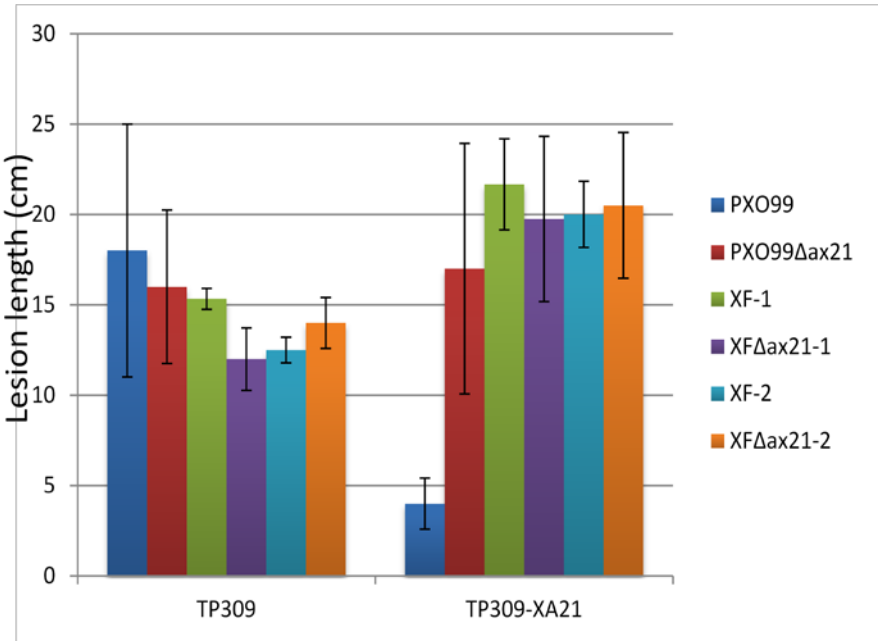

Figure 1. Lesion length on 6 week old TP309, susceptible to *Xoo* PXO99 strain, and TP309-XA21, resistant to PXO99 rice plants inoculated with PXO99Δ*raxST* strain five hours after pretreatments of supernatants. PXO99 and *Xf* indicate wild type of *Xoo* and *Xf* strains, respectively. PXO99Δ*ax21* and *Xf*Δax21 indicates ax21 deletion mutants of *Xoo* and *XF*, respectively. -1 represents supernatants from 8 days incubation culture, -2 represents supernatants from 11 days incubation culture. Each value represents the mean +/- SD.

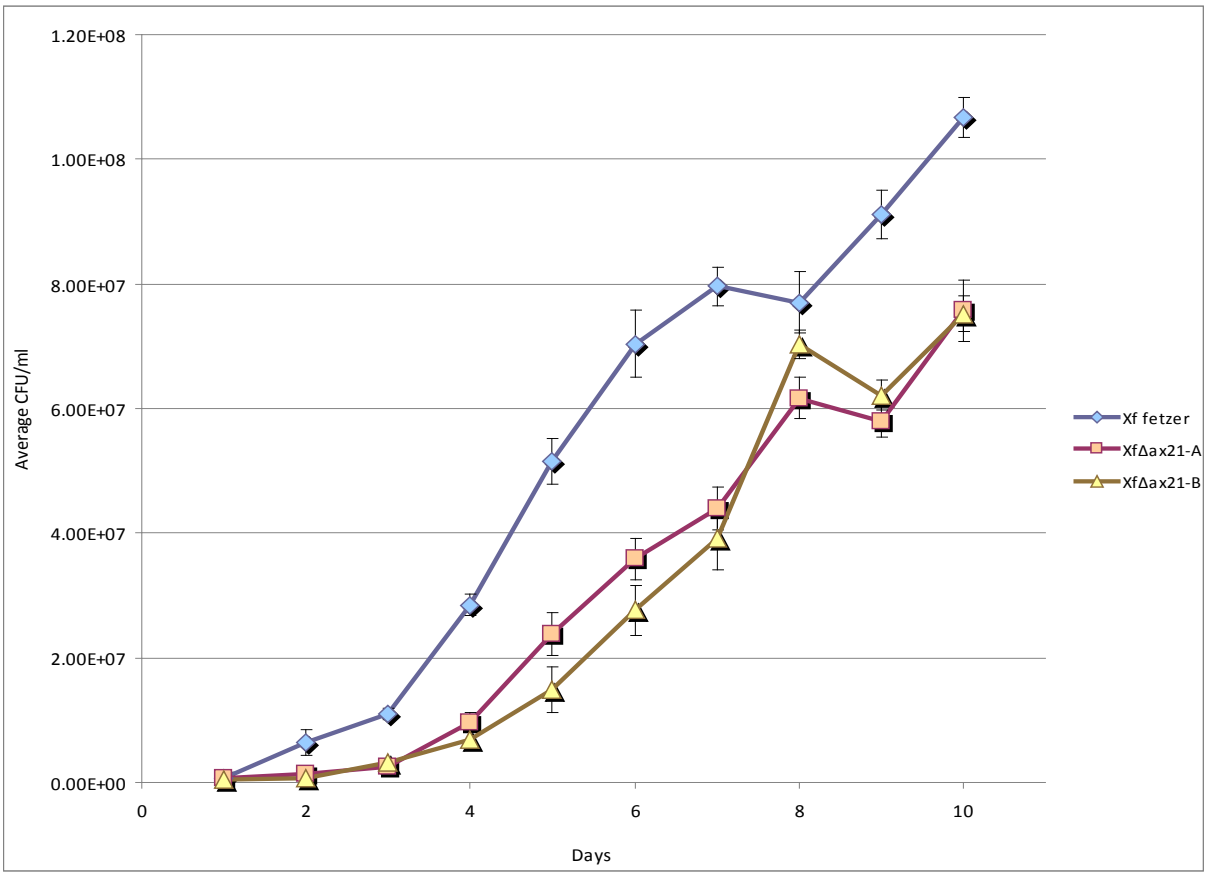

Figure 2. Bacterial growth of wild type *Xf* fetzer, *Xf*Δax21-A, and *Xf*Δax21-B. Values shown are the means of 5 samples +/- error.

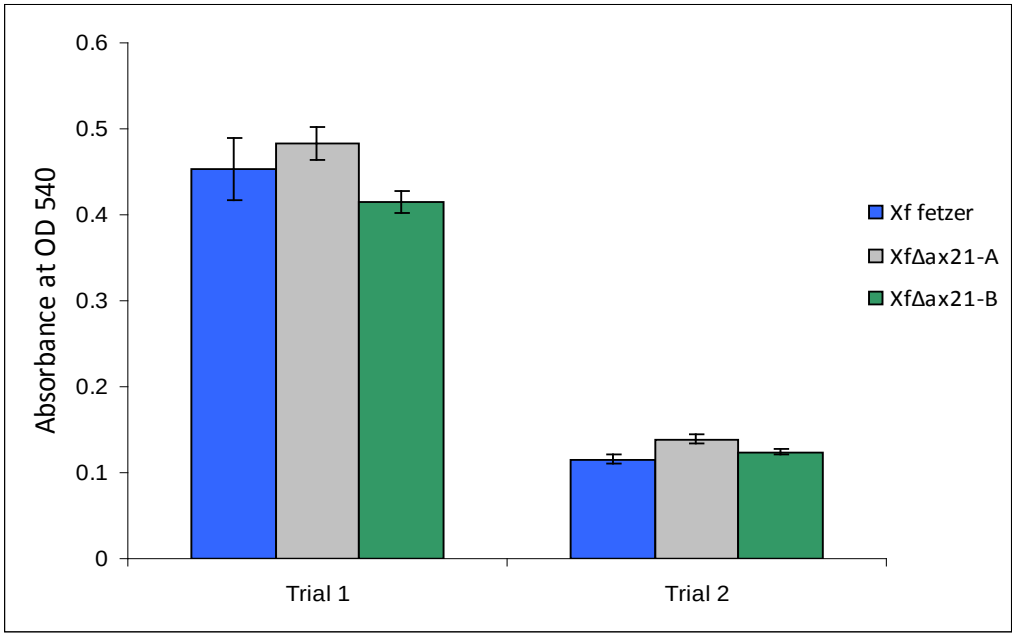

Figure 3. Comparison of biofilm formation in wild-type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B in stationary cultures as determined by the crystal violet staining method. Values shown are the means of 10 samples +/- error.

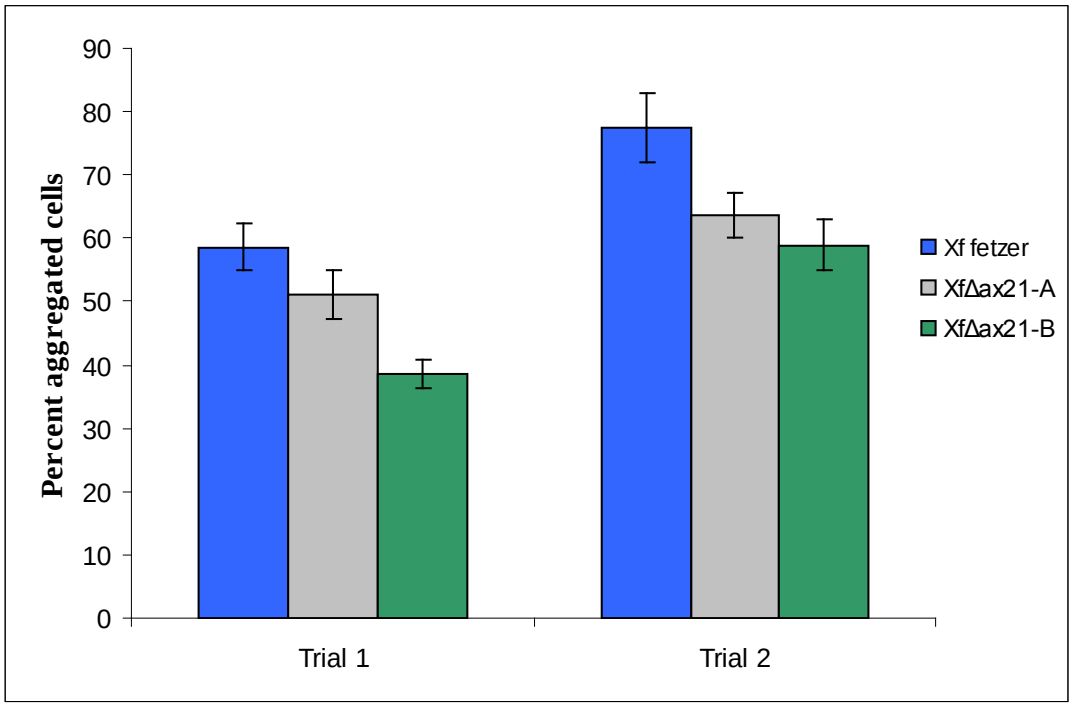

Figure 4. Comparison of percent aggregated cells in wild-type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B. Percentage of aggregated cells was determined as described by Guilhabert and Kirkpatrick, 2005. Values shown are the means of 10 samples +/- error.

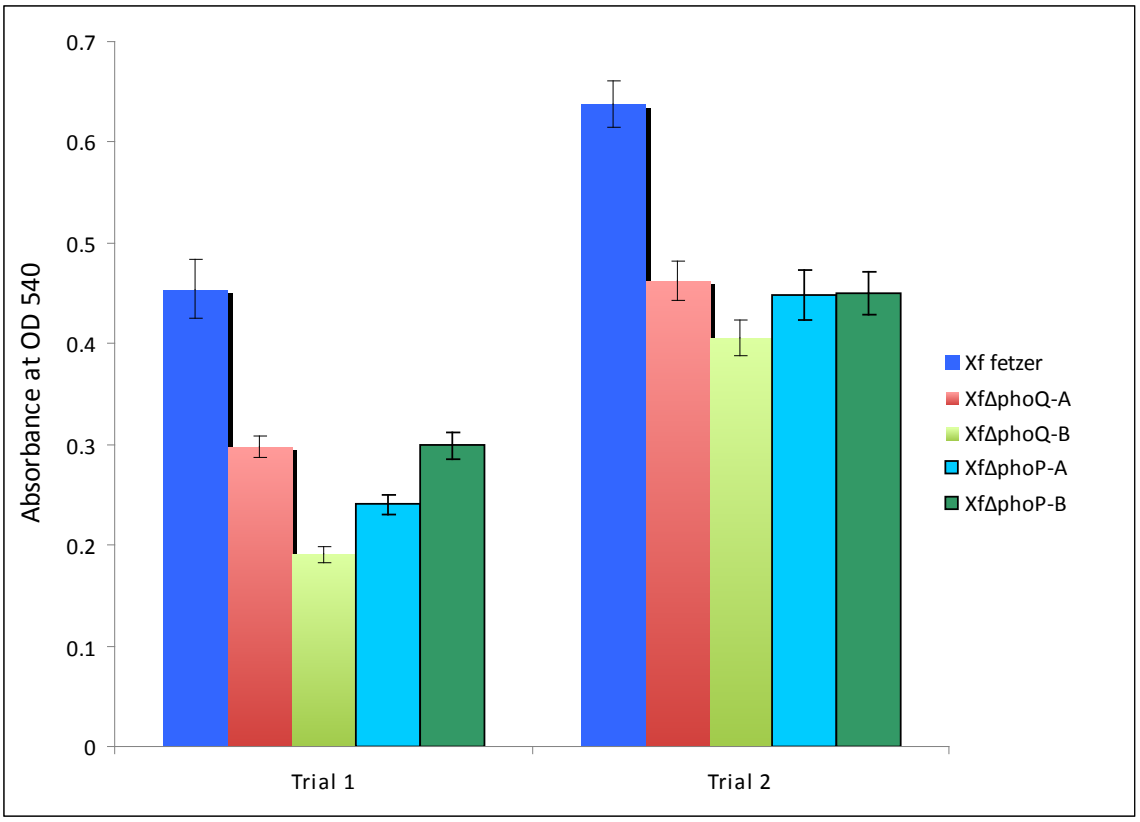

Figure 5. Comparison of biofilm formation by wild type *Xf* Fetzer, *Xf*Δ*phoP*, and *Xf*Δ*phoQ* after 10 days growth in static culture. Values shown are mean +/- standard error.

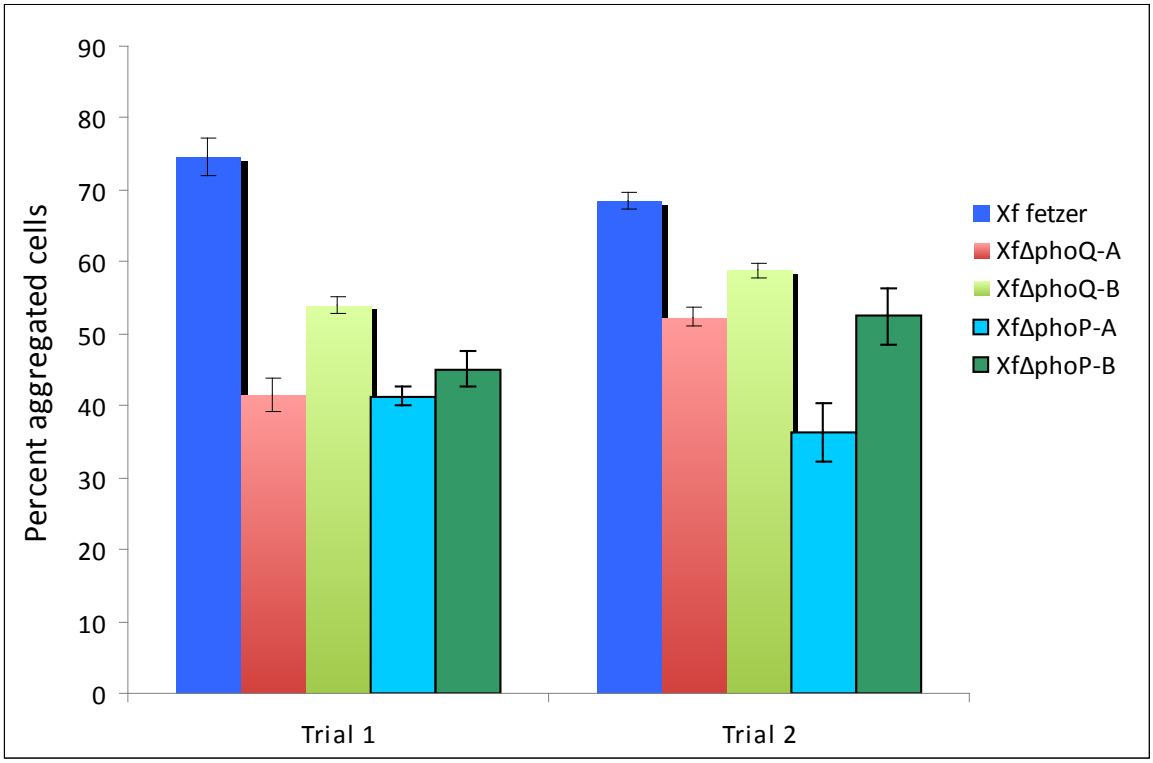

Figure 6. Comparison of percent aggregated cells by wild type *Xf* Fetzer, *Xf* Δ*phoP*, and *Xf* Δ*phoQ* after 10 days growth in static culture. Values shown are mean +/- standard error.

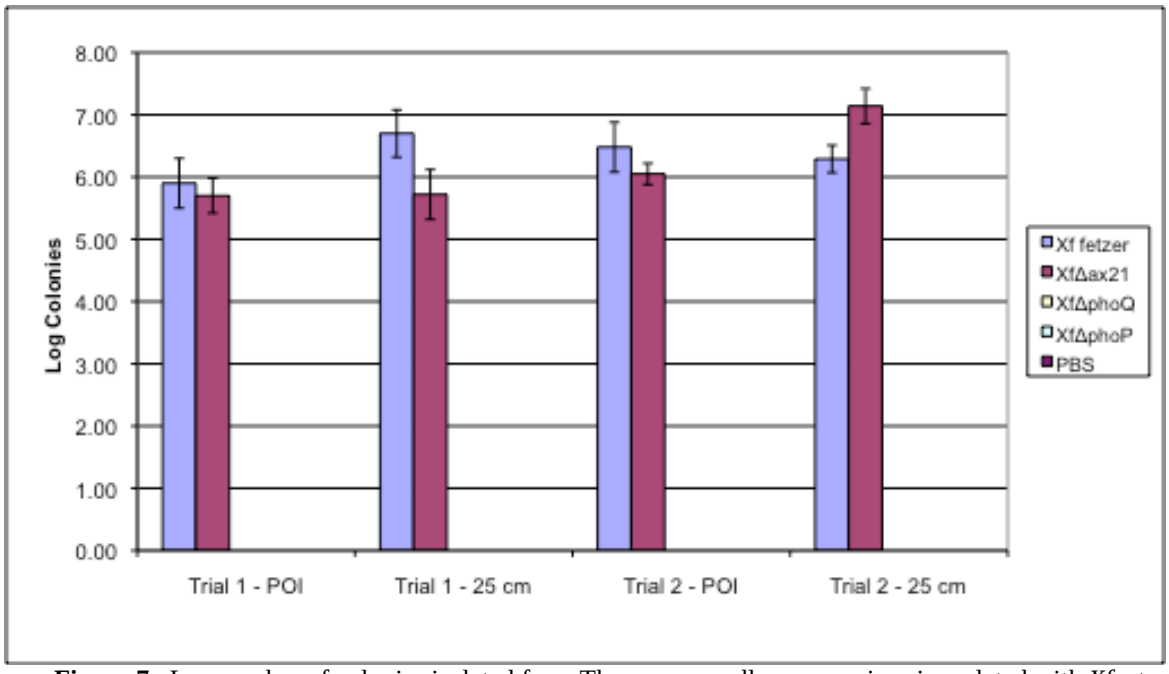

Figure 7. Log number of colonies isolated from Thompson seedless grapevines inoculated with *Xf* wt, mutants or PBS (negative control) 18 weeks post-inoculation. Isolations were taken from a petiole at the point of inoculation and a petiole 25 cm from the point of inoculation. Values shown are mean +/- standard error.

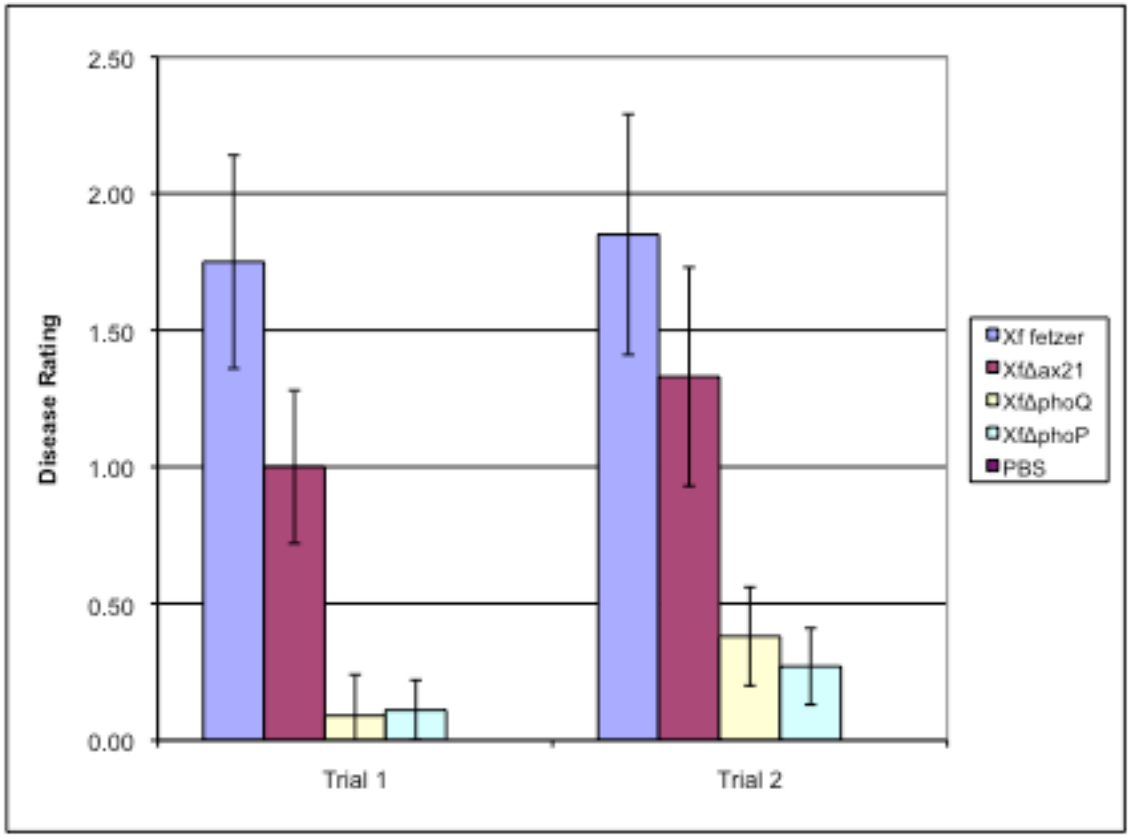

Figure 8: Disease ratings of Thompson seedless grapevines inoculated with *Xf* wt, mutants or PBS (negative control) 18 weeks post-inoculation. Values shown are mean +/- standard error.

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