# METHODOLOGY FOR ASSESSING *Xylella fastidiosa* LIVE CELL TITER IN GRAPEVINE BEARING GENETIC RESISTANCE CONSTRUCTS (Project 140140SA)


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## ABSTRACT

The current field trial of transgenic grapevine (*Vitis vinifera*) tests several genetic constructs, at least some of which appear to operate under distinct mechanisms to provide protection against *Xylella fastidiosa (*Xf*)*, the causative agent of Pierce's disease. A given genetic constructs may be expected to constrain the spread of Xf; another may reduce the number of live Xf cells, relative to dead cells. It is the goal of this project to provide data to distinguish between live and dead Xf cells in infected tissue and thereby assist the researchers and the Product Development Committee in decision making. The central focus is to use a PMA-based strategy to make this distinction by inactivating the DNA from dead Xf cells in the grape tissue. However, we observed that better recovery of Xf genomic DNA is required for the application of PMA to distinguish live/dead bacteria due to the requirement of larger sized amplicons that work in conventional qPCR. Hence, the data provided in this report are from experiments designed to improve the quality of extracted Xf DNA for use as template for longer amplicons in a PMA-based qPCR assay. We provide data here for an improved DNA purification protocol that will allow quantitative PCR analysis of longer amplicons of Xf DNA. The ratio of yields of Xf DNA to grape DNA was found to be constant over a wide range of Xf "titers" in reconstruction experiments using grapevine petioles as the tissue source.


## LAYPERSON SUMMARY

This project is intended to support the current transformed grapevine field trials and future trials that may involve grapevines expressing combinations of genes intended to reduce the impact of Pierce's disease. These field trials are testing the degree of protection provided by various genetic constructions against *Xylella fastidiosa*, the bacterial causative agent of Pierce's Disease. Current analysis methods do not distinguish live Xf from total amount (live or dead). Methods are being developed to provide information on the amount of living Xf bacteria in the inoculated transformed vines compared with wild type control plants.

## INTRODUCTION

The causative agent of Pierce's disease (PD) of grapevine, the bacterium *Xylella fastidiosa* (Xf), is unevenly distributed in the infected vine, and its populations in the plant include both live and dead cells. Greenhouse experiments, and to a limited extent field experiments, have demonstrated that specific genetic constructs inserted into commercial grapevine, *Vitis vinifera*, have strongly protected the vines against increase in inoculated Xf and/or reduced the symptoms of PD. Some of these constructs, inserted in transformed rootstocks, apparently have reduced the susceptibility or severity of symptom development for grafted, untransformed scions (see reports from the laboratories of A.M. Dandekar, D.G. Gilchrist,

B.C. Kirkpatrick, S.E. Lindow, and A.L.T. Powell in the Pierce's Disease Control Program 2013 Symposium Proceedings, http://www.cdfa.ca.gov/pdcp/research.html).

If adequate protection of untransformed scion by transgenic rootstock can be achieved under vineyard conditions, this has the obvious advantage of flexibility by allowing any of many varietals to be the scion-grafted onto a given transformed rootstock. In order to provide the durability needed for a long-lived species such as *Vitis vinifera*, several of the available genetic constructs have been and are being introduced in various combinations into selected grapevine rootstocks. That this "stacking" strategy can be effective is supported by the distinct character of the genetic constructs that have been developed: the various constructs appear to facilitate protection against Xf by different mechanisms, which should provide durability because of the difficulty that Xf should have in simultaneously overcoming two or more distinct transgenic grapevine defenses.

The principal goal of this project is to assess the titers of Xf cells, particularly live Xf cells, in vines in field test plots, especially vines with non-transgenic scions on transgenic rootstock bearing single or stacked gene constructs. The different modes of action for the genetic constructs now being field tested means that different patterns of Xf cell accumulation are to be expected and that the distributions of live Xf cells and dead Xf cells may not be parallel. Our intent is to provide data for evaluating the relative merits of various genetic constructs in reducing the extent of Xf infection of the vine and their suitability for inclusion in protective transgene stacks.

Our selected source of tissue for analysis of titer of Xf cells is the petiole. Petioles can be harvested without much collateral damage to the plant. The tissue is relatively easily disrupted for DNA purification. Xf tagged with GFP was found by confocal microscopy to occur more frequently and to be in higher concentrations in petioles, especially the petiole base, compared to leaf tissue (Gilchrist and Lincoln, 2008). However, not all petioles on a vine will be representative of the Xf cell content of the entire vine or even of a cane, particularly in the early stages of infection. The xylem architecture of the stem is such that not all xylem bundles transport to all petioles. Therefore, some petioles of an infected vine will show a high Xf titer, and others will have only a low or no detected titer. Thus, several petioles must be sampled from a given cane. We also will assess Xf cell accumulation in young grapevine canes.

Two technical issues in this effort are the sensitivity of Xf (live + dead cells) detection and effective assessment of live Xf cell accumulation. Both rely on the exquisite sensitivity of PCR. If the sensitivity is insufficient, it will not be possible to compare the effectiveness of the most effective genetic constructs in their ability to reduce Xf titer. Live cell detection by plating of Xf cells from grapevine tissue extract is not a sensitive or specific option because of low plating efficiency and the lack of a medium selective for Xf.

It is commonly observed that DNA preparations from grapevine tissue contain inhibitors or have alterations in the DNA that interfere with the polymerase chain reaction (PCR). This problem is often partially overcome by diluting the sample before taking an aliquot for PCR, a practice that obviously reduces the sensitivity of detection. A method that removes substances interfering with PCR will allow a larger fraction of the preparation to be submitted to PCR, reducing the detection limit.

We have been using propidium monoazide (PMA) (Taskin et al., 2011; Trivedi et al., 2009), a reagent that is excluded from live bacterial cells but can penetrate dead cells and bind DNA. Visible light irradiation causes PMA to react with the DNA to which it is bound, resulting in interference with PCR amplification. The efficiency of PMA reaction with DNA is not high, so short DNA amplicons (50-150bp), like those used in qPCR are less affected by PMA derivatization than longer amplicons, such that long amplicons will have to be used for PMA qPCR. However, it is the longer amplicons that are most affected in qPCR by contaminants and DNA damage before and during DNA purification.

## OBJECTIVES

Obj. 1. Develop a protocol for assessing live *Xf* accumulation in transgenic grapevines and suited to the evaluation of the relative efficacy of various transgenic constructs

Obj. 2. Use the developed protocol in early 2015 on bud emergence samples from the current field trials of transgenic grapevine

Obj. 3. Advance and standardize the protocol to the point that the protocol could be applied by a designated laboratory to evaluate grapevine lines bearing stacked transgenes

## RESULTS AND DISCUSSION

Our focus in this reporting period has been on testing a variety of DNA purification protocols (Healy et al., 2014; Krizman et al., 2006; Rajakani et al., 2013; Trivedi et al., 2009) and modifications thereof. Several published methods begin with grapevine tissue samples significantly larger than the 50 mg (or even 100 mg) samples available from petioles. Some are very elaborate, requiring many experimental steps. Some call for large dilutions of the preparation before taking an aliquot for analysis. The most effective method that we have found was modified from (Krizman et al., 2006) and takes advantage of charcoal and other agents to recover DNA preparations showing minimal interference during PCR.

Method: Sliced petiole samples are powdered in a 2 mL Eppendorf tube after freezing in liquid nitrogen, using a steel bead and wrist-action tube shaker. The tube shaker can disrupt up to 16 samples in under 2 min. The powdered samples are stored at -80$^0$C or used directly. Hot extraction suspension is added to the frozen powder to rapidly thaw the sample and begin releasing DNA. Extraction suspension contains the cationic detergent cetyltrimethylammonium bromide (CTAB) to denature nucleases and other proteins, and charcoal, polyvinylpyrrolidone, polyvinylpolypyrrolidone, EDTA and sodium metabisulfite to at least partially remove or inactivate various contaminants. Since infected petioles were not available in the Solano County experimental vineyard in recent months, reconstruction experiments were performed by adding various dilutions of Xf DNA in to the initial grape extract. After incubation at 65$^0$C for 20 min, samples are centrifuged to allow recovery of the nearly unpigmented, clear supernatant. The supernatant is extracted with chloroform-isoamyl alcohol. The supernatant is mixed with a half-volume of isopropyl alcohol. The precipitate is collected and further purified using a commercial silica filter method (Zymo Research clean and concentrate kit).

The average $A_{260}/A_{280}$ ratio of DNA recovered by the above method was 2.08+/-0.04, suggesting that nucleic acids were the dominant ultraviolet-absorbing constituent for the preparation. Figure 1 provides measures of the amplification of Xf DNA and of the consistency of recovery from various initial amounts of DNA. DNA standards were prepared by diluting Xf genomic DNA in a DNA solution from uninfected plants (Francis et al., 2008). The DNA standards were introduced early in the process of extraction of DNA from uninfected grapevine petiole slices or, directly into the PCR reaction, creating two standard curves. The data were fit to two lines, each line having the slope expected for quantitative PCR calibration reactions (Fig. 1).

The displacement on the abscissa of the curve for Xf DNA added during DNA purification from the curve for DNA added just before the PCR reaction corresponds to the fractional yield of Xf DNA from the DNA preparation and is calculated to be 18%. That is an 82% loss of Xf DNA through the purification process. However, the fact that the two lines in Fig. 1 are parallel strongly suggests that the ratio of the Xf DNA to grape DNA was invariant over three $\log_{10}$ intervals of Xf DNA concentration and that the amount of Xf DNA recovered was proportional to the amount of Xf DNA present in the original extraction. This is the desired result if quantitative results are to be obtained.

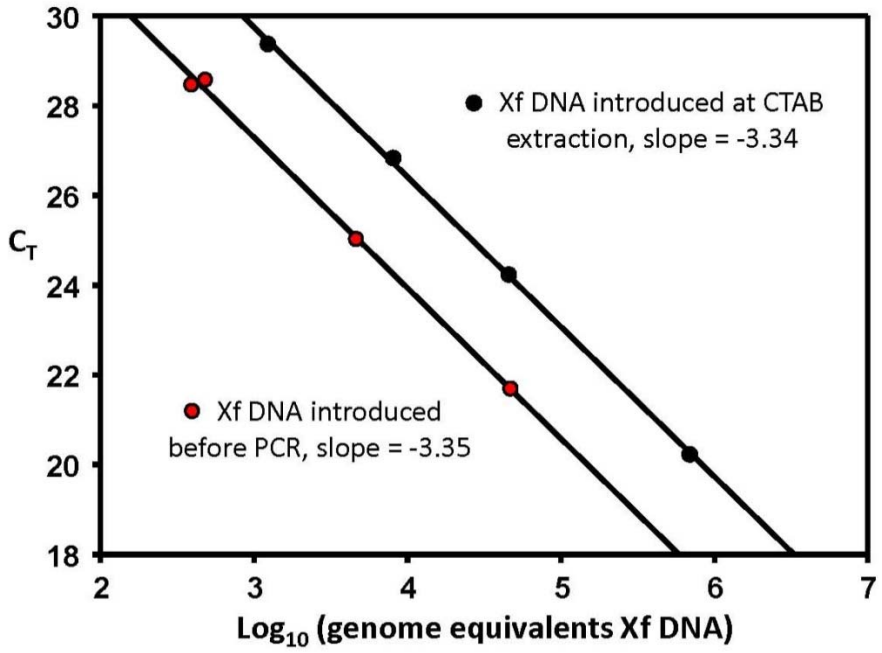


Figure 1. Reconstruction experiment recovering Xf DNA that had been added to extract of petioles from uninfected grapevines. Quantitative PCR samples, in triplicate, were of two types: ● an Xf DNA dilution series prepared in a grape DNA solution was added directly to the PCR reaction well, and ● members of the same Xf DNA dilution series were added to DNA extraction in which powdered grapevine petioles had been dispersed. DNA amounts, on the abscissa, are expressed as Xf genome equivalents, with 100,000 genomes corresponding to 1 ng of Xf DNA. The target is 75 bp amplicon from the 16S intergenic region. (Francis et al., 2006). Quantitative PCR $C_T$ values are on the ordinate. The theoretical slope is -3.32 for a doubling of the amplicon amount at each cycle, compared to the observed -3.34 and -3.35. Standard deviation error bars are not shown because they are shorter than the diameter of the data points.

**CONCLUSIONS**

In this reporting period we developed a procedure for purifying DNA from grapevine petiole slices. The procedure reduced contaminates to a level that allowed quantitative PCR to be performed with aliquots of the preparation that correspond to 10 mg or more of the original petiole material. In a reconstruction experiment, 10-fold dilutions of purified Xf genomic DNA were introduced early in the purification of DNA from grapevine petioles. Quantitative PCR analysis of the DNA preparations revealed that the amounts of Xf DNA detected was a constant fraction of the amounts of Xf DNA introduced, over a three $log_{10}$ interval of Xf DNA amounts added. This should allow comparisons of Xf DNA accumulation, i.e., comparisons of Xf titer, in grapevines bearing different anti-PD genetic constructs. In the propidium monoazide (PMA) methods (Liang et al., 2011), sensitivity to photo-derivatization by propidium monoazide (i.e., in dead Xf cells) increases greatly with amplicon size, since the chance of propidium monoazide derivatization somewhere within the amplicon template sequence increases with sequence size. We will be working to improve the quality of extracted Xf DNA for use as template for longer amplicons in qPCR. The new methods will be applied to petioles of grapevines in the Solano County transgenic trial.

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