Effect of Tissue on the Inoculation and Detection of *Xylella fastidiosa* in the Grapevine

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Abstract: Pierce's disease of the grapevine is caused by a xylem-limited bacterium, *Xylella fastidiosa* (Xf). Susceptible vines die within a few years after infection. However, the mechanism of pathogenesis is not fully understood. It is very important to optimize the timing and positioning of inoculation in host plants to study the infection process. Rooted dormant cuttings of cv. Chardonnay (susceptible vine) were grouped as stem tissue hardening and shoot elongation for needle-inoculation with 20 µl of 2×10^8 cfu ml^{-1} of Xf suspension. The infected plants were sampled 2 and 3 weeks following postinoculation (wpi) at 10 cm above the point of inoculation. Negative controls were prepared with 5 plants from each group with water inoculation. DAS-ELISA was used to detect Xf. Plants with soft stem tissue were not well suited to needle inoculation due to their poorly hardened stems, and bacterial suspension could not be fully supplied to the tissue. However, ELISA worked well with both stem types. Chardonnay cuttings with ≥30 cm long semi-hardwood tissue, sampled 2 wpi, resulted in fast bacterial movement, while ELISA readings dramatically dropped 3 wpi. Samples from the apical end of inoculated shoots had low ELISA readings, likely the result of young xylem development.

Key words: *Xylella fastidiosa*, Pierce's disease, Grapevine, Enzyme-linked immunosorbent assay

Asmada *Xylella fastidiosa*'nın İnokülasyon ve Teşhisinde Doku Tipinin Etkisi

Özet: Asmalarda "Pierce's disease" hastalığına, ksilemde sınırlı bir bakteri olan *Xylella fastidiosa* neden olur. Duyarlı asma çeşitleri infeksiyondan birkaç yıl sonra ölmektedir. Bununla birlikte bakterinin patojenite mekanizması tam olarak anlaşılamamıştır. Bakterinin infeksiyon işlevini anlamak için konukçu bitkide en uygun inokülasyon zamanı ve pozisyonunun seçilmesi çok önemlidir. Bakteriye duyarlı olan cv. Chardonnay varyetesinin köklendirilmiş dormant çelikleri, gövde dokusu sertleşmesi ve sürgün uzunluğuna göre enjektör şırıngası yardımıyla 2×10^8 koloni oluşum birimi (kob) ml^{-1} olan bakteri süspansiyonunun 20 µl'lik kısmıyla inoküle edilmek üzere gruplanmıştır. İnokülasyondan sonra 2 ve 3 hafta aralıkla, inokülasyon noktasının 10 cm yukarısında kalan gövde dokusundan örnekler alınmıştır. Her gruptan beş adet bitkiye saf su inokülasyonu yapılarak negatif kontroller hazırlanmıştır. Bakterinin bitki dokusundaki ilerlemesini teşhisini yapmak için DAS-ELISA yöntemi kullanılmıştır. Odunlaşmamış gövde dokusuna sahip bitkiler, iletim demetleri oluşumu yeterli olmadığından şırınga iğnesiyle inokülasyon yapılmasına uygun bulunmamıştır ve bakteri süspansiyonları dokuya tam olarak verilememiştir. Ancak ELISA her iki gövde yapısıyla iyi çalışmıştır. Yarı odunsu ve ≥30 cm uzunluktaki sürgünlü Chardonnay çeliklerinde, inokülasyondan iki hafta sonra hızlı bakteri hareketinin olduğu saptanmıştır. Ancak 3 hafta sonra yapılan örneklemede, ELISA değerlerinde düşme kaydedilmiştir. İnoküle edilmiş sürgünlerin tepe kısımlarından alınan örneklerdeki genç ksilem dokusunun gelişimi sonucuna bağlı olarak ELISA değerleri düşük bulunmuştur.

Anahtar Sözcükler: *Xylella fastidiosa*, Pierce's disease, Asma, ELISA

Introduction

Pierce's disease (PD) of grapevines is caused by *Xylella fastidiosa* (Xf), a xylem-limited, Gram-negative bacterium (Wells et al., 1987). The bacterium has a wide host range including alfalfa, almond, peach, citrus, forest trees, and other plants that are not economically important (Hopkins, 1989; Purcell and Hopkins, 1996). Strains of Xf are pathogenically specialized, but all multiply in the vascular system, resulting in plugging of the xylem vessels. The plugging and subsequent water stress (Goodwin et al., 1988) produce symptoms that include marginal leaf necrosis, chlorosis, raisining of the fruit,

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and the death of the plant. Disease control is achieved through vector control, removal of symptomatic plants, and eliminating infected nursery stock (Almeida and Pereira, 2001).

Bunch grapes (*Vitis vinifera* and *V.X. labruscana*) are particularly susceptible to Xf. However, grape species from the southern United States, where PD is endemic, are resistant (Clayton 1975; Milholland et al., 1981; Huang et al., 1986). Researchers have examined the multiplication and translocation of Xf in grape species (Hopkins et al., 1974; Hopkins and Thompson, 1981; Fishleder and Walker, 1999). On the other hand, studies on Xf pathogenesis in the grapevine are limited by a lack of understanding regarding where to best inoculate shoots and Xf's slow and inconsistent growth in the host. Xf infection can be verified using standard bacteriological isolation techniques from symptomatic tissues, followed by serological testing of isolated colonies. A limiting factor in studies concerning Xf is the slow growth of the pathogen on solid medium, leading to frequent contamination of culture attempts (Hill and Purcell, 1995). One of the major difficulties faced by those studying the potential pathogenicity of the bacterium is the lack of a good experimental host. Serological and polymerase chain reaction (PCR) tests are now available to detect the bacterium in infected plant tissue, albeit with some limitations (Nome et al., 1980; Buzkan et al., 2003).

The objective of our study was to understand the effect of tissue hardening and shoot length on successful inoculation of Xf in grapevine plants. We aimed to determine the earliest time and the lowest point at which shoots could be inoculated and when Xf could first be detected.

Materials and Methods

Bacterial culture conditions: The greenhouse and laboratory facilities of the Department of Viticulture and Enology, University of California, Davis, CA (USA) were utilized to carry out this research program. The 'Stag's Leap' strain of Xf was isolated from semi-lignified canes of PD-infected potted *Vitis vinifera* cv. Chardonnay, which was maintained in the greenhouse as an inoculum source. Xf containing xylem extracts were taken from surface disinfecting semi-lignified canes and cultured on periwinkle (PW) agar medium (Davis et al., 1981) at 28

°C. Once well established colonies (about 4-5 days) developed, the bacteria were harvested by washing the surface of the agar with deionized water. The concentration of bacterial suspensions was standardized to an O.D. of 0.25 at A_{600}, which was established to be 2×10^8 colony forming units (cfu) ml^{-1} by Minsavage et al. (1994) via dilution plating on PW medium.

Preparation of plant materials: Cuttings of *Vitis vinifera* cv. Chardonnay were used for the experiment. Two node woody dormant cuttings, about 10 cm in length, were rooted in moist wood shavings in the dark at 28 °C and 100% relative humidity. After rooting, cuttings were transplanted into 10 cm^2 pots and placed in a greenhouse at 26 ± 1 °C with a 16 h photoperiod.

Inoculation: The Chardonnay plants were grouped according to length (10-15 cm, 20-25 cm, ≥30 cm) and lignification by observation of their shoots as semi-hardwood, (SHW) and soft (SF) (Table 1). The needle (0.5 mm x 25 mm) puncture procedure was used to inoculate the plants (Hopkins and Adlerz, 1988). A 20 μl droplet of 2×10^8 cfu ml^{-1} bacterial suspension was placed on the plant stem and a dissecting needle was pushed through the drop into the stem. The plants were inoculated at the 2nd or 3rd node above the attachment to the stem that rooted the plant. Water inoculations were carried out on 5 plants for each group of plants as negative controls. The numbers of plants with shoots in the various categories that were inoculated are shown in Table 1.

Stem samples were collected for ELISA processing from the inoculated plants 2 and 3 wpi at 10 cm above the point of inoculum. All samples were placed in plastic bags and kept under cool conditions until ELISA tests were run.

IgG enzyme conjugation and DAS-ELISA test: Xf specific-IgG was supplied by Dr. B.C. Kirkpatrick, Plant Pathology Department, University of California, Davis, CA. IgG was purified by 'HiTrap' protein A/Sepharose HP column. Aliquots of 1 ml of antiserum were passed through the column, allowing the binding of IgG onto the sepharose beads, and the column was washed with 50 ml of 0.01 M phosphate buffer (pH 7.2). Elution was performed with 0.1 M glycine buffer (pH 3.0). One-milliliter fractions were collected, immediately neutralized with 0.04 M Tris-HCl (pH 8.0) and scanned at 280 nm. The fractions containing purified IgG were pooled, diluted

Table 1. Number of Chardonnay plants in each tissue type and shoot length category used to test the efficiency of artificial Xf inoculations. SHW = semi-hardwood shoots. SF = soft shoots

Shoot Size/	≥30 cm		20-25 cm		10-15 cm	
Stem Hardness	Xf Inoculation	H_2O	Xf Inoculation	H_2O	Xf Inoculation	H_2O
SHW	10	5	20	5	12	5
SF	20	5	40	5	12	5

with 0.5X PBS to O.D. A_{280} = 1.4 (final concentration 1 µg µl^{-1}) and stored at –20 °C. IgG was conjugated with peroxidase enzyme (Rowhani et al., 1985).

Double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) was performed as described by Clark and Adams (1977), with slight modifications. Stem samples (0.5 g) in grinding bags were ground in 10 vol (weight/volume; w v^{-1}) of extraction buffer [PBS, 2% polyvinylpyrrolidone (PVP, w v^{-1}) and 0.05% Tween 20] with a "Bioreba" mechanical grinder and stored at –20 °C until processing. The polystyrene microtiter plates were coated with 200 µl of purified IgG at 1 µg µl^{-1} in coating buffer, and incubated at 37 °C for 1 h under constant shaking. The plates were washed 3 times and 200 µl of sample extract was spotted in each well (duplicate wells per sample), followed by incubation at 37 °C for 1.5 h. After subsequent washes as described above, bacterial standards with 10-fold bacterial dilutions (10^{-2}, 0.5 x 10^{-2}, 10^{-3}, 0.5 x 10^{-3}, 10^{-4}, 0.5 x 10^{-4}) were prepared in healthy grapevine extract and added to the plates as a positive control and a means to further quantify Xf levels. After the plates were re-washed, 200 µl of conjugate was placed into each well and incubated. The presence of immobilized enzyme-conjugates was quantitated by 200 µl of solution B, consisting of 41 mM TMB (3,3',5,5'-tetramethylbenzidine), 8.2 mM TBABH (tetrabutylammoniumborohydride), in 50 ml of DMA (N,N-dimethylacetamide) in buffer A (205 mM potassium citrate, pH 4.0, and 3.075 mM H_2O_2) (Frey et al., 2000). The plates were incubated for 30 min at room temperature and the reaction was stopped by 100 µl of 2 M sulfuric acid. The color intensity was measured with a "Multiskan MCC/340" type ELISA reader at A_{450}. ELISA readings were converted to cfu ml^{-1} using the *Delta Soft3* system.

Results

Inoculation efficiency: Two weeks after inoculation, the first samples were taken from the various shoot length and hardening categories. Shoots with SF tissue were more difficult to inoculate. The needle inoculation procedure is designed to use xylem pressure to "suck up" the inoculum after the droplet has been pierced. The calculated amount of bacteria which was sought to be inserted into the Chardonnay plants was about 4 x 10^6 cfu ml^{-1}. The inoculum droplet was not consistently taken up after needle inoculation on SF shoots. However, ELISA worked well with most of the samples. The ELISA readings were converted to colony forming units after comparisons with the Xf in plant sap dilution series on each ELISA plate with the *Delta Soft3* system. About 31% of all the SF shoots had Xf levels equal to or greater than the highest Xf standard (1,900,000 cfu ml^{-1} = 10^{-2}), and all the samples contained Xf at detectable levels (Figure 1). Samples from the SF/20-25 cm shoot category were inoculated more easily and allowed Xf to spread more readily. Samples from shoots in the SF/10-15 cm shoot category were the most difficult to inoculate and had the lowest Xf levels (Figure 1).

The Chardonnay plants with SHW shoots allowed Xf to spread in the shoots with about the same efficiency as the SF shoots, but the droplet inoculation was more readily taken up by the stem. The SHW/≥30 cm long shoots allowed consistent upward movement of Xf at high levels. Two of the shoots in the SHW/20-25 cm category had very low levels of Xf, and Xf levels were inconsistent in this group (Figure 2).

Three weeks after inoculation, another set of SF and SHW shoots from other Chardonnay potted plants were sampled (Figures 3 and 4). It was still possible to detect Xf; however, there was a noticeable drop in bacterial population in SF shoots compared to the 2 wpi samples. The first 6 samples in the SF/≥30 cm long category had
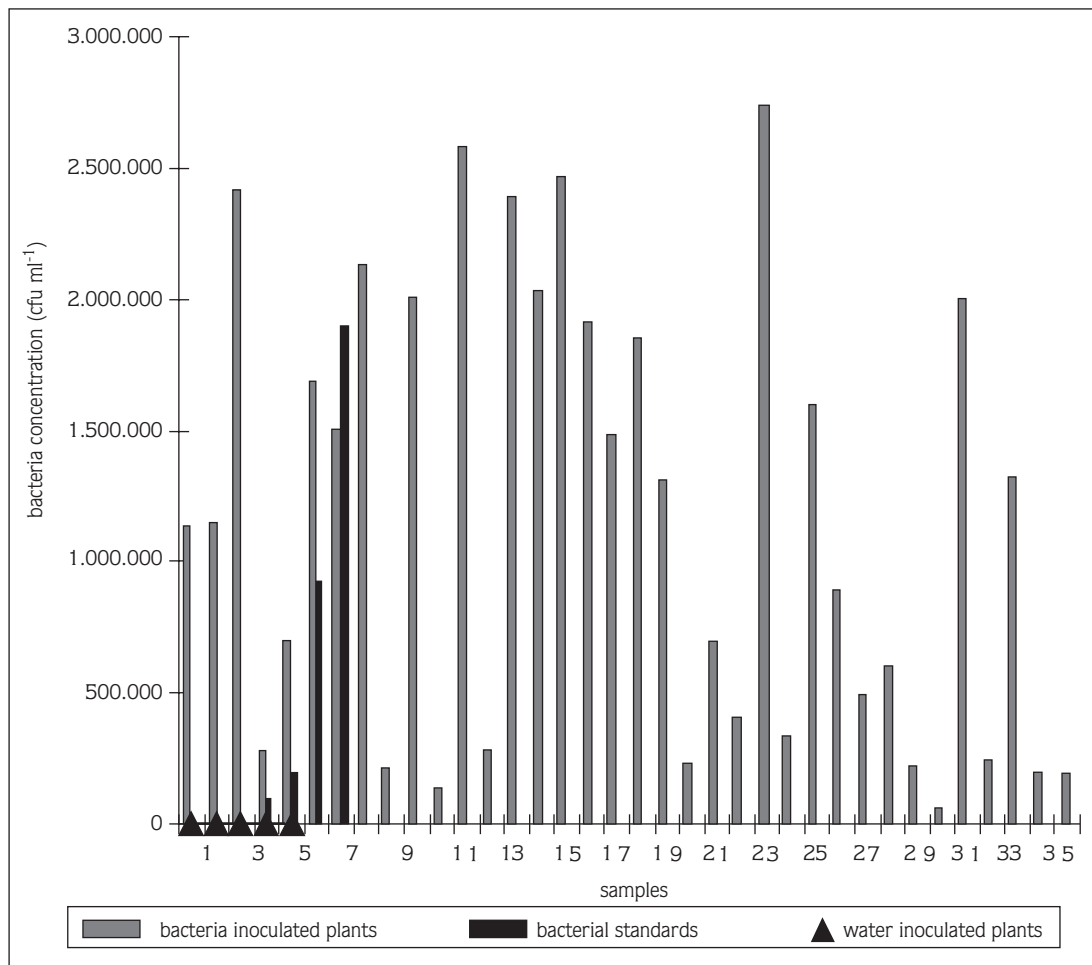
Figure 1. ELISA readings converted to colony forming units per ml of *Xylella fastidiosa* in shoots from potted vines of inoculated Chardonnay. Samples were taken at 10 cm above the point of inoculation and the stem tissue was not hardened (SF). Shoots were categorized into three groups: Samples 1 to 10 = ≥30 cm long; 11 to 30 = 20-25 cm long, and 31 to 36 = 10-15 cm long. Samples were taken 2 weeks after inoculation.

more consistent levels when compared to the 2 wpi samples, but overall had lower levels. Samples from the SF/20-25 cm long category were very high in some cases, but overall were also lower than the comparable 2 wpi samples. Samples in the SF/10-15 cm long category had much lower values when compared to the 2 wpi data.

Bacterial levels were much lower in the SHW shoots 3 wpi. The number of bacteria in 30 cm-long shoots decreased to the value close to the 2 lowest bacterial standards in the test, while ELISA readings of the samples were much higher a week before. A few samples 20-25 cm long kept a bacterial population significantly high compared to other samples, but still below the highest bacterial standard value. Shoots 10-15 cm long had low

bacterial populations. The extra week did not result in greater levels or movement in these shoots (Figure 4).

Symptoms. The first symptoms of PD in Chardonnay plants were visible 10 weeks after inoculation. Plants had mild marginal leaf chlorosis at that time. The symptomatic leaves turned brown and became scorched by 12 to 14 weeks. Water inoculated Chardonnay plants had normal growth and their shoots lignified normally (data not shown).

Discussion

Because Xf infections are usually lethal and kill cultivated host plants within 2-3 years, the diseases are
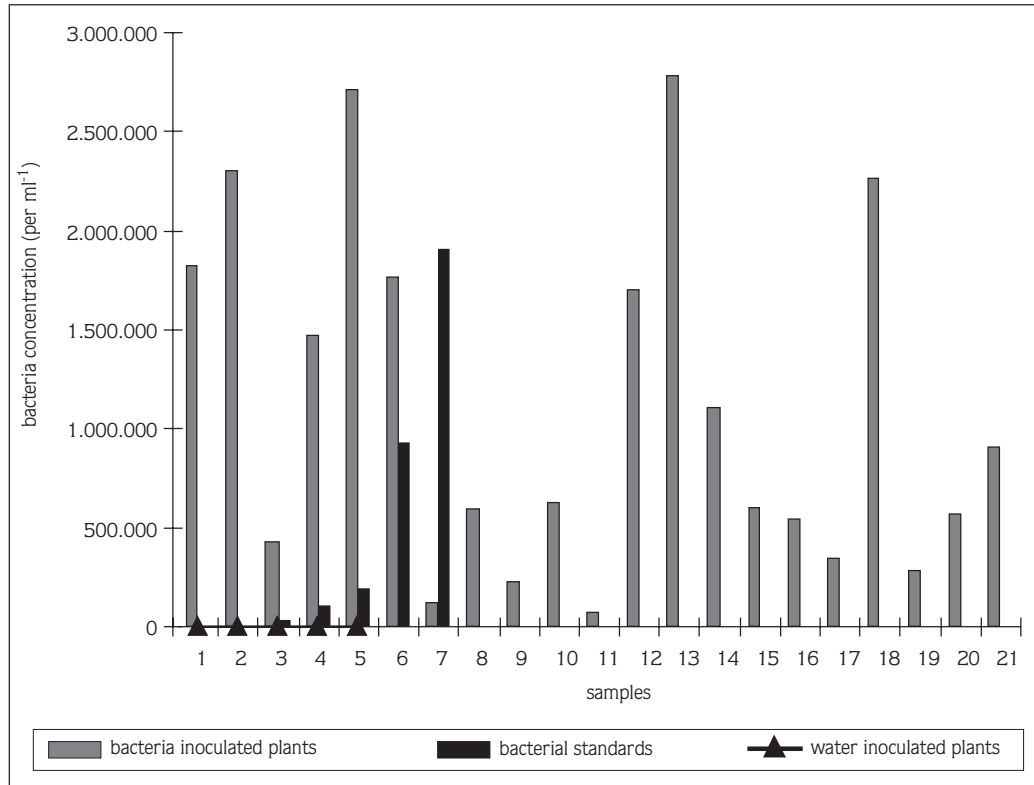
Figure 2. ELISA readings converted to colony forming units per ml of *Xylella fastidiosa* in shoots from potted vines of inoculated Chardonnay. Samples were taken at 10 cm above the point of inoculation and the stem tissue was hardened (semi-hardwood SHW). Shoots were categorized into three groups: Samples 1 to 5 = ≥30 cm long; 6 to 15 = 20-25 cm long; and 16 to 21 = 10-15 cm long (31 to 36). Samples were taken 2 weeks after inoculation.
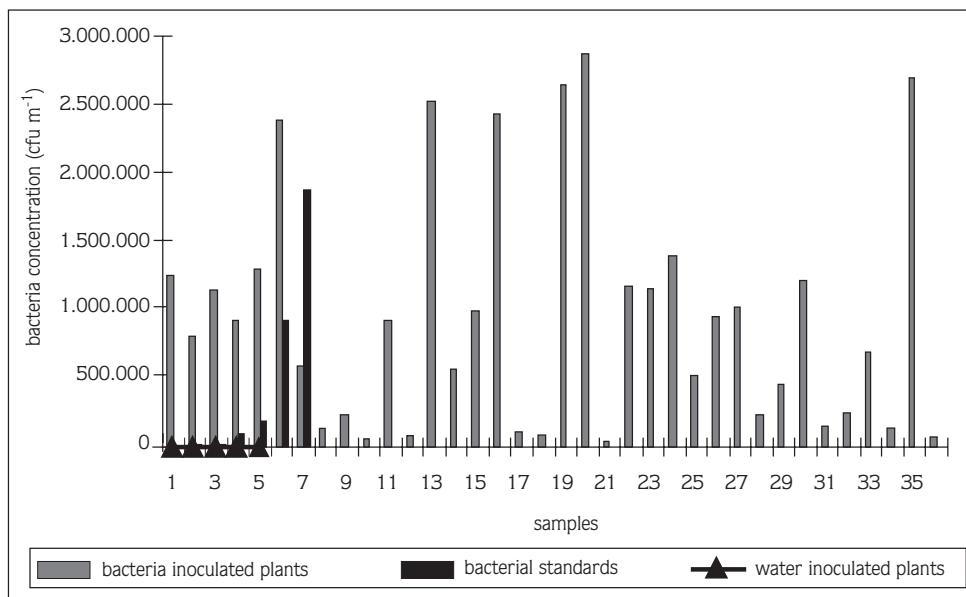

Figure 3. ELISA readings converted to colony forming units per ml of *Xylella fastidiosa* in shoots from potted vines of inoculated Chardonnay. Samples were taken at 10 cm above the point of inoculation and the stem tissue was not hardened (SF). Shoots were categorized into three groups: Samples 1 to 10 = ≥30 cm long; 11 to 30 = 20-25 cm long; and 31 to 36 = 10-15 cm long. Samples were taken 3 weeks after inoculation.
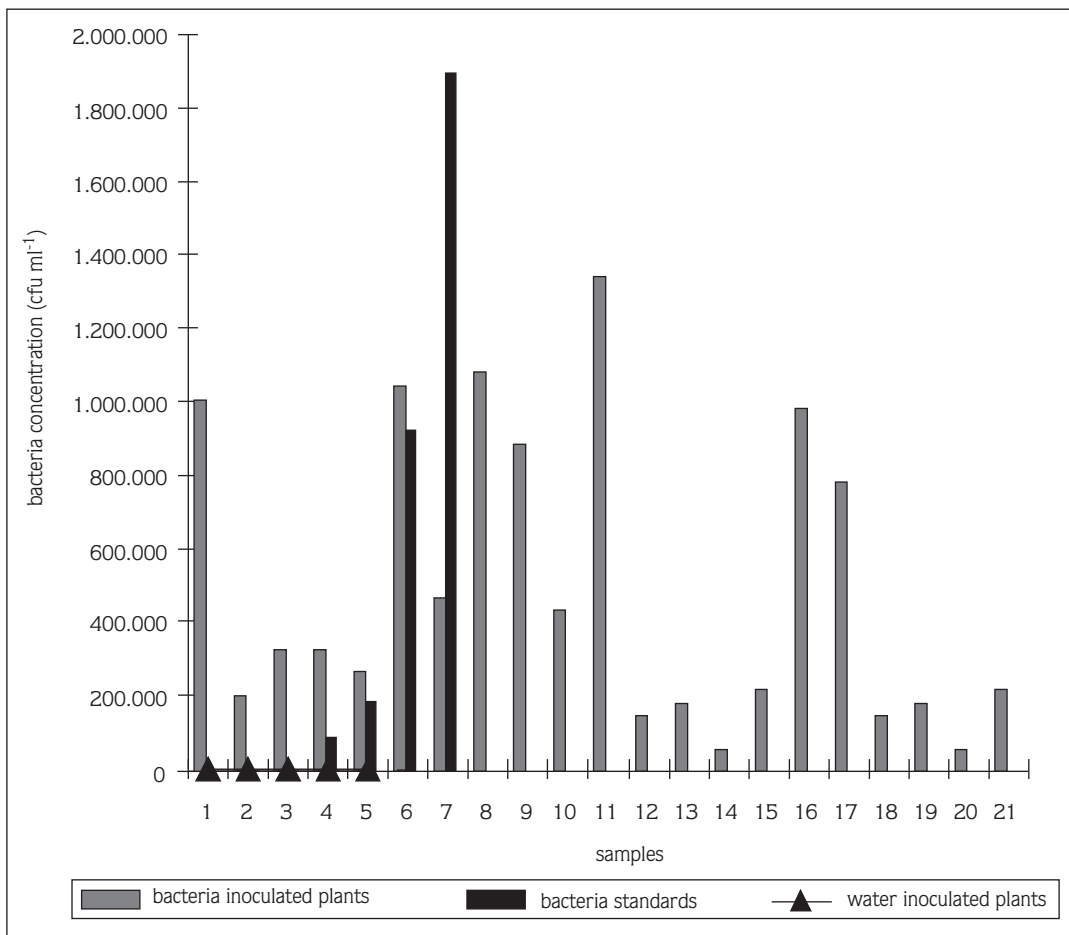
Figure 4. ELISA readings converted to colony forming units per ml of *Xylella fastidiosa* in shoots from potted vines of inoculated Chardonnay. Samples were taken at 10 cm above the point of inoculation and the stem tissue was hardened (semi-hardwood SHW). Shoots were categorized into three groups: Samples 1 to 5 = ≥30 cm long; 6 to 15 = 20-25 cm long; and 16 to 21 = 10-15 cm long (31 to 36). Samples were taken 3 weeks after inoculation.

often self-eliminating. Some diseased plants serve as a sources of inoculum for further spread. However, diseased plants take up valuable space in an orchard, but do not produce marketable fruits, even during the early stages of infection. Planting resistant or tolerant cultivars is one of the best strategies for long-term management of Xf-caused diseases. Innovative research in breeding and genetics is needed to achieve control of diseases caused by Xf. Once disease resistant plants are obtained from breeding programs, they must be tested against Xf pathogenicity. Many researchers have investigated different aspects of the bacterial infection such as multiplication and movement of Xf within the grapevine (Fry and Millholland, 1990; Hill and Purcell, 1995).

We obtained preliminary results to establish whether plant tissue type is essential to perform a successful mechanical inoculation of the bacterium. Efforts to control PD by management of outlying vegetation should take into account the differences among growth stages of plants that serve as a suitable host. The study was designed to determine if relatively succulent grape shoots could be effectively inoculated and to determine how quickly ELISA sampling would detect the bacteria. The results indicated that succulent stem tissue could be effectively inoculated, but that the needle inoculation process did not seem to work as well because less of the inoculum droplet was taken up in the stem. Although xylem tissue was not well developed in green stem tissue,

it was possible to detect the bacteria population at a considerable distance from the point of inoculation. This can be explained as bacteria movement with active growth and water flow in young plants. However, Hopkins (1983) suggested that Xf multiplies better in older inoculated tissues than in younger ones. Older plants may have a greater transpiration flow and be able to transport the bacterium further up the stem compared with younger plants.

Two weeks after inoculation was sufficient to detect Xf with ELISA. Shoot length did not have influence on inoculation efficiency and Xf was detected in almost all tested plants. However, bacterial populations dropped drastically at the second sampling date, which was only 1 week after the first sampling date.

Acknowledgments

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