THE EFFECT OF DORMANT SEASON SURVIVAL OF *XYLELLA FASTIDIOSA* IN GRAPEVINES ON PIERCE'S DISEASE EPIDEMICS IN CALIFORNIA

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ABSTRACT
The two California Pierce's disease (PD) epidemics associated with population outbreaks of the glassy-winged Sharpshooter (GWSS), at Temecula in the mid-1990s and in Kern County, peaking in 2002, differed in the number of vineyards lost and the grapevine varieties affected. In Temecula, almost half of all vineyards of all varieties were lost to PD, whereas in Kern County only the vineyards of two varieties, Redglobe and Crimson Seedless, suffered losses; all the vineyards of the other four varieties were unaffected. A hypothetical explanation of this epidemiological pattern is that in those parts of California where the winters are more severe, dormant-season die-out of *Xylella fastidiosa (Xf)* is more likely, and only the earlier-season inoculations and infections survive the winter. The likelihood of *Xf* die-out is a function of both winter climate and varietal susceptibility. In Kern County, only the most susceptible varieties were affected by secondary (vine to vine) transmission and early season primary transmission (where insect vectors acquire *Xf* from plant sources outside the vineyard) was of little consequence. Through field experiments, this project expands our knowledge of secondary transmission in the southern San Joaquin valley. The benefit to grape producers in this area will be twofold: 1) more accurate assessment of risk of economic loss from PD, and 2) suggestion of new integrated disease-management practices to control PD.

INTRODUCTION
The glassy-winged sharpshooter (GWSS)-associated Pierce's disease (PD) epidemics in Temecula and in Kern County were the first instances of epidemic secondary transmission of PD in California since the Anaheim epidemic of 1885 – 1895. During the intervening 100+ years, losses from PD in California have resulted from primary transmission, and those losses have been economically manageable in most areas. In the General Beale epidemic in Kern county (which has a colder winter climate and longer dormant season than Temecula), only a small percentage of the vineyards were lost, and all of the lost vineyards were planted in only two of the six varieties in the area, Redglobe and Crimson Seedless.

The losses to vineyards of the other four varieties were very small—in most cases less than 1 in 10,000 vines. By contrast, all 12 of the Redglobe vineyards monitored in the General Beale area were significantly damaged, with a range of 2% to over 50% of the vines lost (Hashim, *et al*, 2003). Most of these vineyards were ultimately removed.

Grapevines acquire new *Xylella fastidiosa (Xf)* infections either by primary or secondary transmission. Primary transmission occurs when vector insects acquire the bacterium from source plants outside the vineyard, then fly into the vineyard to infect vines. Secondary transmission occurs when vector insects acquire *Xf* from an infected vine within the vineyard and then transmit the infection to other vines, known as vine-to-vine transmission.

The risk associated with these two kinds of transmission differs. The disease and vine loss pattern associated with primary transmission is linear; that is, a relatively constant number of vines per year become infected, so the yearly accumulation of PD vines increases additively and predictably. By contrast, the pattern of yearly accumulation of PD vines associated with secondary transmission is typically logarithmic, increasing as a multiple of the infected source vines that are present, so entire vineyards can be lost within just a few years.

Secondary transmission cannot begin to occur until that time in the growing season when the bacterial cells in diseased vines have multiplied and moved within the vine; the cells travel from the refuge site, where they survived the dormant season, up into the new growth where vector insects can feed and acquire them. Secondary transmission of infection can then continue until the end of the growing season. However, infection does not equal disease. The phenomenon of over-winter curing of *Xf* infections is well-documented in most viticulture areas of California (Fiel *et al*, 2003). Early-season inoculations can result in infections which survive the dormant season and progress to chronic disease and vine death. Conversely, later-season infections do not become sufficiently established to survive the dormant season, and the vines are free of infection the following year (Fiel *et al*, 2003).

In most viticulture areas of California (Napa and Sonoma Valleys, for example), secondary transmission of infection regularly occurs, but it cannot begin early enough in the season for the infection to survive vine dormancy and progress to chronic PD. In these areas, secondary transmission occurs but does not result in disease.

We propose that in the General Beale area, secondary transmission of infection occurred in all varieties, possibly infecting large numbers of vines in every vineyard. The rate of *Xf* multiplication and movement varies within plant hosts (Hill and Purcell, 1995) and among grapevine varieties. In the most susceptible varieties, Redglobe and Crimson, the rate of bacterial multiplication and movement was faster, so the result was that the bacteria had a window of opportunity some time in mid-season when secondary transmission could progress to disease. Secondary transmission of infections could not occur before this time window, and secondary transmission of *Xf* after this time window did not survive vine dormancy. Thus, in the two susceptible varieties some, but not all, of the secondary infections progressed to chronic disease.

In the resistant varieties, however, by the time secondary transmission could begin, it was too late for the infections to become well enough established to survive vine dormancy, and virtually all of those infections died out, leaving the vines free of disease the following year. This is illustrated in the two hypothetical Figures 1 & 2 below. The position and shape of these two curves can be a function of the severity of winter climate, the length of the growing season, and the varietal susceptibility. Favorable factors (such as a short, mild dormant season) would move the curves toward each other, resulting in a greater probability of overlap —thus a bigger window of opportunity when secondary transmission would result in chronic disease. In the General Beale area, most of the varieties would be "resistant" to secondary transmission of PD (curves shifted apart); thus the vineyards were not lost to disease. Those same varieties, if grown in the Temecula area, would have the curves shifted toward greater overlap, and the varieties could then be lost.

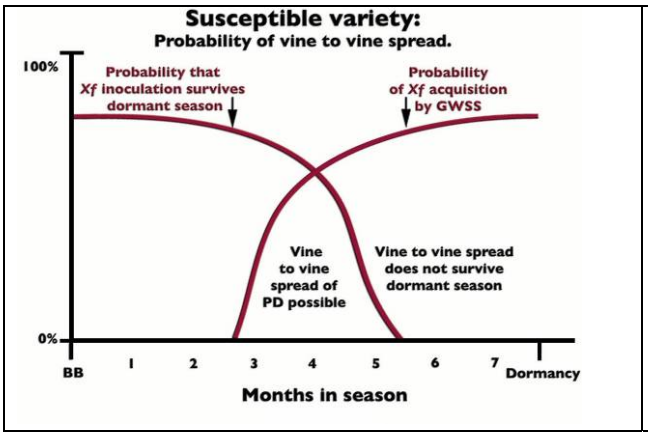

Figure 1.

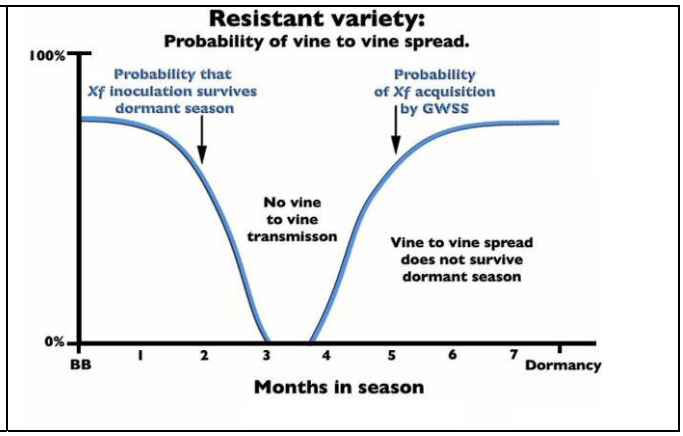

Figure 2.

This project addresses the dynamics of secondary transmission in the southern San Joaquin valley. Previous work (Fiel *et al*, 2003) has examined the left-hand curve, dormant season survival by time of inoculation. However, little is known of the right-hand curve, probability of acquisition by GWSS with regards to time. Because of concerns about the possible transmission of PD to commercial vineyards, it was not possible to pursue the best experimental designs using insects to transmit *Xf*, nor to do the experiments in commercial vineyards in either Kern or Tulare Counties. Perhaps the only possible project site, a 3.2-acre vineyard on the University of California Kearney Research and Extension Center at Parlier, CA, was available, and using this site enabled us to begin experiments that might not otherwise have been done. This site had mature vines of two varieties, Thompson Seedless and Selma Pete (a table/raisin variety similar to Thompson). For the first time, we were able to examine the effect of varietal differences on our theoretical curves.

In addition, 850 mature Thompson vines were cut about 40 cm above the ground and were grafted with Red Globe, Thompson, and Princess in 2005. In three years when these vines are mature enough, other experiments can be done to further understand the influence of varietal differences on secondary transmission and over-winter survival of *Xf*. The projects discussed herein, with other projects that build on these concepts, will help extension advisors and growers devise new integrated disease management practices for PD.

OBJECTIVES

1. Follow over-winter survival of *Xf* associated with time of inoculation by needle-inoculating 20 to 35 vines at a time, of each variety, at twice-a-month intervals for 4 months beginning on May 1, 2005. Confirm all resulting infections by ELISA testing of each vine during the year that they are inoculated. Test all vines in late season 2006 to determine whether the infections persisted over the dormant season.
2. Determine the time of detection of *Xf* in foliage in 2006. In May 2005, 60 vines of each variety would be needle-inoculated. At 2X per month intervals in 2006, all 120 vines to be sampled where *Xf* is most likely to appear in the new foliage to determine when *Xf* is detectible. Test all samples by ELISA, and store a part of each sample at minus 80^0F for possible future PCR testing.

3. Graft 850 mature Thompson vines with 3 varieties of differing PD susceptibility to enable future experiments in this vineyard about the influence of varietal differences on secondary transmission.

RESULTS

Objective 1: The 180 Selma Pete vines used in these over-winter survival experiments were grafted in 2001 about 30 cm above the ground on to mature Thompson vines. These Selma Pete vines, now in their fourth growth year, and another 220 Mature Thompson Seedless vines were needle-inoculated in 2005 at twice-per-month intervals beginning at the May 1 through the middle of August, for a total of eight inoculation times. The inoculated vines were tested in late 2005, and the inoculations were 100% effective in producing infections in the vines. Each vine was inoculated in two places on opposite sides of the vine (different cordons) on first-year growth about 15 cm from old wood. At each inoculation site, both a petiole and the stem were inoculated with droplets containing ca. 10^7 *Xf* cells from a 9-day-old culture. The over-winter survival of the resulting infections is shown in Figure 3.

Objective 2: The samples for testing the time of *Xf* detection in the new foliage in 2006 were petioles taken from the site considered most likely to be where the bacterium would appear first, whenever possible from the base of the cane that was inoculated the previous year. Because each vine had two inoculation sites, two sites were sampled for each vine, and 60 vines produced 120 samples. In many vines, one side of the vine began testing positive several weeks before the other side. Even on August 18, the samples from one side were still testing negative in 24 vines of Thompson and four vines of Selma Pete, respectively.

On June 14, petiole samples were collected from six vines that had tested positive on June 1 (three vines each of Thompson and Selma Pete). One basal petiole was tested from each new shoot, growing from old wood within 15 cm of the trunk. A positive petiole would mean that *Xf* was present in the basal portion of the cane. The Thompson and the Selma Pete had *Xf* in 5% (3 of 58) and 19% (12 of 62) of the canes respectively.

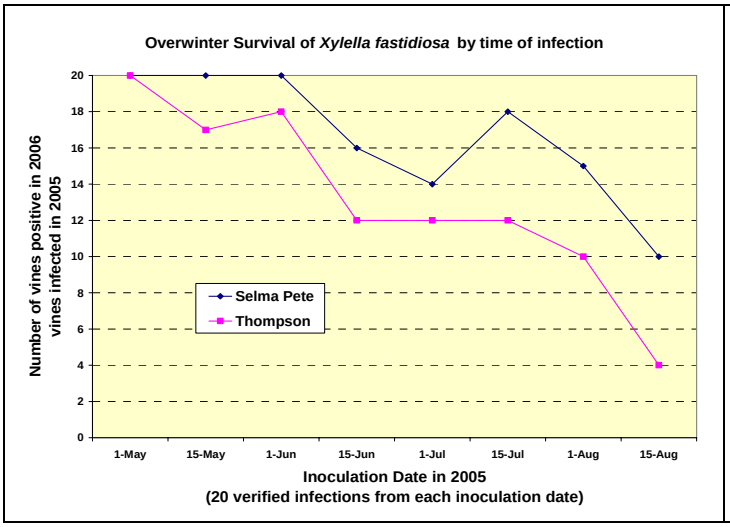

Figure 3.

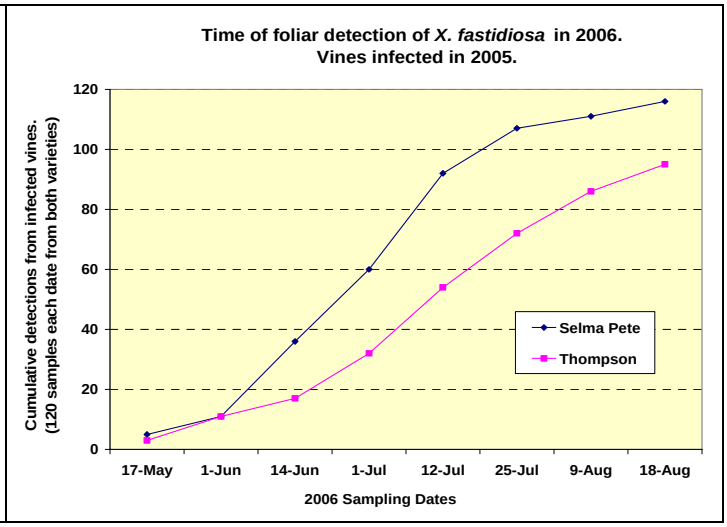

Figure 4.

DISCUSSION

The over-winter survival experiment (Figure 3) was designed to represent the worst-case possibility, and therefore the results do not represent what might occur in an actual field situation, nor do the results agree with previous work at Kearney (Feil et.al., 2003). We chose to inoculate the base of the canes vs. more distal sites because the severity of the GWSS vectored PD epidemics has been in part attributed to the possibility that GWSS can feed (and therefore acquire and inoculate) at the base of the cane. Mid season basal inoculations are more likely to result in infections that survive the winter than more distal inoculation sites. We inoculated each site with a very large number of cells to insure that all inoculations would result in infections. We inoculated multiple millions of bacterial cells per inoculation into the xylem, compared to inoculations by GWSS or another vectors that might introduce a few (<100) cells. Our resultant curves (Figure 3) were skewed far to the left in comparison to previous work at Parlier. Feil et.al. (2003) found that infections resulting from basal insect inoculations in July survived the winter, but none of their August inoculations, whether by insect or needle, or basal or distal, resulted in infections that survived. Our work is, however, the first case of comparing the differences in over-winter survival of *Xf* as a function of varietal susceptibility, supporting the idea that more susceptible varieties result in over-winter survival curves that are shifted to the right. It may be that irregularities in the shape and position of the curves in Figure 3 are the result of using an excessive number of bacterial cells per inoculation. We will address these aspects in future inoculation experiments.

The "time of foliar detection" curves in Figure 4 are probably not affected by the decision to inoculate with a worst-case design, and probably do represent actual field epidemic situations. These curves show a difference between varieties in when the bacteria become detectable in the new growth, and this is consistent with the hypothesis about secondary transmission that is represented in Figures 1 & 2.

Putting together the information from Fiel et.al. (2003) and our Figure 4, we would predict that the window of possibility for secondary transmission that survives the following winter may begin in early June and end by early August. However possibility is not the same as probability, and epidemics are stochastic phenomena. When *Xf* is first detected it is present in only a small part of the total canopy; and it is highly patchy. Also in mid June only a small proportion of the canopy of chronically infected vines have detectible *Xf* in the foliage, where it would be available for vector acquisition. Therefore the target area, both in the vineyard and on the vine, where acquisition feeding might occur in mid June is a very small part of the total vineyard or canopy, especially compared with the target area in August and beyond. Also in mid June to August the target area where an infective vector must feed in order to inoculate a vine with an infection that survives the winter is a small and continuously shrinking portion of the canopy of a vine.

The fact that GWSS can feed at the base of canes in July and August does not speak to the probability that GWSS would prefer to feed at these target sites and search for them preferentially. Furthermore we know of no evidence that in mid summer GWSS prefers a basal feeding site (where either acquisition or inoculation might be successful) over the more available and vigorously growing outer parts of the canes. GWSS flying onto an infected vine in July would have a very small probability of randomly encountering a target feeding site that would result in acquisition. This raises the question why did secondary transmission play such a big role in the Temecula epidemic and in the susceptible varieties in the General Beale epidemic? We propose that the most important epidemiological factor, in addition to the ability of GWSS to feed at the base of the canes, is simply the extraordinarily high numbers of GWSS that occurred in these epidemics. One or a few GWSS landing on a vine may be very unlike to acquire *Xf*, but when hundreds or even thousands of GWSS per vine are feeding and actively moving among the vines, the probability of *Xf* acquisition and transmission by a percentage of these GWSS becomes larger. This may be enough to explain the kind of secondary transmission that was observed. Also the effect of variety on shifting the shape and position of the curves as represented in Figures 1 & 2 may explain the varietal difference observed in the General Beale epidemic.

Figure 4 represents new information. It does not however quantify the probability (vs. the possibility) that GWSS will acquire *Xf* by feeding on an infected vine. Our future efforts will be directed toward determining the geometric features of the target feeding area in an infected vine, and in exploring the behavioral feeding preferences of GWSS in the mid season. This will help to interpret the curves in Figure 4 and to come closer to predicting a more realistic position and shape for the theoretical acquisition curve postulated in Figure 2. The research vineyard at Kearney provides an opportunity to pursue these goals.

CONCLUSIONS

The results of these experiments support the hypothesis for secondary transmission that is represented in Figures 1 & 2 above, namely that the two curves which represent: (1) the probability that an *Xf* infection survives the dormant season, and (2) the probability of *Xf* acquisition by a vector must overlap for secondary transmission of *Xf* to survive dormancy and progress to PD. The experiment concerning time of foliar detection of *Xf* in previously infected vines provides some limits on when such overlap of these curves can begin, and previous work suggests the probable end of the window. We now better understand the severe losses of the two recent Kern and Temecula epidemics, and strategies are emerging for timely, effective, and affordable control practices to predict and avoid such losses in the future. The benefit to grape producers in this area will be twofold: 1) more accurate assessment of risk of economic loss from PD, and 2) suggestion of new PD management practices. For example protecting vines during the window of overlap might reduce or eliminate secondary transmission of PD. Practices that use this epidemiological knowledge may be thought of as Integrated Disease Management, a concept analogous to Integrated Pest Management that has been so widely adopted and successful.

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THE PIT MEMBRANE BARRIER TO *XYLELLA FASTIDIOSA* MOVEMENT IN GRAPEVINES: BIOCHEMICAL AND PHYSIOLOGICAL ANALYSIS

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ABSTRACT
Studies planned for this proposal will (1) examine further the impacts of cell wall-degrading proteins on pit membrane integrity, (2) describe what our uses of the *Xylella fastidiosa* (*Xf*) cell wall-degrading enzymes tell us about the pit membrane polysaccharide network, and (3) specifically examine the relationship between pit membrane disruption, grapevine ethylene production, and xylem water conduit obstruction. Of particular interest because of its potential for identifying a new mechanism for a vine's resistance to PD, will be tests of the role of *Xf* cell wall xyloglucan-degrading endo-β-1,4-glucanases (EGases) in increasing the pit membrane's porosity and efforts to identify natural pant proteins that are inhibitors of those EGases.

This is a new project, approved in Spring 2006, with funding beginning July 1. Dr. Alonso Pérez-Donoso, who had recently finished his Ph.D. work in our laboratory was to have been the primary bench scientist in the project. However, he was offered a faculty position in Santiago, Chile and left to assume that position in early Spring 2006. Therefore, progress toward meeting our objectives has been slow. We are fortunate in that Dr. Quang Sun will be taking a position as a postdoctoral researcher in the project, beginning October 1, 2006. We anticipate rapid progress on Objectives 2 and 3 once Dr. Sun has become comfortable with his new laboratory environment. We have been able to begin testing of xyloglucanase-inhibiting proteins (XGIPs) on *Xf* EGase activity. Unfortunately, no inhibition was detected.

INTRODUCTION
For five years, Labavitch and the listed collaborators have been testing a model proposed to describe the development of Pierce's Disease (PD) in grapevines (Labavitch et al., 2001, 2002; Labavitch and Matthews, 2003; Labavitch et al., 2004, 2005; Pérez-Donoso, 2006; Pérez-Donoso et al., 2006). Findings reported in the last two PD Symposia strongly suggest that enzymes, likely produced by *Xylella fastidiosa (Xf)* resident in xylem water-conducting cells (also Roper et al. 2004) are important contributors to the escape of the pathogen from the vessels into which it has been introduced by GWSS, thus initiating its systemic spread through the vine and the subsequent development of PD symptoms. However, observations made only in the past year have suggested that seasonal changes in normal grapevine development may also contribute to the systemic spread of *Xf*, beginning in late Spring. These observations may be linked to those made by collaborators Rost, Matthews et al. (Thorne et al., 2006) suggesting that relatively long xylem conduits, likely to be of primary xylem origin, may allow relatively long distance passage (i.e., the length of 2-3 internodes) of *Xf* into grape leaves. While this pathway is not likely to facilitate long distance systemic spread of the pathogen through stems, it may facilitate rapid movement from stems into which *Xf* has moved, into leaves where disease symptoms then become evident. Work planned for this project will examine aspects of these reports, with a strong focus on factors that might affect the integrity of the pit membranes in grapevine xylem water conduits.

OBJECTIVES
1. Characterize the biochemical action of *Xf* EGase, *in vitro* and *in planta* and determine if it is inhibited by plant proteins that have been identified as xyloglucan-specific endoglucanase (EGase)-inhibiting proteins.
2. Examine the full range of effects on grapevine pit membrane porosity that result from introduction of cell wall-degrading polygalacturonase (PG) and EGase.
3. Repeat our 2005 observations of a late Spring, dramatic increase in the porosity of grapevine pit membranes.

RESULTS
Objective 1. Characterization of the biochemical action of *Xf* EGase, *in vitro* and *in planta* and determine if it is inhibited by plant proteins that have been identified as xyloglucan-specific endoglucanase (EGase)-inhibiting proteins. We have reported that the introduction of PG and EGase to the xylem of explanted grapevine stems causes breakdown of pit membrane structure (see the report for the project "The contribution of the pectin-degrading enzyme polygalacturonase (PG) in transmission of *Xf* to grape