BIOLOGICAL, CULTURAL, AND CHEMICAL MANAGEMENT OF PIERCE'S DISEASE

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INTRODUCTION

The systemic movement of *Xylella fastidiosa* (*Xf*) within the plant xylem system is essential to this bacterium's ability to cause disease and probably for its indefinite survival in natural environments. Numerous microscopic studies of plants affected by Pierce's disease revealed high concentrations of bacteria in some xylem cells, but it is notable that in all of these studies, adjacent xylem elements are often devoid of bacteria. The important basic question of how the bacteria move from cell to cell is still unanswered.

Our initial hypothesis is that bacterial multiplication is an important requisite for cell-to-cell movement. Do *Xf* populations in nonsystemic hosts reach high or low densities within infected cells? Do *Xf* populations in systemic hosts with low populations of *Xf* (as determined by dilution plating) attain high populations in few cells or lower populations in many cells? We will examine the behavior of *Xf* in nonsystemic hosts such as willow and mugwort and in plants with low, but systemic populations, such as blackberry. The occlusion of xylem cells with *Xf* in willow, for example, would illustrate that bacterial aggregates that completely fill xylem cells is not sufficient for systemic movement.

The *Xf* oleander strain is not systemic in grape and vice versa (A.H. Purcell et al., unpublished data). The typical fate of *Xf* in most woody plant species is to multiply without systemic movement (A.H. Purcell, unpublished). We will investigate if this is true for the oleander strain in grape and the grape strain in oleander. Both of these strains are systemic in their pathological hosts but not in the opposite host. We will seek to identify whether oleander strains multiply in grape using dilution plating on solid culture medium (PW) and confocal fluorescent microscopy.

A recent development that aids the study of bacterial movements in plants is the emergence of scanning confocal laser microscopy (SCLM). The new techniques to study bacterial biofilms could provide valuable information on the distribution of *Xf* within xylem tissue. The application of the SCLM coupled with image analysis techniques permits the study of living, fully hydrated microbial biofilms.

Success in introducing novel genes into *Xf* (objective 4) to create new but grape-virulent strains with reporter gene constructs allowing easy *Xf* detection in grape tissues would greatly facilitate studies of *Xf* movement in plants. Such a system could enable *Xf* detection with SCLM in plants without fixing, dehydrating, staining, or otherwise preparing plant specimens. Thus the same tissues could be examined repeatedly to follow *Xf* movements, especially those events associated with the cell to cell movements that are critical to disease. If *Xf* can be genetically engineered to express the green fluorescent protein (GFP) gene, the movement of bacteria could be followed through the plant similar to the methods used in studies on the movement of *Erwinia amylovora* in apple. Our use of GFP-mutants for histological studies of movements or of biofilm formation will depend upon success of objective 4.

An understanding of biofilms may also help explain *Xf* movement and pathogenicity. A biofilm is an aggregate of attached cells produced when bacteria adhere to a surface, initiate glycocalyx (exopolysaccharide) production and form microcolonies. *Xf* appears to produce biofilms that are unique compared to other documented biofilms in that they are inhabited only by a

single bacterial species and occur within plant xylem and insect guts. Others have speculated the matrix material surrounding aggregations of *Xf* within plants improves the bacterium's extraction of nutrients and provide physical protection. We suspect that biofilm formation is also critical to *Xf*'s movement from cell to cell within plants and necessary for its survival within the vector foregut, from which it is transmitted to plants by insects. We examine *Xf*'s occurrence within a spectrum of host plants - from those in which it multiplies rapidly but does not exhibit systemic spread (willow), to those in which it multiplies and moves but does not reach high enough levels to cause disease, to pathological hosts such as grape. We will also develop methods to produce and examine biofilms under in vitro conditions so as to be able to experimentally manipulate environmental conditions and determine their effects on biofilm formation. If we are successful in developing *Xf* transformation protocols (objective 4), we should be able to provide conclusive genetic evidence for the potential role of biofilms in plant pathogenesis or insect transmission by knocking out biofilm biosynthesis gene(s) using transposon mutagenesis.

OBJECTIVES

1. Understand how *Xf* moves, and the patterns of its movement, in systemic (grape, blackberry) and non-systemic (willow) plant hosts using microscopy. (AHP, PCA, BCK)

2. Understand how temperature influences the movement and survival of *Xf* and the incidence and/or severity of PD. (AHP)

3. Determine whether vegetation barriers between riparian areas and vineyards and/or insecticide-treated "trap crops" at the vineyard edge can reduce the incidence of PD. (AHP, EAW, BCK, MAW)

4. Develop transformation / transposon mutagenesis systems for *Xf* using existing or novel bacterial transformation vectors. Use *Xf* mutants to identify bacterial genes that mediate plant pathogenicity, movement, or insect attachment. (BCK)

5. Isolate and identify endophytic bacteria that systemically colonize grapevine. Develop methods to genetically transform grape endophytes to express anti-*Xf* peptides. (BCK)

6. Develop a genetic map to *Xf* resistance using *V. vinifera* x (*V. rupestris* x *M. rotundifolia*) seedling populations and AFLP (amplified fragment length polymorphism) markers, identifying resistance markers, and possible identification of resistance genes. Utilize DNA markers for resistance to rapidly introgress *Xf* resistance into several *V. vinifera* winegrapes and/or utilize genetic engineering procedures (when available) to move above identified *Xf* resistance genes into winegrapes. (AW)

7. The purpose of this objective is to:
   a. Determine the resistance of 10 grape genotypes to PD after mechanical inoculation and natural infection with *Xf*. Elucidate the xylem chemistry of these grape genotypes and statistically correlate both chemical profiles and specific molecular markers to PD resistance. (PCA, MAW)

   b. Determine the resistance of common host plants (willow, resistant; blackberry, susceptible) to *Xf* and discern the relationship of specific chemical profiles to resistance. Utilize these techniques to examine resistance mechanisms of resistant seedlings identified in 6a. (PCA, AHP)

   c. Validate the influence of chemical profiles and specific chemical markers on the growth and survival of *Xf* by tests in in-vitro culture. (PCA)

RESULTS AND CONCLUSIONS

Objective 1: Understand how *Xylella fastidiosa* moves, and the patterns of its movement, in systemic (grape, blackberry) and non-systemic (willow) plant hosts using microscopy (Purcell)

Confocal microscopy of red willow noculated with *Xf* showed that *Xf* can multiply to very high levels within individual xylem elements but does not move to adjacent cells in non-systemic hosts. Thus, the low populations of *Xf* recovered from

willow by culturing represent many *Xf* cells within a very few colonized cells rather than fewer numbers of *Xf* in a larger number of cells. High magnification scanning electron microscope and modified staining methods of *Xf* in grape revealed "tentacle-like" structures (fimbriae) at the narrow ends and division plane of dividing *Xf* cells collected from expressed xylem sap of infected grape but not when collected from cultured *Xf* cells from PW medium. *Xf* aggregated on the surface of xylem vessels in PD-infected grape petioles as well as dormant infected grape tissue (green and woody stem pieces) early or late in colonization of grape xylem. The bacteria were embedded in a matrix of fibers that covered them in a net-like fashion. This matrix of *Xf* cells and extracellular materials (a biofilm) adhered to the xylem cell walls. The fimbriae of attached *Xf* connected only in contact with plant tissues. Biofilm formation appears to be a consistent feature of colonization of grape by *Xf*. Cells of *Xf* grown in a new culture provided by Brazilian collaborators produced abundant extracellular fibrils but not fimbriae, and these cells attached strongly to glass, unlike cells grown in traditional *Xf* media (PW). Our findings suggest that *Xf* produces attachment structures only under specific environmental cues.

Objective 2. Understand how temperature influences the movement and survival of *Xylella fastidiosa* and the incidence and/or severity of PD (Purcell)

Field inoculations of grape at different times of the year at Oakville (1997), Davis (1997-98), Fresno (1998-99) and Hopland (1999) consistently confirmed that infections during April through May and to a much lesser in June resulted in persistent infections of *Xf* the following year; whereas most June, July or August inoculations resulted in non-persistent infections unless the base of canes were inoculated. At the Hopland site, recovery of early-season infected vines was the first evidence of possible climate-mediated recovery of PD-infected vines that was not explainable by pruning eliminating the *Xf* infections. Inoculations of vines during April 2000 in San Luis Obispo County and Santa Barbara County suggest that cool temperatures (not exceeding 16 ^{0}C) following inoculation may not allow systemic infection by *Xf*. The highest populations of *Xf* occurred at the bases of symptomatic canes and decreased towards the tip of sampled canes in experimentally inoculated or naturally infected vines.

Experimental freezing of dormant, PD-infected potted vines cured the plants of PD at rates of 0 to 70% after exposures to temperatures ranging from -2 to -10 ^{0}C for 3 successive freezing exposures. Cane segments treated identically at the same time never recovered, suggesting that some aspect of plant physiology, rather than the direct action of freezing alone, kills *Xf* by freezing in grape xylem. The survival of *Xf* after freezing in various liquid media further supports this view.

Objective 3: Determine whether vegetation barriers or trap crops can reduce the incidence of PD in riparian areas. (Weber)

Two trap crop trials have been established on either side of a large vineyard in Napa. One trial borders the Napa River, the other Milliken Creek. Vines are spaced 9 feet (rows) by 5 feet (in-row). In each trial, St. George rootstock is planted at the ends of adjacent rows to create the trap crop treatments. Trap crop treatments include the first 6 vines in 12 adjacent rows. In each trial, there are three replicate trap crop planting and three control plantings where Chardonnay or Pinot Noir are planted to the end of the row. The vineyard was planted in May 1999. By October 2000, trap crop vines along the Napa River had reached the upper trellis wire and had filled in well. They were pruned in December along with the rest of the vineyard leaving wood on the "fruiting" wire. On the Milliken Creek side, deer did considerable grazing damage throughout the year and the vines did not develop as well.

Admire (soil-applied imidacloprid) was applied to trap crop vines in November 1999. Due to concerns about its efficacy, this will be replaced with a spring 2001 treatment of Provado (foliar-applied imidacloprid). BGSS were monitored in 2000 in both trials using yellow sticky cards placed at the ends of rows. BGSS were detected in all treatments, although counts were relatively low. Per-trap catch totals ranged from 2 to 23 BGSS for the trapping period March-October. A PD disease survey was conducted in both trials in Sept. 2000 extending approximately 40 vines into the vineyard. Only 5 vines were found showing PD symptoms. Monitoring and mapping will continue in 2001.

Objective 4: Develop transformation and transposon mutagenesis for *Xylella fastidiosa* (Kirkpatrick)

Procedures were developed to successfully introduce the transposon, Tn5, into two different strains of *Xf*. Electroporation conditions that were similar to those that have been used to electroporate DNA into several *Xanthomonas* species were found to be efficacious for electroporating DNA into *Xf*. Several attempts to transpose *Xf* with two different Tn5 and two Tn10 suicide constructs failed to produce transposed *Xf* cells. However, using identical electroporation conditions, we obtained several hundred Tn5 mutants of both the Fetzer and Temecula *Xf* strains using a "transposome" complex composed

of a transposase protein/DNA complex containing Tn5. Southern blot analysis showed these were random, single, Tn5 inserts throughout the *Xf* genome. Sequence analysis of *Xf* genomic DNA that flanked the Tn5 insertion identified several genes that were found in the CVC strain of *Xf*. We now have approximately 500 Tn5 mutants stored at –80; inoculation of grapevines and other analyses of the mutants will begin in a few months after several thousand mutants have been obtained.

Three, 1.8 kb *Xf* plasmids were cloned and sequenced from the UCLA *Xf* strain. The largest open reading frame (ORF) on these small *Xf* plasmids had significant homology with another phage replicase gene, suggesting this *Xf* ORF encodes a plasmid replicase gene. Initial cloning of these plasmids probably interrupted the promoter for this ORF and we have since recloned these plasmids at another location on the plasmid. We are now introducing the Kan^R gene from the Tn5 construct described above, which we know is expressed in *Xf*, into the cloned *Xf* plasmids. In this manner we hope to construct an *Xf/ E. coli* shuttle vector that will greatly facilitate molecular genetic analyses of *Xf*.

Objective 5: Isolate and identify endophytic bacteria that systemically colonize grapevine xylem. Identify natural, or genetically engineer, endophytes to be antagonist to *Xylella fastidiosa* (Kirkpatrick)

Several hundred bacterial isolates were obtained from both healthy and *Xf*-infected grapevines located in Napa and Yolo counties. Specific grapevines of two cultivars were sampled bimonthly during 1999 and 2000. In addition, healthy appearing grapevines that were growing in the middle of vineyards that were decimated by PD were also randomly sampled. Four different media were used to cultivate the bacteria, however no significant differences in the types or numbers of bacteria were observed using the different media. Gram stains were performed on all of the isolates so that the appropriate Biolog plates can be used to initially identify the isolates, at least to the genus level. Beginning in the spring these isolates will be pin-prick inoculated into grapevines growing in the greenhouse. After several weeks, attempts will be made to recover the bacteria from sections of xylem that are 2 or 3cm from the point of inoculation. Any bacteria that are found to systemically colonize grapevine xylem in reasonable concentrations will be tested for natural antagonism towards *Xf*. We are now screening a random peptide library for synthetic peptides that are inhibitory to *Xf*. If these peptides are identified, attempts will be made to genetically engineer grape endophytes to express these peptides within grapevine xylem.

Objective 6: Genetics of Resistance to *Xylella fastidiosa* (Walker)

A Design II mating design with a set of 6 females by a set of 6 male parents, from which we will select sets of seedlings to study the inheritance of *Xf* resistance, is completed. The mean expression of resistance within a given female across all males and similar comparisons of males across females will allow us to draw conclusions about the inheritance of *Xf* resistance.

We define *Xf* resistance as the ability of a genotype to limit the movement of *Xf*, particularly in a downward direction, and have tested a set of resistant and susceptible individuals to determine whether this definition is valid. The known susceptible genotypes were *V. rupestris* 'A. de Serres' (the female parent of the 89 population), Chardonnay and the *V. rupestris* x *M rotundifolia* genotype 8909-19, potentially resistant genotypes were 8909-04 and 8909-11, and the resistant genotypes were 890915 and 890917. After 4 weeks, *Xf* was easily detected in the three susceptible genotypes. By 16 weeks, the differences among resistant and susceptible genotypes are very clear in terms of both symptom expression in leaves and unevenly lignified stems and ELISA readings. ELISA is cheaper, faster, quantifiable and more simple than PCR detection of *Xf*, and can reliably detect 10,000 cfu/ml of *Xf* in ground plant sap. 150 samples with duplicate readings can be processed in a day at a material cost of $0.28 per plant sample. IC-PCR detection is 10 more sensitive, but is not quantifiable and costs about $1.44 per sample. Nested PCR gave very good results and is 100 times more sensitive than IC-PCR, but few samples can be run per day, and the cost of materials and labor is high ($2.88 per sample). We also examined spot-PCR which would allow several hundred samples to be run per day, but the sensitivity is equivalent to ELISA and cost $2.70 per sample. (All costs exclude labor).

We are mapping *Xf* resistance in the *V. rupestris* X *M. rotundifolia* 9621 population (previously used for *Xiphinema index* resistance). We have AFLP marker data on about 70 of the 150 individuals and are testing 4 replicates from each of these 150. *Xf* resistance data from 70 individuals are complete and the others are due for analysis in about 4 months. We plan to place *Xf* resistance on our existing AFLP based map. If the resistance trait does not place on the existing map, we have seeds for a second mapping population based on a cross of the 8909-15 X 8909-19, a resistant by susceptible genotype which will allow greater power in mapping the resistance genes. However, this seedling population will have to be grown out from seed, propagated and screened for resistance and marker information. We also have (8909-08 and 8909-23) to Chardonnay, which should also segregate widely.

We bench-grafted Chardonnay on each of the following rootstocks: AXR#1, St. George, 3309C, 101-14Mgt, Schwarzmann, 44-53 Malegue, Riparia Gloire, 1616C, Lenoir, 5BB, 5C, Börner, 110R, 1103P, Harmony, Freedom, and Ramsey, and Chardonnay (control), Riesling, Sylvaner, Chenin blanc, and Colombard. Own-rooted Chardonnay, Riesling, Sylvaner, Chenin blanc and Colombard were also inoculated. We have evaluated all three reps for PD expression and ELISA sampling to determine the extent of downward movement is continuing. The vines were cut back to basal buds, are now re-growing and will once again be evaluated for symptoms and by ELISA.

Objective 7: Determine whether xylem chemical composition is involved with PD resistance or susceptiblity in grape varieties and common *Xylella fastidiosa* plant hosts (Andersen)

In an effort to determine whether xylem fluid chemistry is related to resistance to *Xylella* we investigated the chemistry of *Vitis* genotypes covering a wide range of resistance/tolerance. Xylem fluid chemistry of 10 grape genotypes belonging to 5 species has been characterized, although only the amino acid data was presented. Total amino acids varied over 4-fold, and many amino acids only occurred in less than 10uM or trace quantities. A complete correlation with resistance will await compilation of organic acid and sugar data. Total amino acids in xylem fluid collected from Chardonnay grafted on 4 different rootstocks showed an effect of rootstock and of infection with *Xf*. Xylem fluid of Chardonnay on 3309 rootstock tended to be most dilute of the rootstocks and *Xf* infection increased total amino acids in xylem fluid of Chardonnay on all rootstocks except SO4. Arginine was the amino acid that showed the biggest increase in amino acids with *Xf* infection. A more complete analysis of the effect of xylem chemistry of grape genotypes on resistance to *Xf* and the change in chemistry with *Xf* infection awaits further research.

An investigation is underway to determine whether resistance to *Xf* is influenced by xylem fluid chemistry across plant species common to both Florida and California. Xylem fluid chemistry of 32 host plants species/cultivars was analyzed and natural *Xf* (resistant/susceptible) was noted by PCR analysis. About 50% of the plant species/cultivars were *Xylella* negative. Those that were *Xylella*-negative were mechanically inoculated with *Xf* during the fall of 1999. After 4 weeks only one species was positive, *Vitis rotundifolia* (wild grape). Each of the *Xylella* negative species/cultivars have also been sampled fall 2000 and are currently being analyzed via PCR. Those species that still do not harbor *Xylella* may be inoculated with different strains for confirmation of resistance to multiple *Xylella* strains.

In vitro nutrient requirements of *Xylella* have been studied for 3 months without much success. A UCLA strain of *Xf* was subjected to 3 months of experimentation using Chang and Donaldson's chemically defined media. This UCLA strain grew well on PD or PW+ but did not grow on the chemically defined media. We switched strains to ATCC 35881 and tested this strain on all the media. The result was that it grew well on all the media and it even grew slowly on a media consisting of glutamine as the only amino acid source. Thus, nutritional fastidiousness is extremely strain dependent. Additional experiments will be performed with a different strain (probably the Temecula strain) after consultation with B. Kirkpatrick, S. Purcell and A. Walker.

Lytic peptides were extremely effective against *Xylella*. They act by disrupting cell membrane integrity of bacteria but not eucaryotic cells of higher animals. Cecropins were one to three orders more effective than tetracycline. *Xf* incubated with Cecropin A and B at 1 uM resulted in 100% inhibition of growth; incubation with *Xylella* at 0.1 uM resulted in 95% inhibition. Indolicidin was a fairly strong inhibitor of *Xf* with 100% inhibition at 9.5 uM. Magainin II was followed by Magainin I in potency. For tetracycline 100% inhibition was achieved at 112 uM. These compounds may serve as a potent naturally occurring pesticide against *Xylella*.

CDFA Objective 1: Determine the effects of lethal and sub-lethal doses of insecticides on insect transmission of *Xylella fastidiosa* to plants (Purcell)

Preliminary transmission tests to characterize GWSS' transmission of *Xf* to grape suggested that some adult GWSS were very poor transmitters. We are investigating several unidentified bacteria isolated from plants and sharpshooters as possible antagonists to *Xf* transmission by GWSS. Experiments confirmed the prediction that molting stops GWSS' transmission of *Xf*. GWSS experimentally transmitted *Xf* to woody stems (scaly bark) of grapevines at rates estimated at 6% per insect per day, while GWSS exposed to green shoots of the same aged vines transmitted at rates of about 7%.

Doses of the systemic insecticide imidacloprid applied to soil (Admire^{R}) that killed from 40% to 60% of GWSS on potted grape within 24 hours or smaller, "sub-lethal dosages" drastically reduced sharpshooter feeding and movements. The efficacy of all dosages mimicking field rates of Admire peaked 3 weeks after application, then decreased. Lab experiments

with GWSS having a choice or no-choice of treated or untreated grape produced no evidence that imidacloprid repelled GWSS, except at perhaps extremely high doses not attained with full label rates of application. Sub-lethal or slightly toxic doses prolonged GWSS survival compared to untreated controls but greatly reduced the ability of GWSS to jump or fly. Experiments to test the effects of Admire treatments on GWSS of *Xf* were inconclusive because of low rates of transmission of *Xf* to untreated controls.

CDFA Objective 2: Evaluate potential *Xylella fastidiosa* bactericides and developments to introduce these materials into grapevines (Kirkpatrick)

Plant microelements such as zinc, copper, manganese and iron, as well as three antibiotics were tested for inhibition against *Xf* in vitro. Tetracycline was the most effective antibiotic and zinc was the most toxic microelement. Both prophylactic and therapeutic field plots were established in Napa and Temecula vineyards during 1999 and 2000. Prophylactic materials being evaluated included 3 inducers of systemic acquired resistance (SAR) and 4 microelement formulations. Therapeutic materials include several formulations of microelements and 2 antibiotics. All prophylactic field plots were mapped for PD each fall, however no new infections were found in either the treated or control vines in 2000. Bactericides were applied as foliar sprays, as materials packed into hollow nylon DP screws or in drilled holes packed with bactericides that were suspended in agarose and the ends of the holes were sealed with DP screws. Several of the drilled through/DP screw treated vines did not show any PD symptoms following treatment, however these vines were also severely pruned following treatment. We know from Purcell's work that severe pruning can produce vigorous growth in the season following pruning but many of these vines later develop PD in the second year. Thus final assessment of the efficacy of these bactericide treated vines will be made in the summer of 2001. An injection machine that is widely used for injecting avocado trees was found to work well for injecting vines in the spring but less effective in the fall. Several potted plant experiments were performed using soil drenches of microelements as therapeutic or prophylactic bactericides. Although significant phytoxicity occurred with some of the materials, managanese and zinc treatments may have some potential. A custom-made pressure bomb was purchased and used to express xylem sap from 1-meter long grapevine canes that were treated with various microelements. The expressed xylem sap was analyzed for microelement concentrations with the assistance of Peter Andersen. Surprisingly high concentrations of zinc and managanese were found in the xylem sap of grapevines treated with amino acid chelates of these elements. Additional experiments are now being done to determine whether the xylem sap is actually toxic to *Xf* or if the ions are too tightly bound.