XYLEM CHEMISTRY MEDIATION OF RESISTANCE TO PIERCE'S DISEASE

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INTRODUCTION

Xylella fastidiosa (*Xf*) is a Gram-negative xylem-limited bacterium that causes Pierce's disease (PD), plum leaf scald, almond leaf scorch, phony peach disease and many other diseases. For many plant species there is no resistant germplasm. Xylem vessels may be filled with exopolysaccharides produced by the bacterium, and pectins, gums and tyloses produced by the plant. There has been considerable interest in the production of exopolysaccharides as a component of disease symptomatology, whereas the formation of gums and tyloses has sometimes been considered an adaptive host plant response to infection (Fry and Milholland 1990). The resultant plant/bacterium interaction between bacterium and plants results in xylem dysfunction, water stress and leaf necrosis, which are characteristic of PD.

Cell multiplication, formation of aggregates and biofilm may be early components of PD. The stimuli for cell multiplication and the formation of aggregates and biofilm may involve specific plant/bacterium interactions and may involve the plant nutrient status of xylem fluid. With the recent development of simple chemically-defined media for *Xf*, it is possible to study the effects of nutrition *in vitro*. We have found that certain chemically-defined media (3G10R and CHARD2) developed in our laboratory promote the development of aggregates and biofilm. The chemistry of xylem fluid typically consists of 95-98% water; amino acids, organic acids, sugars and inorganic ions are the major components of total osmolality. Chemical profiles developed from xylem chemistry of *Vitis* spp. are also being used to test whether xylem chemistry, and specifically xylem nutrient status is related to PD-resistance/tolerance. The chemistry of xylem fluid may be a function of temperature, fertilization and diurnal/temporal alterations (Andersen and Brodbeck 1989 a b, 1991, Andersen et al., 1995). The manipulation of xylem chemistry, whether it is based on the primary compounds or proteins in xylem fluid, is one possible method to affect PD-resistance. An alternative would be the development of transgenic plants with genes encoding for the production of lytic peptides. Transgenic *V. vinifera* cv. Thompson Seedless grapevines have been developed (Scorza et al. 1996) and the technology for the production of these plants has been patented (Scorza and Gray 2001).

A hypothetical model was proposed to explain how *Xf* adheres to xylem vessels (Leite et al., 2002). In this model, a presumed negatively charged surface of *Xf* could be attributed to the presence of sulfur in outer membrane proteins (OMPs). Interaction between bacteria and the formation of aggregates can be facilitated by the formation of disulfide bonds between OMPs. Adhesion may occur between the bacterial cell surface and the negatively charged entities of the xylem vessel wall. Adhesion, in this hypothetical model, may possibly result from the interaction of this negatively charged xylem walls to negatively charged bacterial cell surface via calcium and magnesium bridges (Leite et al., 2002). The presence of *Xf* OMPs with sulfur-containing amino acids (cysteine and methionine) residues with domains localized in the outer membrane region, are being investigated.

OBJECTIVES

1. Determine the relationship between xylem chemistry and resistance of grape *Vitis* spp genotypes to PD after mechanical inoculation with *Xf*.
2. Examine the influence of nutrients on colony number, optical density, aggregation and biofilm formation of *Xf*.
3. In-vitro experiments with cecropin B to evaluate the efficacy against *Xf* and study of stability of this lytic peptide in artificial media and in xylem fluid.
4. Determine the influence of *Xf* cell surface chemistry during the early stages of *Xf* aggregation and biofilm formation.

RESULTS AND CONCLUSIONS

Mechanical inoculation of vines with *Xf* UCLA PD strain during the summer of 2002 produced an average 62 % inoculation efficiency as determined by PCR. Inoculation efficiency varied between 40 and 89% on ten different grape genotypes; however there was no consistent relationship between the known susceptibility of each grape genotype to *Xf* and percentage inoculation success. The percentage of plants positive for *Xf* was much reduced (0 to 43 %) after the winter 2002/2003, perhaps as a result of low winter temperatures (15-20 F). The percentage PCR positive was highest for *V. vinifera* cv. Chenin blanc. We have sampled xylem fluid to test whether or not chemistry is affected by the presence of *Xf*.

Chemically-defined media were developed from the chemistry of xylem fluid (CHARD2 and 3G10-R) (Figure 1). The utility of these xylem chemistry-based media was examined for the PD strains UCLA and STL. All new media were compared to

XF-26 (Chang and Donaldson, 1993) and two genomics-based media (Lemos et al., 2003). The formulation of CHARD2 and 3G10-R were based on the chemistry of the xylem fluid of a PD-susceptible grape genotype *Vitis vinifera* cv. Chardonnay. We have found that growth of *Xf* requires only one amino acid and a much smaller number of compounds than in currently available chemically-defined media. Aggregation, colony size and biofilm formation of *Xf* was mediated by nutrients in the growth media. Our results support the contention that the chemistry of media (or xylem fluid) can greatly affect the behavior of *Xf*. Liquid media of CHARD2 and 3G10-R increased the capacity of *Xf* to form cell aggregates and biofilm, reducing the number of cells in the planktonic state (Figure 1).

Colony growth of two strains of *Xf* (UCLA-PD and STL-PD) after 48 hours of incubation in xylem fluid was determined for *V. rotundifolia* (Michx). cv. Noble (PD-resistant) and *V. vinifera* L. cv. Chardonnay (PD-susceptible). The concentration of total amino acids in xylem fluid was 3.1 to 3.7, and 1.0 to 1.8 mM for *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay, respectively; glutamine generally accounted for more than 80 % of the profile for both species. Number of *Xf* colony forming units was promoted or inhibited depending on the interaction of *Xf* strain *Vitis* species, source of xylem fluid. Since no treatment completely inhibited colony growth, the bacteriocidal effect of xylem fluid plus lytic peptide was tested. The minimum inhibitory concentration (MIC) for 100% inhibition of *Xf* in agar culture was: cecropin A or B $\leq$ 1 μM, indolicidin 9.5-47.0 μM, magainin II > 80 μM, tetracycline $\leq$ 100 μM and lysozyme > 1000 μM. The activity of cecropin B in xylem fluid of *V. rotundifolia* cv. Noble was progressively reduced with time. There was a substantial amount of bacterial growth at 2 or 10 μM concentrations of cecropin A and cecropin B only after 24 hour duration of cecropin and xylem fluid; shorter time intervals did not degrade the cercopins and kill the bacteria. Cecropin B was less efficient in killing large-sized colonies compared to small colonies (Figure 2). This result suggested that *Xf* cell aggregates may serve as a protective mechanism, whereby external cells are attacked by cecropin B while the internal cells are protected. Tricine SDS-PAGE gel electrophoresis of cecropin B (10 μM) in xylem fluid of *V. vinifera* cv. Chardonnay showed that cecropin B degraded substantially and completely after 96 hours in xylem fluid (Figure 3). Similar result was obtained when cecropin B was exposed to protein phase of xylem fluid. Treatments with cecropin B (10 μM) with the filtrate phase did not show a reduction in the intensity of the cecropin B band after 24 hours, and it was still detected after 96 hours. No degradation of cecropin B was observed (even after 96 hours) when mixed with xylem fluid that had been previously boiled (100 ºC) suggesting that xylem fluid proteins were denatured.

The presence of OMPs of *Xf* with sulfur-containing amino acids was investigated. We verified the presence of OMPs with sulfur-containing amino acids by SDS-PAGE gel using specific dye for thiol groups, 5-iodoacetamidofluorescein (IAF) (Figure 4). Different strains of *Xf* from different hosts showed distinct patterns of OMPs with sulfur-containing amino acids. The results indicate the presence of thiol groups in several OMPs of *Xf*. In this SDS-PAGE gel, serum bovine albumin and the molecular mass marker were not labeled by IAF, indicating the specificity of dye by sulfur residues.

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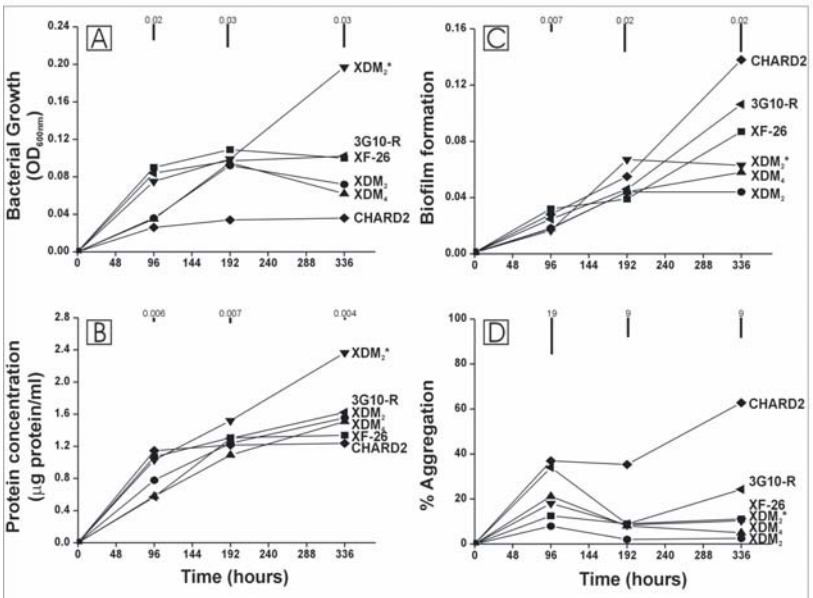

Figure 1 – Growth curves of *Xylella fastidiosa* in chemically-defined media over a period 336 hours at 28ºC. A - OD_{600nm}; B- Protein concentration; C - Biofilm formation and D – Percentage of aggregation. Bars above each data point correspond to lsd, 0.05.

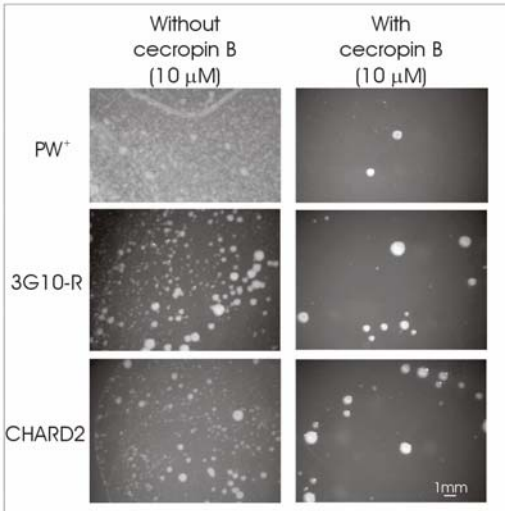

Figure 2 : Colony size of *Xylella fastidiosa* after incubation in cecropin B (10 µM) for 1 hour . Before the treatments *X. fastidiosa* cells were grown in liquid 3G10-R, CHARD2 and PW+ media We assumed that planktonic cells or small-sized aggregates would generate small-sized colonies (0.1mm in diameter); large-aggregates would promote large-sized colonies (>0.1 mm). Bar= 1 mm.

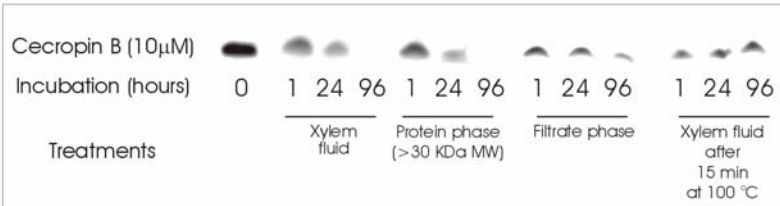

Figure 3: Tricine -SDS-PAGE of lytic peptide after incubation in xylem fluid from *Vitis vinifera* cv. Chardonnay. Cecropin B (10 µM) treated with xylem fluid, protein phase (components of xylem fluid with >30 KDa MW), filtrate phase (components of xylem fluid with <30 KDa MW) and boiled xylem fluid at 0, 1, 24 and 96 h of incubation. Lane MW corresponds to standard molecular weight: myosin (120 KDa), BSA (84 KDa), ovalbumin (60 KDa), carbonic anhydrase (39 KDa), trypsin inhibitor (28 KDa) and lysozyme (18 KDa).

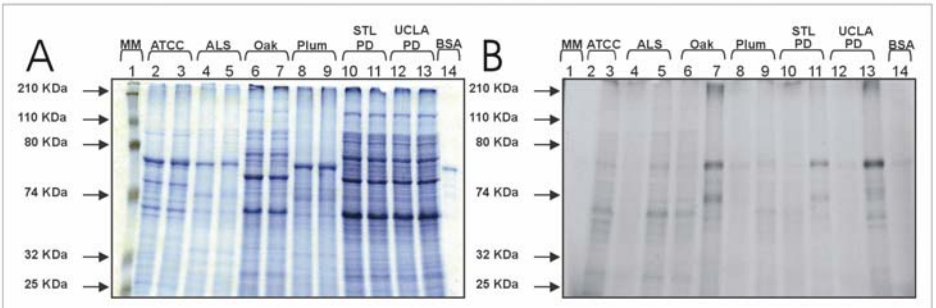

Figure 4: SDS -PAGE gels of surface proteins from different strains of *Xylella fastidiosa* : (A) Gel stained with Coomassie brilliant blue of proteins obtained after cell lysis (lanes 2-13). (B) Proteins isolated from the 5 -iodoacetamidofluorescein (5 -IAF)-labeled cells (lanes 2, 4, 6, 8, 10, and 12) and after treatment with dithiotheitrol (DTT) (lanes 3, 5, 7, 9, 11, and 13). 5-IAF labels sulfur containing amino acids in outer membrane proteins. DTT cleaves disulfide bonds of outer membrane proteins. Notice that proteins bands displayed represent proteins that were localized outside of *X. fastidiosa* cells (facing the environment). Bovine serum albumine (BSA) was used as control (lane14). Prestained molecular mass markers are shown on first lane (MM).