MAGNETIC RESONANCE IMAGING: A NON-DESTRUCTIVE APPROACH FOR DETECTION OF XYLEM BLOCKAGES IN *XYLELLA FASTIDIOSA* INFECTED GRAPEVINES

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ABSTRACT
It is conventionally thought that multiplication of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*) within xylem vessels is the sole factor responsible for the blockage of water movement in grapevines (*Vitis vinifera* L.) affected by Pierce's disease (PD). However, results from our studies have provided substantial support for the idea that vessel obstructions, and likely other aspects of the PD syndrome, result from the grapevine's active responses to the presence of *Xf*, rather than to the direct action of the bacterium. The use of magnetic resonance imaging (^{1}H-MRI) to observe the distribution of water within the xylem has allowed us to test the role of the plant hormone ethylene in promoting xylem obstruction development, and the consequent reduction in vine water transport. In both infected and ethylene-exposed plants, MRI shows that an important proportion of the xylem vessels become progressively air-embolized (the magnetic signal is lost from these vessels) after the treatments. The loss of xylem water-transporting function, assessed by MRI, has been also correlated with a decrease in stem specific hydraulic conductivities (K_S) and the presence of tyloses in the lumen of water conduits. We propose that ethylene may be involved in a series of cellular events that allows the plant to sense the presence of *Xf* and stimulates a plant response that includes the production of tyloses and gels, perhaps in an effort to slow systemic movement of the bacteria.

INTRODUCTION
Results from PD research programs led by Matthews, Rost and Labavitch (reported in 2001, 2002, 2003 and 2004 in San Diego) support the idea that obstructions in the vine's water-transporting xylem tissue develop rapidly post-inoculation, before an appreciable bacterial population has been established. Thus, careful analysis of the timing of changes in xylem element anatomy and function relative to *Xf* introduction, as well as to external symptoms of disease development, is important for establishing reliable indicators of the "stage" of PD development. The analyses done thus far have been based on destructive tissue sampling. Such sampling can be particularly "blind" when it is done on vines in which (based on our earlier results) internal PD symptoms are present but external, visible symptoms, are not yet present. In the report of the year 1 work of our study (Shackel and Labavitch, 2003), the success of Mr. Pérez and Dr. Walton in imaging non-functional vessels in the stems of PD-infected and ethylene-treated grapevine stems was demonstrated. In this report we elaborate on those studies, showing the correlation of reduced vine water transport capacity with the locations of PD and ethylene effects on vessel functionality, as determined by MRI analysis. In addition, because interpretation of the true meaning of the MRIs in relationship to the anatomy and functioning of vessels is a crucial aspect of our work, we have described the approach that we are taking to efficiently derive information about the extent of changes in xylem water conducting capacity that can be deduced from MRIs. Our experimental work is organized around the hypothetical model for the development of the disease described in Figure 1.

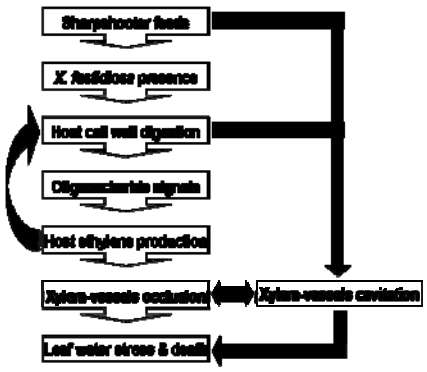


Figure 1. Hypothetical model for PD development. PD starts with infection caused by the glassy-winged sharpshooter's introduction of *Xf* locally (i.e., into one or a few vessels). Once *Xf* is in the xylem the bacteria become systemic, which implies that *Xf* must be able to cross (digest away?) the cell wall in the pit membranes that separate two neighboring vessels. The digestion of the cell wall by bacterial enzymes would generate transient oligosaccharides with biological activity. The presence of these oligosaccharides is detected by the plant triggering a series of defensive responses, including a raise in ethylene production. Ethylene has been shown to induce tylose formation. Cavitation of vessels may be also important for the disruption of water transport in the plant. Cavitations may happen during insect feeding or during PD progression. The "bottom line" of our thinking is that PD is primarily caused by the grapevine's responses (local and systemic) to *Xf* presence.

OBJECTIVES

1. Optimize the use of MRI (Magnetic Resonance Imaging) and to spatially visualize altered water movement in grapevines.
2. Test correlations of observed vascular system obstructions (based on grapevine dissection and microscopy techniques) with predictions based on MRI data.
3. Use MRI to follow the development of grapevine obstructions over time in vines infected with *Xf* or treated with ethylene, bacterial wall-degrading enzymes or plant cell wall oligosaccharides, all of which may be important intermediates in regulating the vine's response to infection and the eventual development of PD symptoms.
4. Use NMR imaging to determine whether localized xylem cavitation occurs at the site and time of *Xf* inoculation or introduction by the glassy-winged sharpshooter.

RESULTS

Objective 1: Optimization of MRI for visualizing water transport deficiencies in PD-infected grapevines.

Magnetic Resonance Imaging (MRI) allows us to visualize, non-destructively, vessels that are functional and full of movable water. Functional vessels appear as small bright circles in an MRI view of the stem cross-section; non-functional vessels lack water and appear as dark spots in the area of the stem where water conduits are found (the magnetic signal is lost from cavitated vessels). Cavitation of xylem vessels is of potential importance in PD development. Our analysis can reveal vessels that have cavitated. In Figure 2 we can see the presence of functional vessels in an intact stem, empty vessels after the stem is severed to cause cavitation, and the re-filling of cavitated vessel with pressurized water. On the other hand, MRI can make no clear distinction between pure water, a saline solution (KCl), and a pectin gel. Figure 3 shows that their signal intensities are quite similar. This suggests that MRI cannot differentiate between vessels filled with regular xylem sap and vessels filled with pectic materials from plant gels and tyloses.

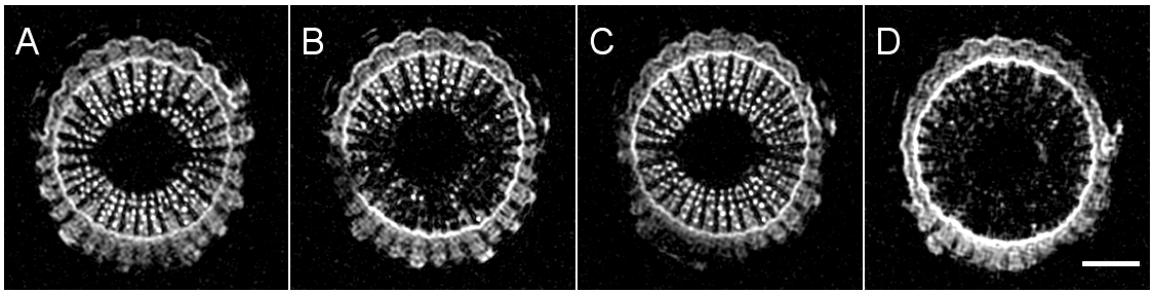


Figure 2. (A) MRI of an intact stem segment in a healthy shoot. (B) Image of the same stem portion after an important part of the cross section below has been severed, thus causing cavitation of many vessels. (C) The same stem segment after it has been refilled with water. (D) Stem segment after flushing with air to completely empty the xylem vessels. Scale bar = 1 mm.

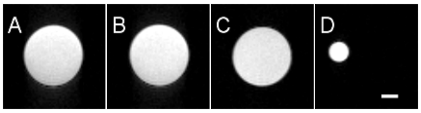
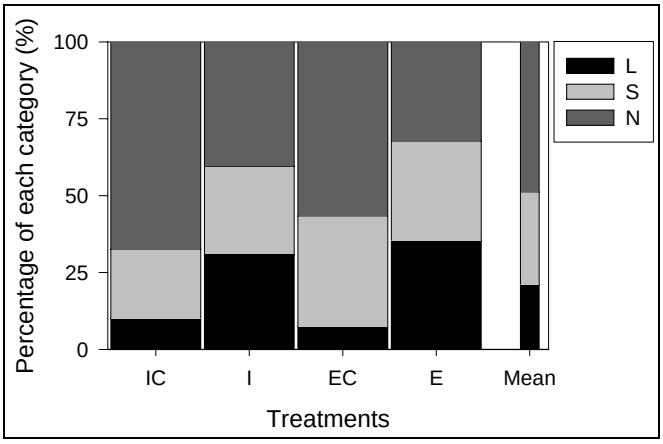


Figure 3. Glass tubes containing either (A) distilled water, (B) 10 mM KCl solution, or (C) a pectin gel, were put in the magnet at the same time and imaged. (D) Small glass capillary filled with a pectin gel next to an empty glass tube that does not appear in the image. The signal intensities ± 1SD were 195.4 ± 15.2, 202.8 ± 12.2, and 196.1 ± 4.4 for A, B, and C, respectively. Scale bar = 1 mm.

MRI has been used to assess the xylem function of control (buffer-inoculated) and infected (*Xf*-inoculated) vines up to seven months after treatment. MRIs of the control vines show well defined xylem, in which individual vessels can be clearly observed. As in previous experiments, stem cross section MRIs of infected plants show that major sectors of the xylem appear dark, indicating that they are no longer water-filled. Furthermore, MRIs of plants infected with *Xf* become less sharp, making it more difficult to discriminate structure, particularly of individual, probably still functional, vessels (see also results for Objectives 2 and 3). Efforts to explain this will be a feature of the work as this project continues. MRI also has been used to follow changes in the functionality of the xylem of plants exposed to ethylene in enclosed chambers (10 mg L^{-1} of air for 48 hours). This experiment has allowed us to confirm that, after seven months of exposure to ethylene, gassed plants show progressive xylem disruption along the stem (see also results for Objective 3). The images taken along the vines in these experiments were classified in three categories. If the xylem disc appeared full of bright vessel, the image was categorized as "normal" (N). However, if the image was showing one or few small dark spots, in which a few vessels were missing, the image was categorized as "small" (S). If one or more evident dark spots were present, compromising an important area of the xylem, the image was categorized as "large" (L). The Likelihood ratio (Chi-square) test was used to analyze the distribution of the proportions of each category (Figure 4) across the treatments. Inoculated and ethylene-treated vines showed a higher proportion of the "L" category and a reduction in the proportion of the "N" category compared with the controls. The results of the test indicate that there is a significantly

different distribution in the proportion of each category (p = 0.0002) among the group treatments (Figure 4). A correspondence analysis (Figure 5) confirmed that inoculated and ethylene-treated vines are more closely associated with the presence of "large" dark spots in the xylem; whereas the control groups are clearly associated with "normal"-looking MRIs.

Figure 4. The mosaic plot depicts the percentages of the image categories normal (N), small (S), and large (L) for each treatment. The treatments were labeled IC (control for inoculation), I (inoculated), EC (control for ethylene) and E (ethylene-gassed). Treatments E and I show a higher percentage of "L" and a lower percentage of "N" than the controls. The narrow bar to the right is the mean category percentage across all treatments. The distribution of the categories proportions for the treatments was analyzed using the Likelihood Ratio Chi-square test, which concluded that the treatments have different pattern distributions (*p*=0.0002). Sampling size was 49, 42, 53 and 59 internode images for IC, I, EC and E respectively.



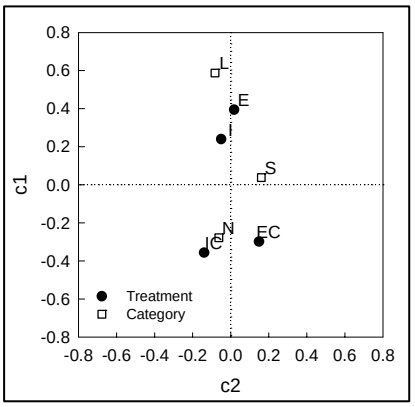


Figure 5. The Correspondence Analysis for the proportions of categories (N, S, and L) across treatments (E, EC, I, and IC) indicates that most of the variation (92%) of the response variable (category) happens in the c1-axis. L, S and N categories align respectively from the positive (top) to the negative range of c1-axis, establishing the directionality of the response. Thus the treatments located in the c1-axis positive range (E and I) are associated with the "L" category, and treatments in the negative range (EC and IC) are associated with the "N" category. The c2-axis explains only about 8% of the variability in the category variable. "L" and "N" are located fairly neutrally in the c2-axis whereas "S" locates in the positive range. Thus treatments in c2-axis positive range, like EC, are more associated to the "S" category.

Objective 2: To test for correlations of observed vascular system obstructions with predictions based on MRI data
MRI is capable of showing xylem disruption and non-functional vessels well before external symptoms appear in infected plants. Dark spots, indicative of vessel embolisms, can be observed in an image of an infected vine at a basal internode where leaf symptoms of PD are apparent (Figure 6A). Closer to the stem apex, at a point where the leaves show no sign of PD symptoms, MRI can also reveal the presence of extensive cavitations in the xylem (Figure 6B). Compare these images with that for a healthy vine (Figure 2A) in which the xylem appears as a full disc of bright vessels. Conventional (destructive) optic microscopy of stem sections has shown that dark spots seen with MRI are frequently associated with the presence of tyloses (Figure 7) and gels (Figure 8) filling the lumen of the water conduits.

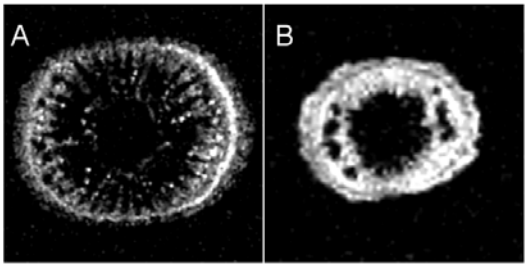


Figure 6. MRI of a PD-infected stem (A) in a basal internode and (B) closer to the apex. Bright spots between the central pith (dark) and the ring of vascular cambium show functional vessels. (B) Dark pockets within the vascular tissue indicate areas in which vessels are not water-filled (compare the image to the healthy stem in Fig. 2A).

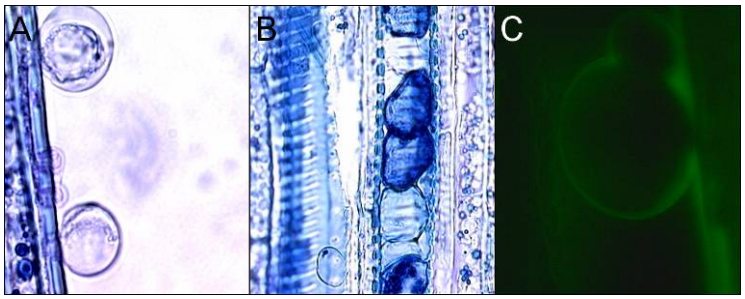


Figure 7. Tyloses are balloon-like outgrowths from living parenchyma cells that expand into adjacent vessels and permanently plug them. Tyloses are often associated with dark spots in MRIs of infected and ethylene-exposed vines. (A) New tyloses bulging into a vessel from neighboring xylem ray parenchyma (100X). (B) Tyloses can completely fill the vessel lumen (40X). (C) The use of a green fluorescent dye (coriphosphin O) allows visualization of the pectic nature of tyloses' newly synthesized cell wall (100X).

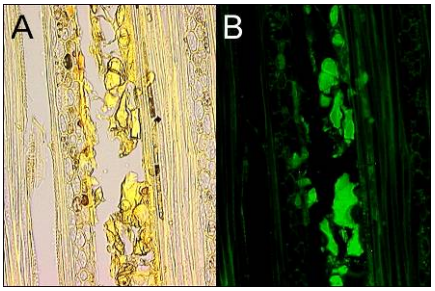


Figure 8. (A) Vessels of infected and ethylene-exposed vines are also filled with amorphous materials (10X). (B) This amorphous material stains intensely with coriphosphin O, which is a strong indication that they probably correspond to pectin gels (10X).

The impression of a loss in xylem function that is given by the MRIs of *Xf*-inoculated and ethylene-gassed vines should indicate that there will be a decrease in the hydraulic conductivity of internodes (K_S) (Figure 9). This is a destructive technique to measure the rate of movement of pressurized water through stem segments. Whole stems of the treated vines also showed an increase in the hydraulic resistivity (ρ_S, the reciprocal of conductivity) relative to the controls (Figure 10), although this difference was statistically significant only for the ethylene experiment. The lack of statistical difference in the inoculation experiment is mainly due to the great variability found in the hydraulic resistivity of inoculated plants. In turn, this might be explained because these vines were in a gradation of early stages of PD infection when examined (they were not showing external symptoms). While we have found a general correlation between the MRIs showing localized areas of empty vessels and reduced hydraulic conductivity in regions of infected stems, the correlations are not perfect. This is due to at least two factors that will be tested more fully in our continuing work. First, an empty vessel shown in the MRI at one level in the plant's stem could be the result of a vessel obstruction or cavitation above or below the point on the stem where the MRI observation was made. Hence, there may be no impediment to water flow in the empty vessel that is being imaged. Second, while cavitation may be an important factor in PD development, because the tests of water conductivity are carried out using water under pressure, cavitated vessels will be re-filled during the test and no reduction in water flux would be revealed.

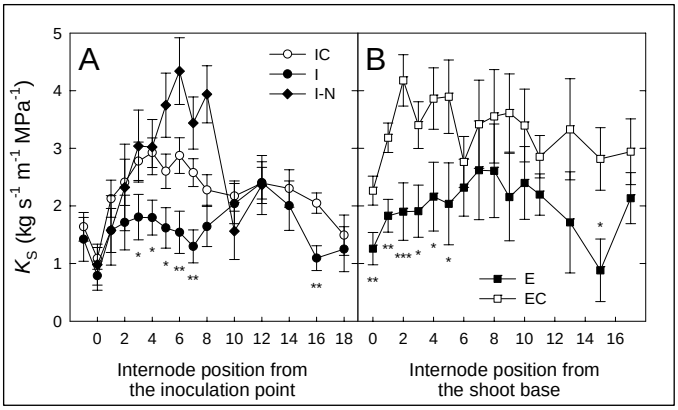


Figure 9. Specific hydraulic conductivities (K_S) for individual internodes (±1 SE) in grapevines stems. (A) *Xf*-inoculated vines showing xylem disruptions detected by MRI (I), inoculated vines with normal xylem appearance (I-N), and controls (IC). The comparison was only made between "I" and "IC". (B) Vines exposed to ethylene (E) and controls (EC). A one-sided ANOVA test was used to determine whether the treatments "I" and "E" had significantly lower means than "IC" and "EC". The symbols *, **, and *** indicate statistical significance at a given internode position with a probability inferior to 0.05, 0.01 and 0.001 respectively.

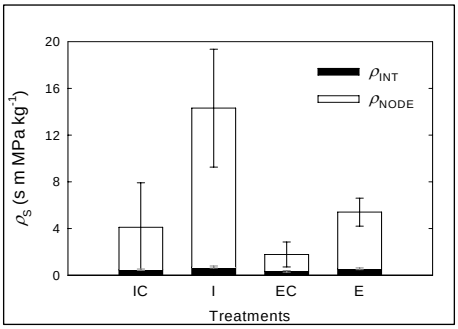


Figure 10. Whole-stem specific hydraulic resistivities (ρ_S) for vines inoculated with *Xf* and exposed to ethylene. Total bar height represents ρ_S ± 1 SE (in black). ρ_S components, ρ_{NODE} and ρ_{INT}, are also shown (± 1 SE in gray). The nodes are a major component of ρ_S (the reciprocal of conductivity). It can be noted that ρ_S is about 3-fold higher for stems of infected plants than for controls, even when infected plants have no external symptoms. This observation agrees with the information provided by MRI.

Objective 3: Use of MRI to follow the development of grapevine obstructions over time in vines infected with Xf or treated with ethylene.

MRI has confirmed that the dark sectors in the xylem of inoculated (Figure 11) and ethylene-gassed grapevines (Figure 12) which are found after seven months of treatment, start to develop gradually, progressively increasing in size after imposing the treatments. Initial signs of embolisms in the xylem can be seen 20 to 50 days after treating the vines, as can be seen in Figure 12, which shows two internodes in independent experiments, imaged over a period of about 40 or 60 days after treatment with ethylene. We expect to perform similar experiments to test the proposed role for cell wall-degrading enzymes and oligosaccharides as regulators of the plant response to PD infection.

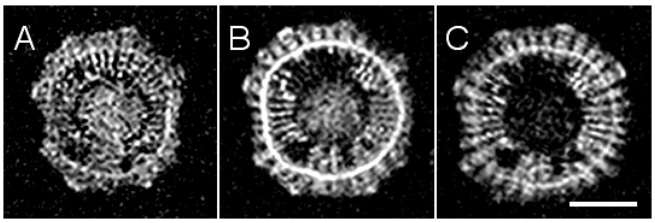


Figure 11. Temporal image sequence of a *Xf*-inoculated vine. Images were taken at the same internode (A) 18, (B) 54, and (C) 97 days after inoculation (September 2003). The progressive development of dark spots due to the presence of embolized vessels is clear from A to C. Scale bar = 1 mm.

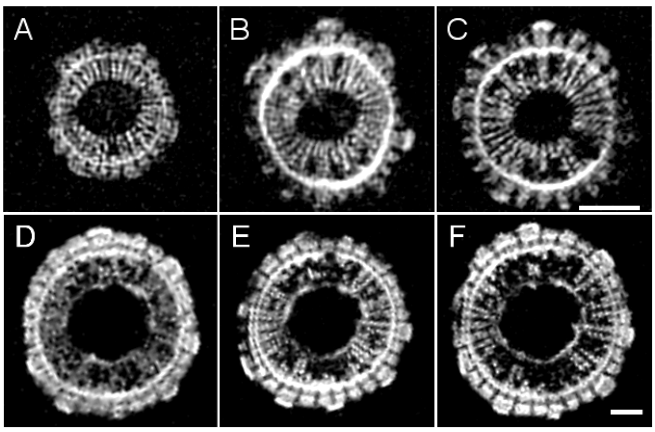


Figure 12. Temporal image sequences for two vines from two different ethylene-gassing experiments (A to C and D to F). Images were taken at the same internodes (A) 19, (B) 47, and (C) 61 days after treatment (September 2003); and (D) 10, (E) 19, and (F) 39 days after treatment (January 2003). The progressive development of dark spots due to the presence of embolized vessels is clear from left to right in both image sequences. Scale bars = 1 mm.

Objective 4: Use of MRI to determine whether localized xylem cavitation occurs at the site and time of Xf inoculation or introduction by the glassy-winged sharpshooter.

Both control-inoculated and *Xf*-inoculated vines show cavitated sectors in the xylem at the inoculation point, even seven months after treatment (Figure 13). Inoculation-related cavitations can be seen up to two internodes above the inoculation site. We have started the use of a glass micro-capillary probe (similar in size to the Sharpshooter's stylet) to mimic insect feeding, and we started studies of real insect feeding during 2005, in collaboration with Dr. Elaine Backus' group.

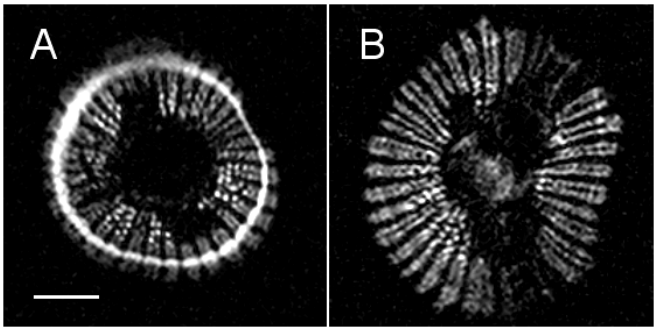


Figure 13. Images taken at the inoculation sites of a (A) buffer-inoculated and a (B) *Xf*-inoculated vine. Both vines were inoculated using a syringe needle to puncture the stem throughout a droplet of "inoculum" until reaching the xylem. The difference in water potential between the xylem and the atmosphere allowed the "inoculum" to be introduced into the xylem. The cavitation of vessels associated with the inoculation event extends above and below the inoculation site; and it can be seen even seven moths after inoculation. Scale bar = 1 mm.

CONCLUSIONS

We expect that our combined approach (use of non-destructive and destructive methods) to study xylem function will determine which kind of disruption (tyloses, pectin gels, or air embolisms) exists predominantly in PD-infected stems; as well as its developmental progression during the different stages of the disease.

REFERENCES

Clearwater, M. J., and C. J. Clark. 2003. *In vivo* magnetic resonance imaging of xylem vessel contents in woody lianas. Plant, Cell and Environment 26: 1205-1214.

Fry, S. M., and R. D. Milholland. 1990. Response of resistant, tolerant and susceptible grapevine tissues to invasion by the Pierce's bacterium, *Xylella fastidiosa*. Phytopathology 80: 66-69.

Holbrook, N. M., E. T. Ahrens, M. J. Burns, and M. A. Zwieniecki. 2001. *In vivo* observation of cavitation and embolism repair using magnetic resonance imaging. Plant Physiol. 126: 27-31.

Lo Gullo, M. A., P. Trifilo, and F. Raimondo. 2000. Hydraulic architecture and water relations of *Spartium junceum* branches affected by a mycoplasm disease. Plant, Cell and Environment 23: 1079-1088.

McElrone, A. J., J. L. Sherald, and I. N. Forseth. 2003. Interactive effects of water stress and xylem-limited bacterial infection on the water relations of a host vine. J. Exp. Bot. 54: 419-430.

Melotto, E., L. C. Greve, and J. M. Labavitch. 1994. Cell wall metabolism in ripening fruit. VII. Biologically active pectin oligomers in ripening tomato (*Lycopersicon esculentum* Mill.) fruits. Plant Physiology 106: 575-581.

Newbanks, D., A. Bosch, and M. H. Zimmermann. 1983. Evidence of xylem dysfunction by embolization in Dutch elm disease. Phytopathology 73: 1060-1063.

Ridley, B. L., M. A. O'Neill, and D. Mohnen. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 57: 929-967.

Shackel K. A., and J. M. Labavitch. 2003. Magnetic resonance imaging: A non-destructive approach for detecting xylem blockages in *Xylella fastidiosa*-infected grapevines. 2003 Pierce's Disease Research Symposium. p. 33-35.

Stevenson, J. F., M. A. Matthews, L. C. Greve, J. M. Labavitch, and T. L. Rost. 2004. Grapevine susceptibility to Pierce's disease II: Progression of anatomical symptoms. American Journal of Enology and Viticulture 55: 238-245.

VanderMolen, G. E., J. M. Labavitch, L. L. Strand, and J. E. DeVay. 1983. Pathogen-induced vascular gels: ethylene as a host intermediate. Physiologia Plantarum 59: 573-580.

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