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

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## INTRODUCTION

Results from Pierce's disease (PD) research programs led by Matthews, Rost and Labavitch (reported in 2001, 2002 and 2003 in San Diego) have provided substantial support for the idea that obstructions in the vine's water-transporting xylem tissue develop rapidly post-inoculation, before an appreciable bacterial population has been established. The results also strongly suggest that these obstructions, and likely other aspects of the PD "syndrome", result from the grapevine's active responses to the presence of *X. fastidiosa* (*Xf*), rather than to direct "action" by the bacterium. 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 symptoms of PD 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 that locations of reduced vine water transport capacity, as determined by non-destructive MRI analysis, is correlated with the locations of PD and ethylene effects on vessel functionality (destructive analysis). In addition, because interpretation of the meaning of the MRIs with respect to the anatomy and functioning of vessels is a crucial aspect of our work, we have described the methodology used to validate our approach to obtaining the relevant information from the MRIs.

## 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 *X. fastidiosa* 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 *X. fastidiosa* inoculation or introduction by the glassy-winged sharpshooter.

## RESULTS

***Optimization of the Use of MRI for Visualizing Water Transport Deficiencies in PD-Infected Grapevines.***

Progress on this objective has been delayed because a supplier for a key electronic element of the new MRI probe that has been designed for use with grapevines no longer provided a key part. The parts are all now available and development of the new probe is underway. We are proceeding with the testing of aspects of the PD model using the NMR instrument in its more conventional configuration.

***MRI Will Show Non-functional Sections in the Xylem of a PD-infectedGgrapevine Stem.***

Usually the techniques to evaluate xylem function are destructive. Magnetic Resonance Imaging (MRI) allows us to visualize vessels that are functional and full of movable water. Functional vessels appear as bright spots 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-conducting cells are found. Figures 2a & 2b show the difference in the distributions of functional vessels in an infected vine at a point where leaf symptoms of PD are apparent (Figure 2a) and nearer to the stem apex at a point where the leaves show no sign of PD symptoms (Figure 2b). Compare these images with that for a healthy vine (Figure 3a). Cavitation of xylem vessels is also of

potential importance in PD development. Our analysis can reveal vessels that have cavitated. Figure 3 shows functional vessels in an intact stem, and empty vessels after the stem is severed to cause cavitation, and that cavitated vessels can be re-filled with water under pressure. When we have the optimized MRI probe we will develop a series of image sets taken along the lengths of vines at intervals following water (control) and *Xf* inoculation to give a time course of PD development. However, at this point we do not have images for a full time course.

MRI is capable of showing xylem disruption and non-functional vessels well before external symptoms appear in infected plants. Figures 4 and 5 show images for the length of control (buffer-innoculated) and infected (*X. fastidiosa*-innoculated) vines six months after inoculation. MRIs of the control-inoculated vine show defined xylem rays, in which individual vessels can be clearly observed. As in previous experiments, stem cross section MRIs of infected plants (Figure 5) show that major sectors of the xylem appear dark, indicating that they are no longer water-filled (Note: the magnetic signal is lost in cavitated vessels). Furthermore, MRIs of plants infected with *Xf* become less sharp, making it more difficult to discriminate structure, particularly of individual, probably still functional, vessels. 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 ppm for 48 hours). We previously described the progressive development in time of "dark sectors" in the xylem of ethylene-gassed, presumably indicating vessels no longer involved in water transport. This new set of experiments has allowed us to confirm that, after 6 months of exposure to ethylene, gassed plants show progressive xylem disruption along the stem (Figure 6). Most of the damage is localized close to nodes/internodes that had just developed in the stem growth tip at the time of ethylene treatment and had then expanded in the intervening six months prior to our observations. The MRIs show "dark sectors" in those internodes. These sectors decrease are less extensive in internodes below and above the internodes that were in the growth tip at the time of treatment; that is, internodes formed after the time of treatment and already partially elongated, respectively when ethylene as applied. As in *Xf*-infected plants, MRIs of ethylene-treated plants are less sharp than images of control plants (Figure 6).

The impression of a loss in xylem function that is given by the MRIs of *Xf*-inoculated and ethylene-gassed vines can be correlated with a decrease in the hydraulic conductivity of internodes. This is tested by determining the rate of movement of pressurized water through stem segments (Figure 7). Similarly, stems of treated vines showed an increase in the hydraulic resistivity (the inverse of conductivity) relative to the controls (Figure 8), 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 there is some 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. There may be no actual impediment to water flow in the empty vessel at the level at which it is being imaged. Thus, a test of water flux at the imaged level may reveal no water flux difficulty. 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. Destructive anatomical work will define which kind of vessel disruption (tylose, gel or air embolism) exists in stems with non-functional vessels as revealed by MRI.

A more quantitative analysis of the MRIs has been attempted in order to characterize objectively the presence of "dark sectors" in the images. For this purpose, the MRIs were processed and analyzed using the ImageJ program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/ij). First, the number of functional vessels ($\boldsymbol{N_V}$) was counted in the MRIs of inoculated and control vines (like the one in Figure 9a), based on the assumption that a bright (hence, water-filled) vessel was functional. Next, the xylem-cross sectional area ($\boldsymbol{A_x}$) was measured by isolating in the MRIs (Figure 9b) the ring of tissue that is usually occupied by the xylem. Then, the digital image of the xylem-ring was converted to a binary image (Figure 9c) using a built-in algorithm in ImageJ, in which all the pixels above a set grey intensity threshold are black and the pixels below this value remain white, and the functional xylem-cross sectional area ($\boldsymbol{A_f}$) was determined by measuring the black area. To confirm that the threshold area correctly estimated $A_f$,,the area of individual functional vessels was selected by hand and measured in a series of MRIs, some with clearly delimited vessel images and others with less distinct ("fuzzy") images such as those often seen when PD-infected grapevine stems are examined. The images from infected vines often do not show vessels as bright or darks spots, rather the images of individual vessels are fuzzy, making determination of vessel functional status difficult. The area of functional xylem measured manually was then correlated with the number of functional vessels (Figure 10), and with the results of the automated routine (Figure11). The regressions confirmed that both the number of functional vessels and the threshold areas depicted in the binary images, are excellent estimators of $A_f$. Preliminary results of the quantitative analysis described above, in which all the images for an individual plant were averaged; indicate that *Xf*-inoculated vines have a lower mean density of functional vessels (Table 1) than that of controls. Figures 12 and 13 show that the vessel density also correlates positively with the hydraulic conductivity for whole stems, suggesting that the visual assessment of MRIs conveys information about the actual water movement capacity of grapevine stems. Principal components ellipses ($p = 0.5$) in Figures 12 and 13 show that, in both, inoculated and control vines, the hydraulic conductivity for the whole stem is a function of the vessel density, but infected the vines tend to localize

clearly in the lower range of that response. We have shown that cavitated vessels that are air-filled can be re-filled (including restoring an image showing that they are water-filled, see Figure 3). However, attempts at refilling segments of PD-infected stems that showed "dark sectors" in the MRIs generally failed. This indicates that "dark sectors" in MRIs of infected vines are likely a sign of a relatively permanent deterioration of the water movement capacity in the stem, probably a consequence of tylose formation and/or vascular gel development.

**Table 1.** Mean values for calculated functional vessel densities in healthy and infected grapevine stems.

| | Vessel density ± 1 SE | |
|---|---|---|
| Treatment | $N_V/A_x$ | $N_V/A_f$ |
| Control | 63.03 ± 4.81 | 124.88 ±11.93 |
| *Xf*-inoculation | 49.78 ± 4.81 | 93.25 ±11.93 |

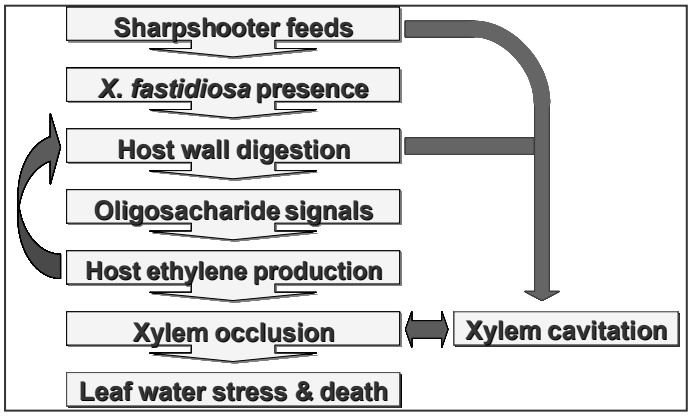



**Figure 1**. Hypothetical model for PD development. PD starts with a local infection caused by the glassy-winged sharpshooter's introduction of *Xf* locally (i.e., into one or a few vessels). Once *Xf* is in the xylem the bacteria become systemic, which implies that *Xf* must be able to cross (digest 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.

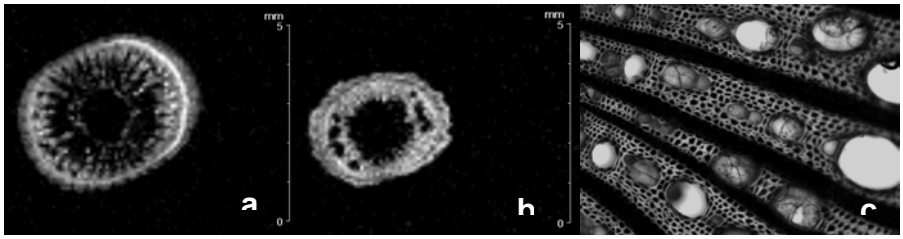



**Figure 2**. MRI of a PD-infected stem in a basal internode (a), and closer to the apex (b). Bright spots between the central pith (dark) and the ring of vascular cambium show functional vessels. Image b shows dark pockets within the vascular tissue that indicate areas in which vessels are not water-filled (compare the image to the healthy stem in Figure 3a). Tyloses (cellular-physical blockages of the vessels) are often associated with dark spots in MRIs of infected xylem, Tyloses are shown as accumulations of dark, bubble-like structures in vessel seen in the light microscope of an infected stem (c).

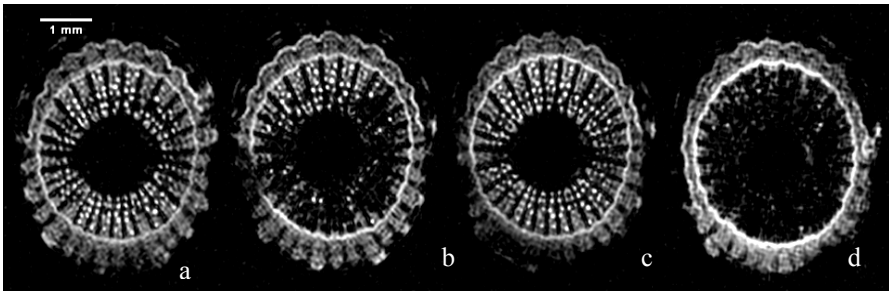



**Figure 3.** (a) MRI of an intact stem segment in a healthy shoot. (b) Image of the same stem portion after an important part of the cross section below has been severed, thus causing cavitation of many vessels. (c) The same stem segment after it has been refilled with water. (d) Stem segment after flushing with air to completely empty the xylem vessels.

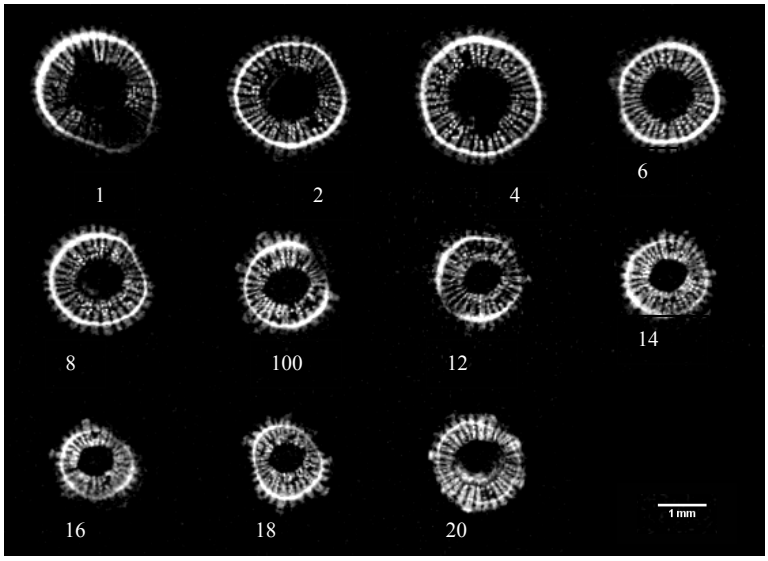
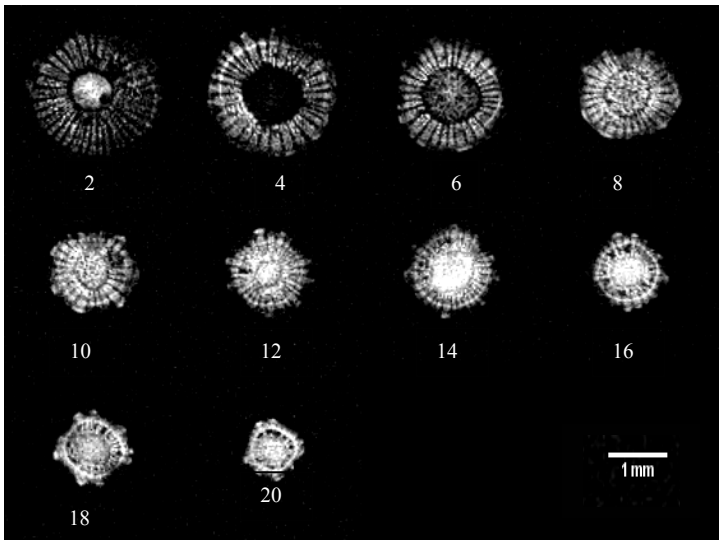



**Figure 4**. Stem cross section MRIs of a Control (water-inoculated) plant. The numbers indicate the internode position, counting from the base of the stem. In internodes 1-3 it is possible to observe the disruption of the xylem caused by the needle inoculation. The xylem disk looks normal in the other internodes. Note that individual vessels are easily observed as bright spots.

**Figure 5**. Stem cross section MRIs of an infected plant. This plant was not showing external symptoms after 6 moths of inoculation. The effect of needle inoculation can be seen in internode 2. Dark sectors of embolized vessels can be observed from internodes 10 to 20. Note that in this image it is more difficult to distinguish anatomical features and individual vessel than in MRIs of a Control plant (Figure 4).



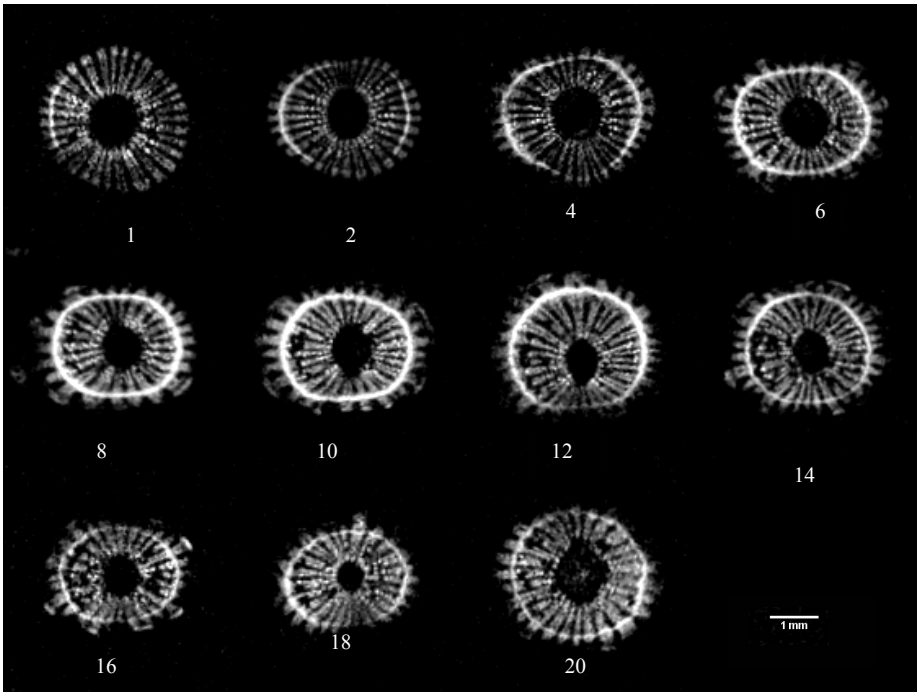




**Figure 6**. Stem cross MRIs of a plant exposed to ethylene. Numbers indicate the position of the internodes, numbered from the base of the stem. “Dark spots” that show non-functional vessels can be seen increasing in size from the base of the stem. The xylem disk appears to be compromised the most at internode 16, which was approximately the youngest internode in the stem (i.e., in the growing tip) at the time of ethylene treatment.

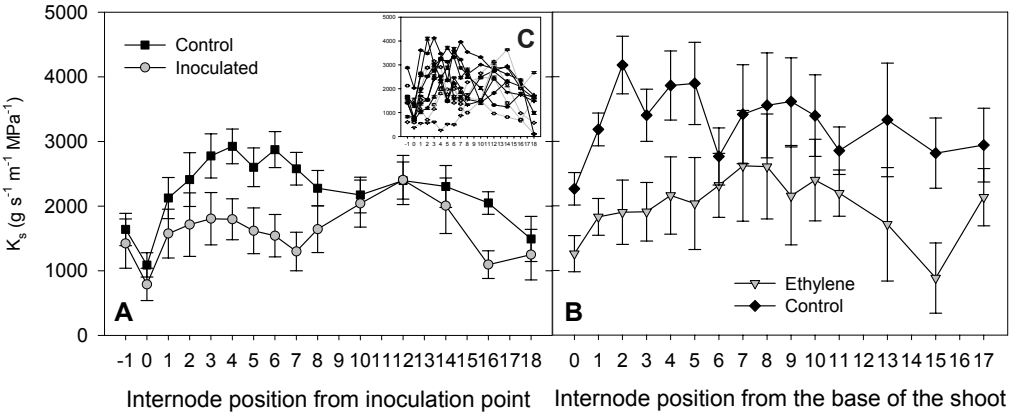



**Figure 7.** Specific hydraulic conductivities ($K_s$) for individual internodes of vines (a) inoculated with *Xf* and (b) exposed to ethylene (± 1 SE). Control plants show maximum $K_s$ in middle third of the stem. In contrast, infected plants show a decrease in $K_s$ in the middle portion of the stem. Panel (c) shows $K_s$ ± 1 SD for all the plants analyzed in the inoculation experiment. Although the variation among different plants is high, the error associated with the measurements is negligible. **Note:** These measurements reflect the contribution of water flowing through cavitated vessels because the embolized vessels are filled by the pressurized water that is used in the test.

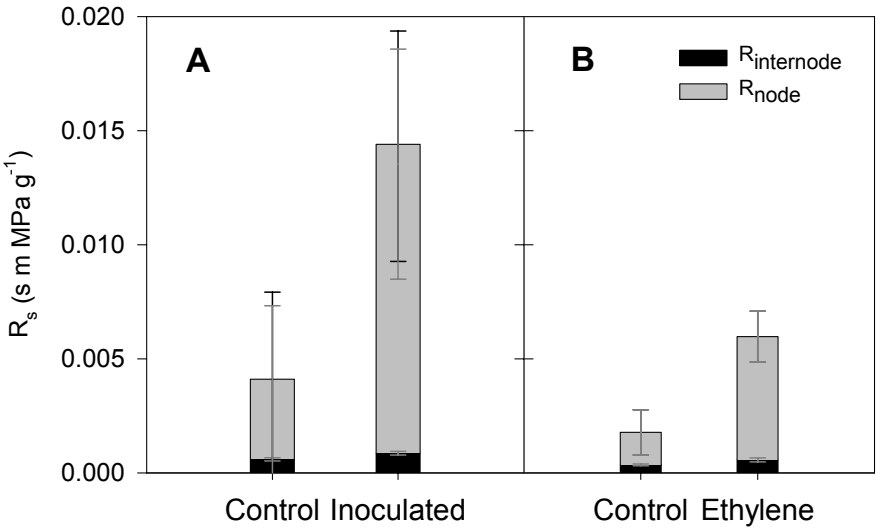



**Figure 8.** Specific hydraulic resistivity ($R_s$) for (a) vines inoculated with *Xf* and (b) exposed to ethylene. Total bar height represents $R_s$ ± 1 SE (in black). $R_s$ components, $R_{node}$ and $R_{internode}$, are also shown (± 1 SE in gray). The nodes are a major component of stem hydraulic resistivity (the inverse of conductivity). It can be noted that $R_s$ 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.

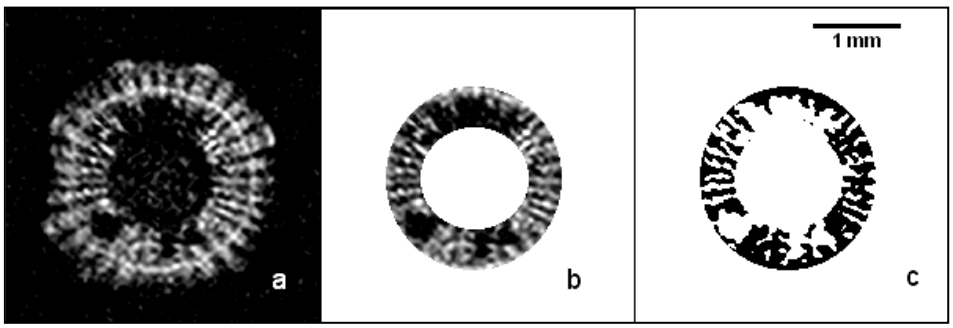



**Figure 9.** Example of the digital processing and analysis performed on MRIs to evaluate quantitatively the development of dark spots. (a) Original cross section MRI of an infected plant showing dark spots. Individual functional vessels are counted using this type of image. (b) Isolation and quantification of the cross sectional area of the stem that is normally xylem tissue ($A_x$). (c) Binary analysis of the xylem ring to determine the area of functional xylem ($A_f$), the black area represents the pixels that are above the threshold defined as the minimum value for a water-filled pixel. The program allows us to vary the threshold value.

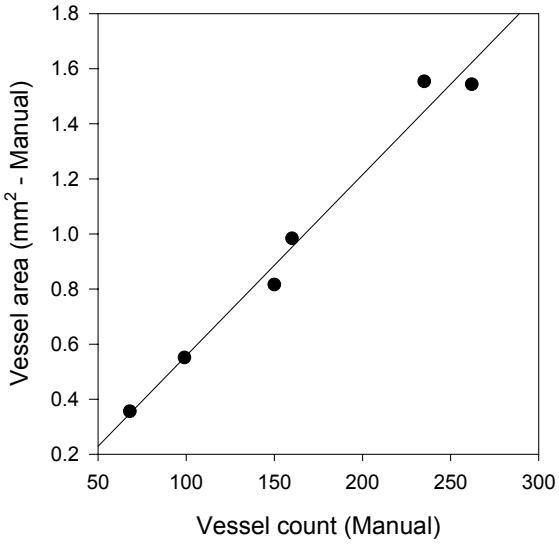



**Figure 10.** The number of functional vessel (vessel count) is a good predictor of the total area occupied by those vessels. Individual vessel areas were marked on the digitized MRI and summed automatically by ImageJ. Linear regression line $r^2 = 0.98$.

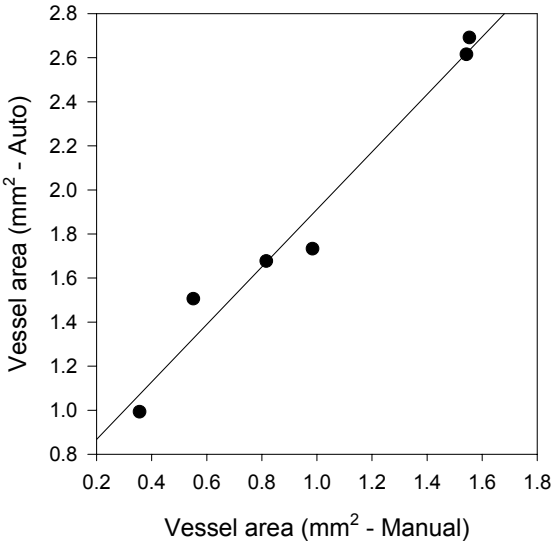



**Figure 11.** The area of functional xylem (the summation of the areas of individual vessels, see Figure 10 legend) is well correlated with the area calculated using an automated algorithm ($r^2 = 0.97$). $A_f$ is the area calculated using the algorithm.

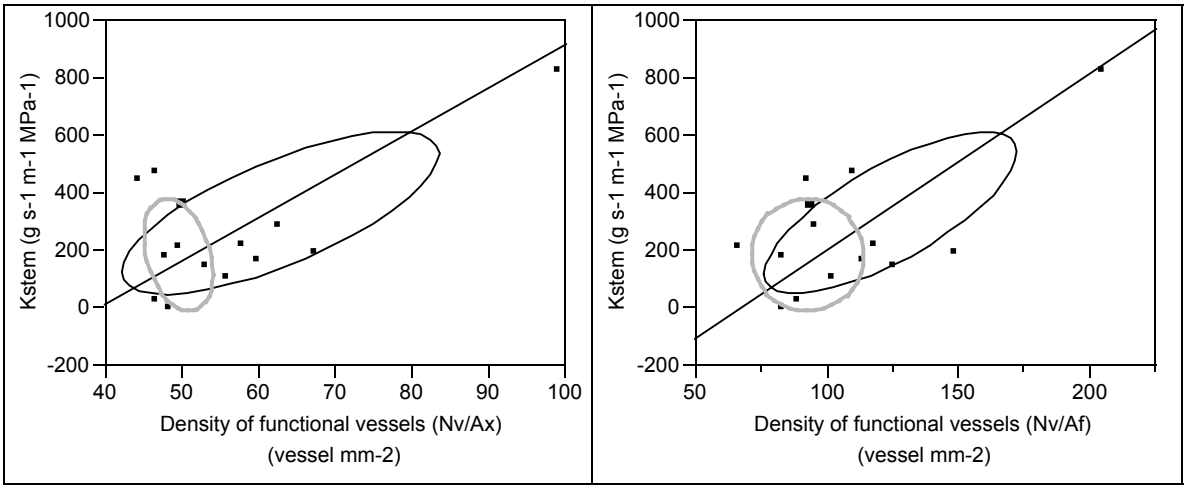



**Figure 12.** Principal component analysis plotting stem conductivity (y-axis) *vs* functional vessel density calculated as vessel number divided by total xylem area (x-axis). Ellipses enclose values for healthy vines (dashed, light line) and infected vines (heavy, grey line).

**Figure 13.** As in the Figure 12 legend, except that functional vessel density is calculated as vessel number divided by functional xylem area.

## CONCLUSIONS

MRI will be a powerful adjunct to other, more conventional approaches for characterizing the changes that occur in grapevine xylem following introduction of *Xf*.

## REFERENCES

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