Identifying Factors Mediating Resistance to Almond Leaf Scorch Disease

Project No.: **08-PATH8-Kirkpatrick**

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Objectives:

1) Identify the biochemical and anatomical properties that eliminate *Xf* infections in resistant almond cultivates.

Interpretive Summary:

Almond Leaf Scorch (ALS) disease is caused by the bacterium *Xylella fastidiosa* (*Xf*) which occupies a particular niche in plant xylem vessels. Previous work on ALS showed striking differences of disease incidence among cultivars. Disease incidence was higher in the Peerless and Nonpareil varieties while virtually no disease occurred in Carmel and Butte varieties. More recent research studying relative resistance of 10 commercial almond varieties showed that following mechanical inoculation of *Xf*, the bacteria readily moved and caused disease in ALL varieties. However, several of the varieties emerged disease and pathogen free after overwintering the year following inoculation.

The focus of this research was to determine if certain components of xylem sap or anatomical xylem structures mediate resistance to ALS over winter months. Identifying differences between resistant and susceptible cultivars could point to factors useful for controlling the disease.

Xylem sap was extracted from 2 ALS susceptible varieties, Peerless and Sonora, and 2 resistant varieties, Butte and Carmel. Xylem sap was extracted from these trees over winter months for two years and once in a later month for comparison. Xyelm sap samples from different cultivars for each month were subjected to several analyses where we measured pH and osmolarity, concentrations of sugars, minerals, organic acids and polyphenolic content. We also looked for anatomical differences in the xylem

vessels between Peerless (ALS susceptible) and Butte (ALS resistant) varieties. We completed one bioassay designed to determine *Xf* survival in xylem sap from winter months in the different almond cultivars. This is the first report of these analyses on xylem sap from almond trees.

We found statistical differences in xylem sap polyphenolic content between ALS resistant and ALS susceptible varieties over winter months in both years. Polyphenolic concentration was significantly higher in Butte compared to Sonora in February 2008 and later in December 2008. In January 2009, Carmel exhibited significantly higher polyphenolics than Sonora, Peerless, and Butte. Butte also had higher polyphenolics than Peerless. Carmel continued to have higher concentrations of polyphenols than Peerless in February 2009. Polyphenolics are associated with plant defenses and may reduce *Xf* survival in resistant almond varieties during this season.

Our analyses of pH, osmolarity, concentrations of sugars, calcium, magnesium, and organic acids showed that these components were not significantly different among resistant and susceptible almond varieties. Furthermore, we found no differences in the cross sectional area between xylem vessels of Peerless (ALS susceptible) and Butte (ALS resistant) varieties. Therefore these constituents are most likely not involved in mediating resistance to ALS.

Results of the bioassay did not show a clear correlation between higher polyphenolic content and *Xf* survival *in vitro*. Protein profiles of xylem sap samples revealed one or two proteins which may be associated with only resistant almond varieties. We are attempting to identify these proteins however our success will depend on the availability of sequences or similar proteins found in other plant genomes.

Materials and Methods:

Isolation of ALS strains of *Xf* from infected trees

ALS strain of *Xf* used for bioassays was isolated from infected 'Peerless' or 'Sonora' almond trees in the same orchard as the healthy trees used for xylem sap collection.

Sample varieties and isolation of xylem sap from almond branches

Four trees of each cultivar, Butte, Carmel, Peerless, and Sonora were chosen for xylem sap sampling. These represent two susceptible varieties (Peerless and Sonora) and two resistant varieties (Butte and Carmel). Originally two trees per variety were chosen, but in February of 2008 two more trees per variety were added to obtain better statistical results. The two additional Sonora trees were slightly older than trees 1 and 2. The Peerless trees obtained for reps 3 and 4 were about 5 years younger. Despite age difference all trees were located in the same area at the Armstong facility in Davis.

Xylem sap was expressed on a monthly basis over winter months. In 2007-2008 xylem sap was collected in November, January, February, March, and July. In 2008-2009 xylem sap was collected in November, December, January, February, March and April. Xylem fluid was expressed using a specially designed pressure chamber (PMS Instruments, Corvallis, OR). Collection tubes containing expressed sap were put directly on ice to slow the oxidation process.

Osmolarity and pH measurements

On the same day that sap was extracted, three pH measurements were made from 3 different branches from each tree (Corning pH meter140). These measurements were averaged to obtain a pH value for xylem sap from each sample tree. Xylem sap samples were then frozen at -20C. Two to three osmolarity measurements per tree per month were made using a 5500 vapor pressure osmometer (Wescor Inc., UT).

Folin-Ciocalteau method for total phenolic compounds in xylem sap

Total polyphenol concentration was measured using a modified Folin Ciocalteau micro method (Waterhouse, 2001). Results were statistically analyzed as reported below.

Sugar and minerals testing/organic acids

Xylem sap samples from November 2007, January, February, March and July 2008 were sent to the Davis ANR Analytical Lab where sap concentrations of fructose, glucose, sucrose, calcium, magnesium, and iron were measured. Similar samples were sent to University of Florida for organic acid analysis.

Protein profiles

Xylem sap protein profiles were generated for each sample tree/month in the following manner. Xylem sap proteins were precipitated with cold acetone and centrifuged at 5000rpm for 20 minutes. Dry protein pellets were dissolved in SDS loading buffer and boiled for 5 minutes. Samples were run on a 12% Tris-HCL acrylamide gel for approximately 2 hours. Protein gels were stained with SYPRO Ruby protein gel stain overnight. Gels were visualized and photographed under UV light. Individual protein profiles were visually compared for band differences.

Xylem vessel cross sectional measurements

Four trees each of Butte (resistant) and Peerless (susceptible) varieties were chosen for xylem vessel measurements. These trees were all planted in the field in spring 2005 and are about 3 ½ years old. Every two months one branch from each tree was cut for sampling. Cut branches were 7/16” -3/4” in diameter at the base. Thin sections and xylem vessel measurements were made at 4 locations i) the base of the branch ii) 2” from the base and iii) 4” from the base of the branch iv) 6” from the base. Thin sections of wood from each location were dyed with Toludine Blue O pH 4.4. Toludine blue O stains some types of polysaccharides and usually stains phloem and tyloses.
Thin sections were observed using bright field microscopy at 20X and digital pictures were taken of spring and summer xylem vessels. Almond wood has two types of xylem vessels: spring and summer. Spring vessels are bigger, presumably for better water transport in the spring. Cross-sectional areas of spring and summer vessels were measured and compared using a program called Image J.

Bioassay: *Xf* survival analysis

The *Xf* growth and survival bioassays consisted of several tubes of filter sterilized xylem sap from trees sampled in January and February 2008, sterile water, SCP buffer, and PW medium. These tubes were inoculated with 10^5- 10^6 *Xf* cells per tube. In order to monitor *Xf* survival over 24 hours, small aliquots were removed from each tube every

two hours and plated onto PWG media plates. These plates were incubated at 28°C until *Xf* colonies were large enough to count, approximately 14 days.

Statistics

Statistics for sugars, minerals, pH, osmolalrity, and phenolics were done using a repeated measures model in the SAS 9.1 software. Pair-wise comparisons between varieties for each month were done using a Tukey's HSD test.

Results and Discussion:

Results of total phenolic analysis

Phenolic compounds are associated with pant defenses and the presence of phenolic compounds in xylem sap could affect *Xf* survival. Possibly higher constitutive levels expressed in resistant varieties during winter months could be associated with lower *Xf* survival during this time.

We found that polyphenolic content in xylem sap varied across months. Average total polyphenolic concentrations were significantly higher in at least one of the resistant varieties during winter months two years in a row. In February 2008 (**Figure 1**) and December 2008 (**Figure 2**) Butte had significantly higher polyphenolic concentration than Sonora. In January 2009, Carmel exhibited significantly higher polyphenolics than Sonora, Peerless, and Butte. Butte also had higher polyphenolics than Peerless (**Figure 2**). Carmel continued to have higher concentrations of polyphenol than Peerless in February 2009 (**Figure 2**).

Pierce's disease (PD) of grapevine, also caused by *Xylella fastidiosa*, can be cured by a phenomenon called "cold curing". This occurs when a grapevine infected with PD in the summer is cured of the disease when the vine overwinters in a cold climate. Xylem sap collected from grapevines during winter in a cold climate was found to have higher polyphenolic concentrations than xylem sap collected from grapevines overwintering in a more temperate area (M. Meyer unpublished data). This evidence suggests that heightened levels of polyphenols may be influencing *Xf* survival in xylem vessels over winter.

Figure 1.

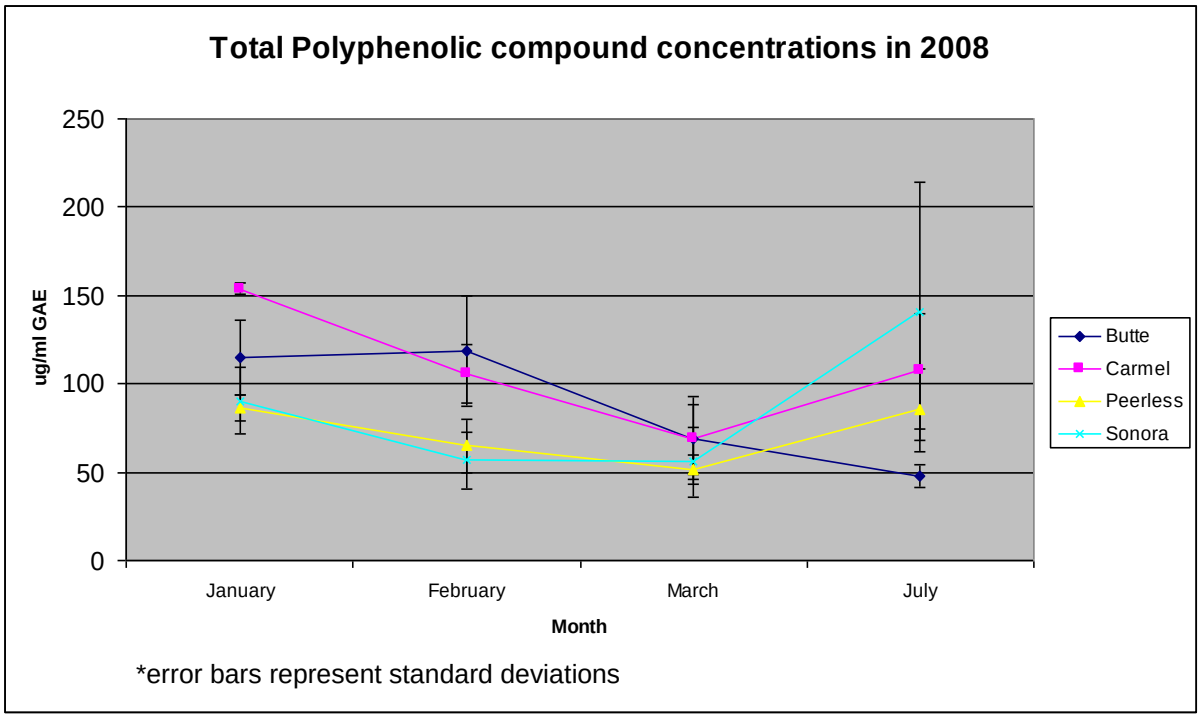
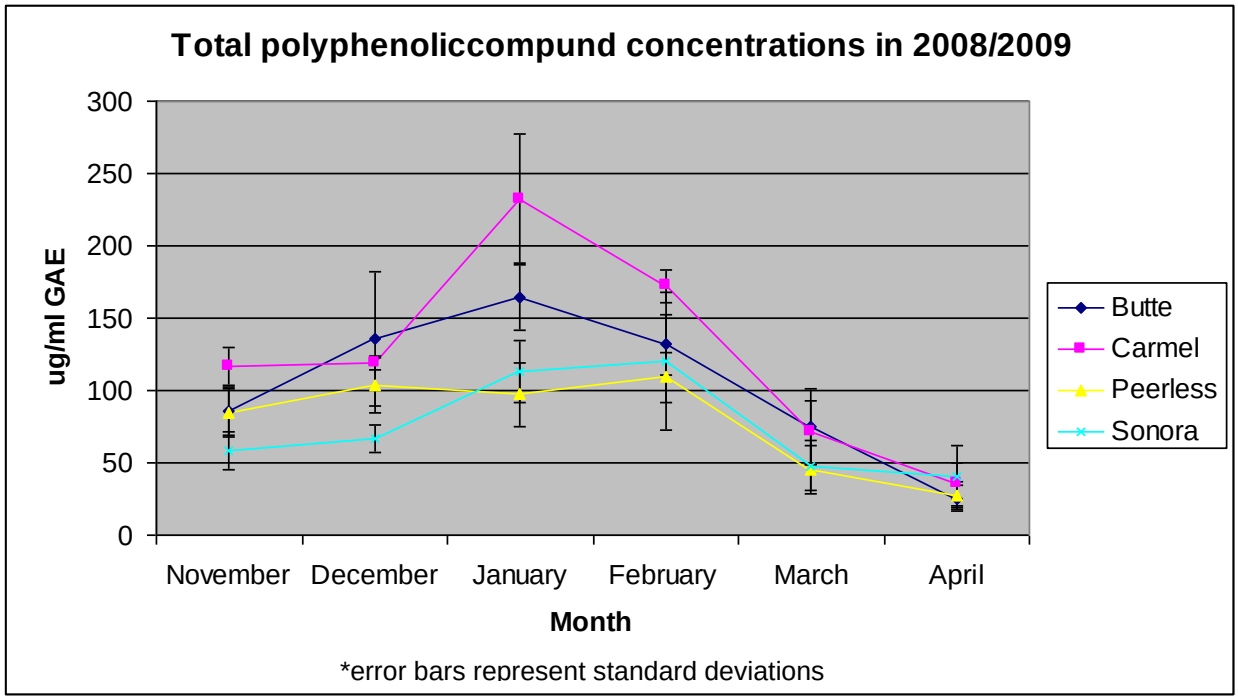


Figure 2.



Results of pH and osmolarity

Average pH values for all xylem sap samples from all months were between 5.1- 6.5 units (**Figures 3 and 4**). In xylem sap sampled over winter 2008-2009, susceptible varieties had significantly higher pH values for April than resistant varieties. However, in other months a difference in pH was not significant between susceptible and resistant varieties. This suggests that xylem sap pH probably does not play a role in ALS resistance. Furthermore, fluctuations in pH were different for the two years sampled indicating pH changes may be more affected by rainfall or other environmental factors.

Components of osmolarity include sugars and salts. Analysis of sugars show that fructose, glucose, and sucrose content also increased into the summer, potentially accounting for the osmolarity increase.

In sap sampled over winter 2007/2008, osmolarity increased steadily from January to July in all varieties (**Figure 5**). In our second year of sampling osmolarity also increased from winter to spring (**Figure 6**). Although **Figure 5** shows osmolarity higher in the Carmel variety for January and February, only osmolarity for Carmel in February was statistically higher than Sonora in February. Differences in osmolarities during winter and spring 2008/2009 were NOT statistically significant, even though **Figure 4** shows resistant and susceptible varieties diverging in January, February and March.

During 2007/2008 high osmolarities in July might reflect the measured increase in sugar concentrations at that time. We would expect that osmolarity might decrease in spring months due an influx of water and dilution of solutes. In March 2008 measured sugars did decrease at this time but osmolarities either increased or remained similar to February levels. In this case, sugar and mineral content did not correlate well to increasing osmolarity. Sugar and mineral content were not measured for xylem sap in winter 2008/2009 so we could not compare results to 2007/2008.

Figure 3.

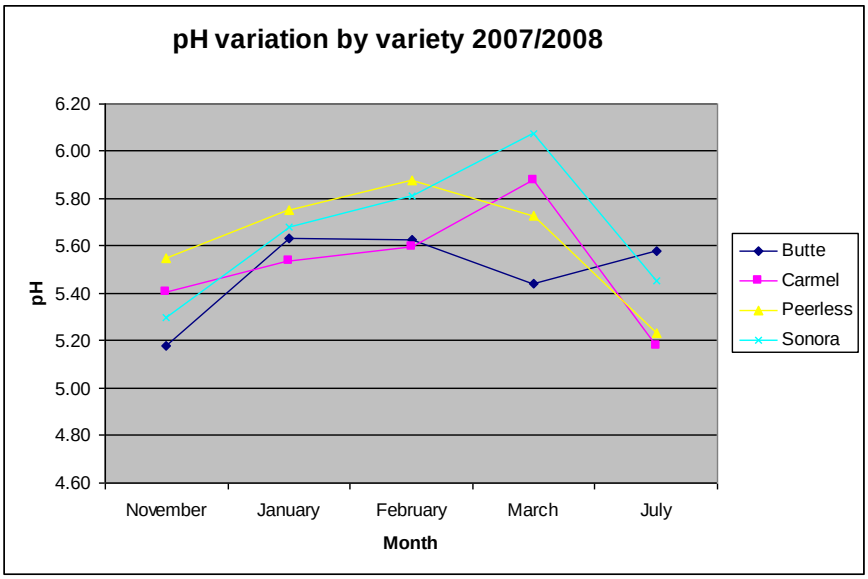


Figure 4.

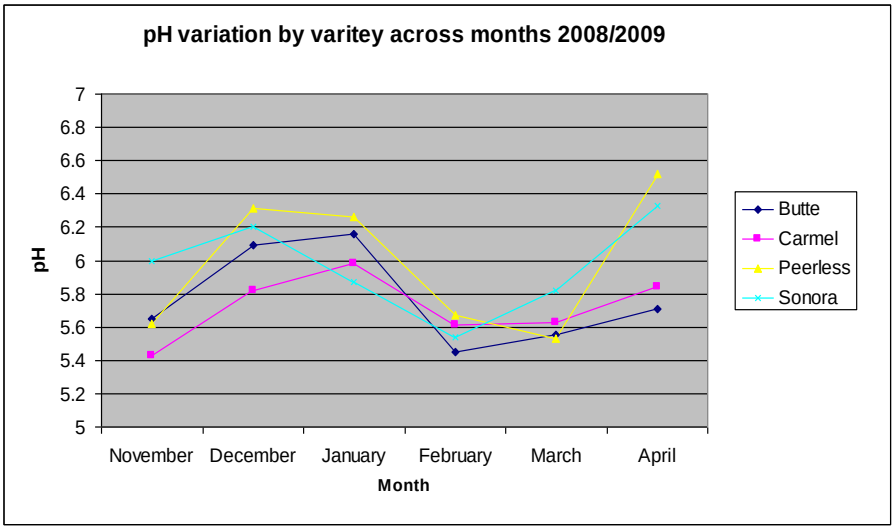


Figure 5.

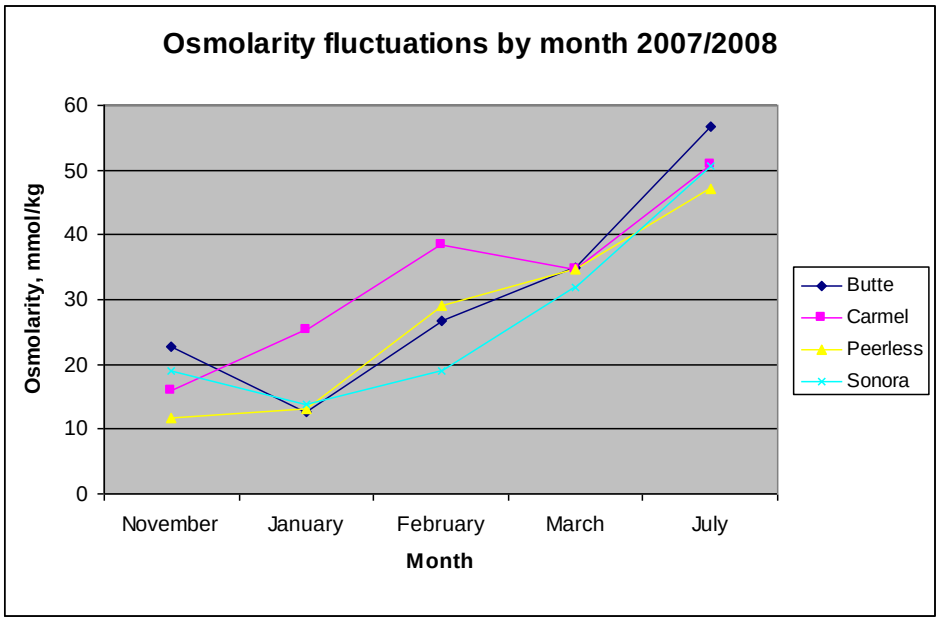
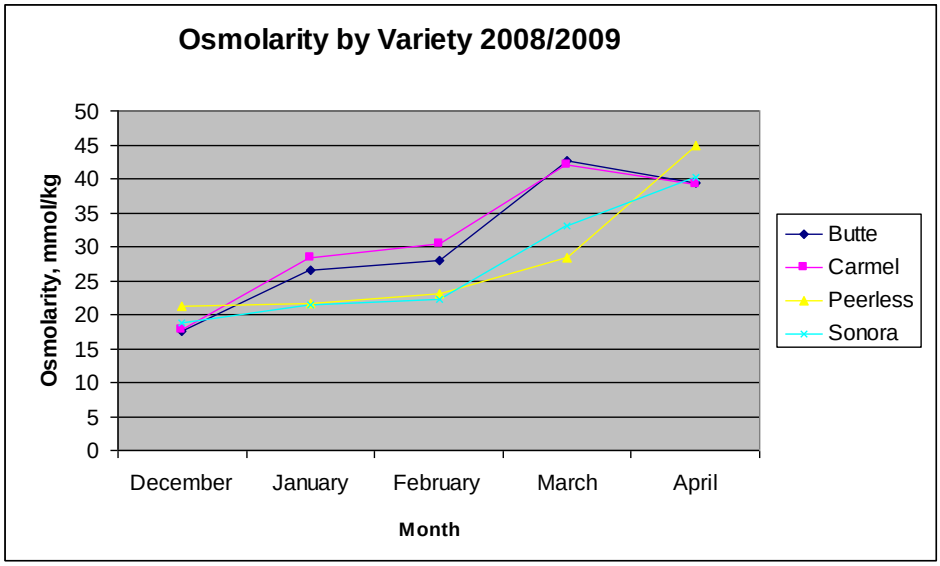


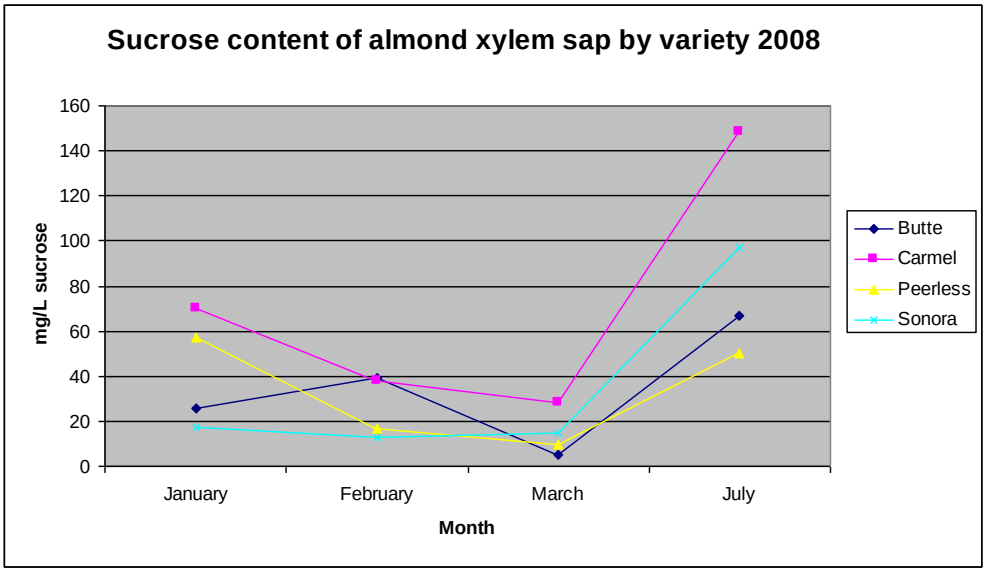
Figure 6.



Results of sugar and mineral analysis for xylem sap collection 2007-2008

Our analyses of sucrose, fructose, glucose, calcium, and magnesium content of almond xylem sap show that there are fluctuations in these xylem sap components over the winter months (**Figures 7-11**). However, there were few statistically significant differences among almond varieties in any given month. We found no clear correlation between sugar or mineral concentrations and resistance or susceptibly to ALS. In March 2008, glucose, sucrose and fructose levels decreased, possibly due to increasing water content in xylem sap in the spring. By July, all sugar concentrations had increased. Furthermore, concentrations of fructose and glucose were approximately10-80 times greater than sucrose concentrations. Soluble calcium and magnesium concentrations among all varieties fluctuated similarly.

Figure 7.



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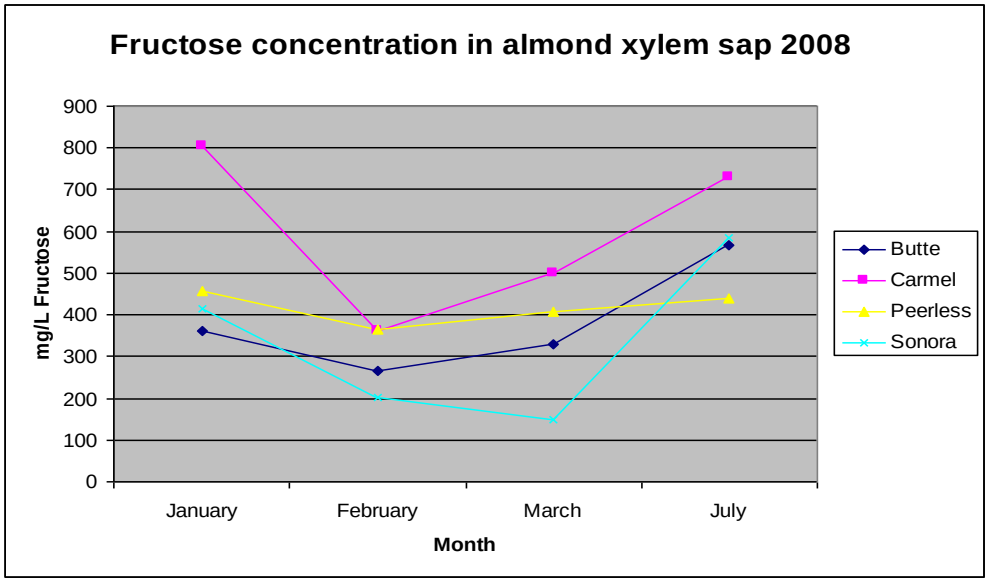

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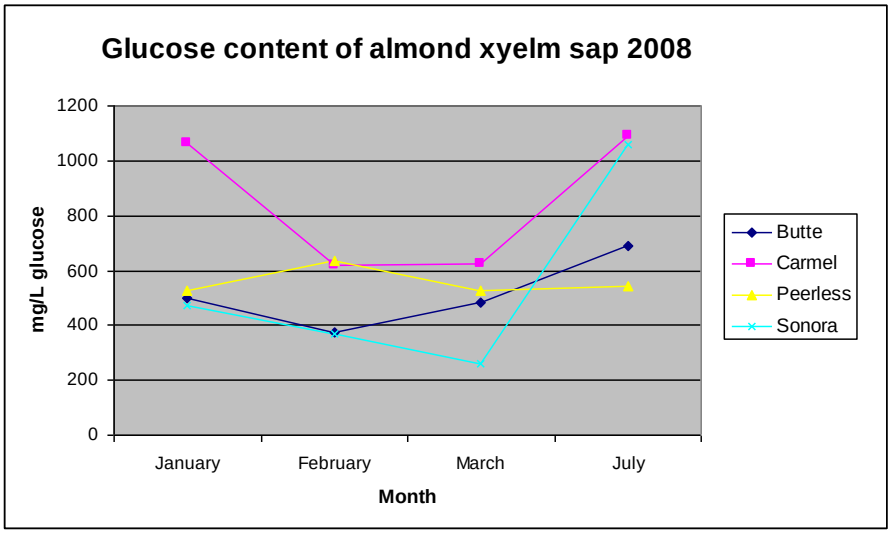

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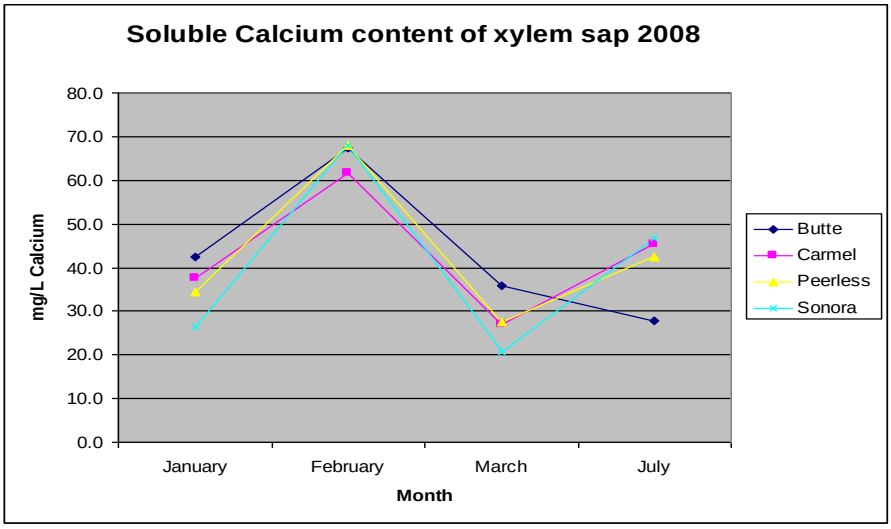

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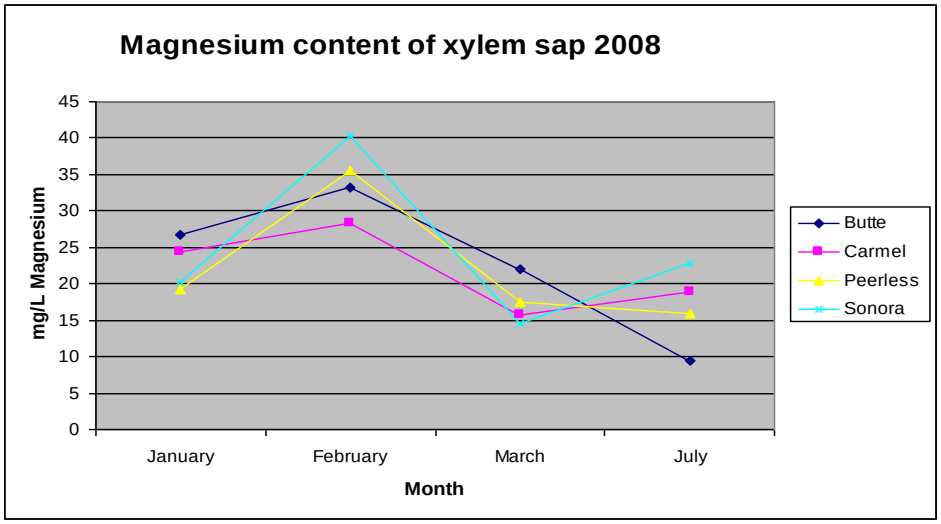


Microscopy of xylem vessels in resistant and susceptible varieties

We hypothesized that susceptible almond cultivars might have smaller xylem vessels that would more easily become clogged with *X. fastidiosa* bacteria. However, we found no statistically significant differences between cross-sectional areas of xylem vessels in Peerless (ALS susceptible) and Butte (ALS resistant) almond varieties. Vessels of the susceptible variety were not thinner and therefore would not have a greater chance of becoming clogged with *Xf* than vessels of the resistant variety. Spring and summer xylem vessels were analyzed separately because summer vessels have areas approximately 5-7 times smaller than spring vessels.

Figure 12.

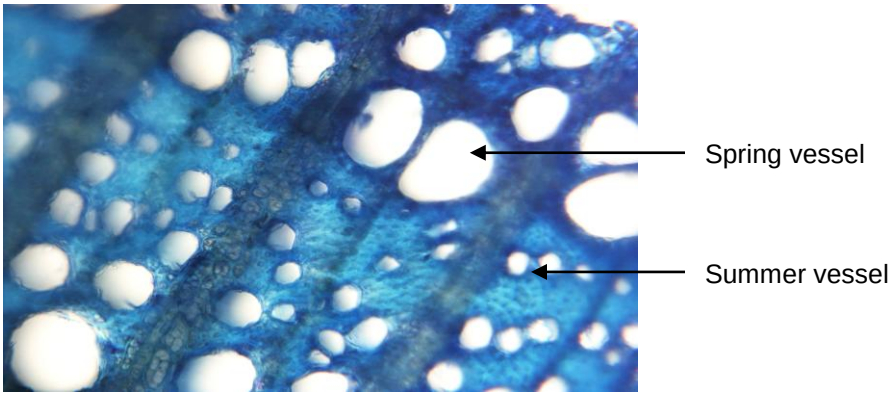
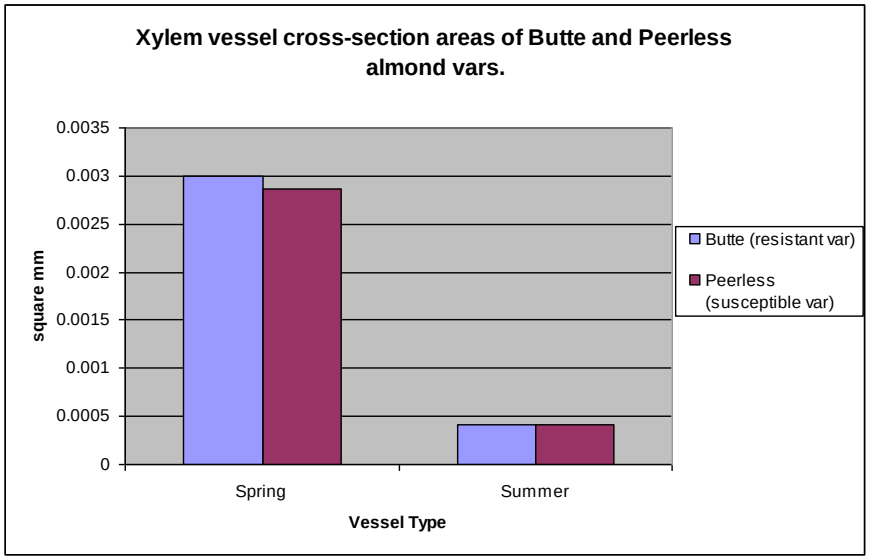


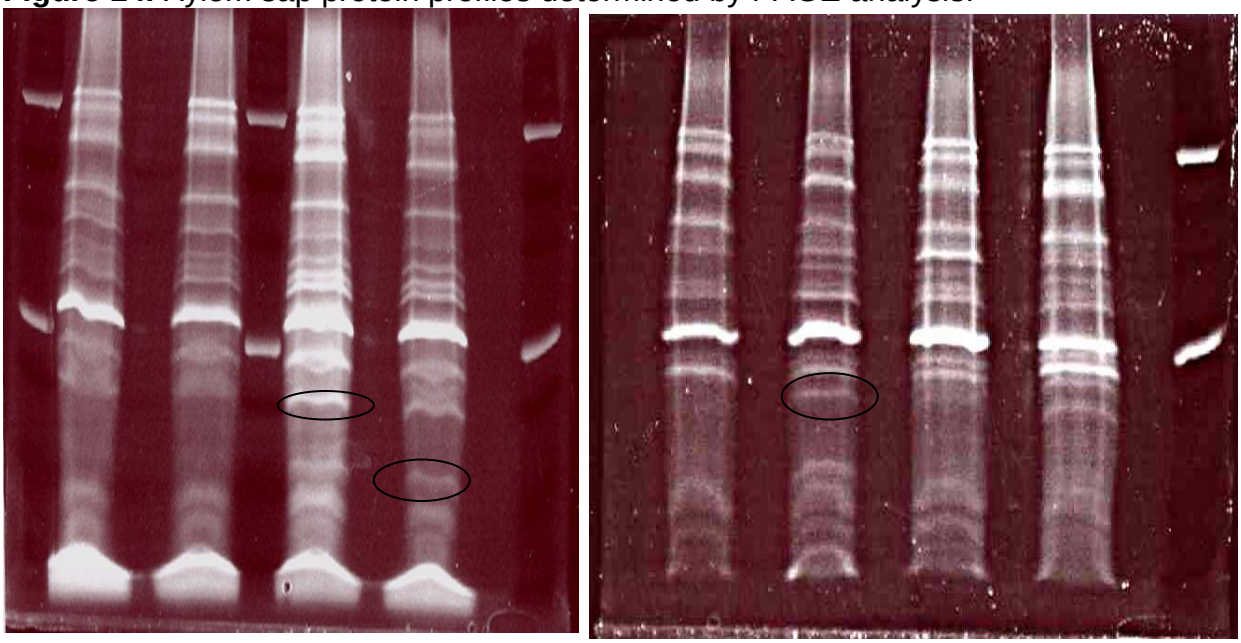
Figure 13.



Protein profiles of almond xylem sap

Protein profiles for Sonora (ALS susceptible) and Butte (ALS resistant) xylem sap in February 2008 are shown in **Figure 14**. All xylem sap profiles had between 11-16 protein bands with the brightest, largest band in the middle at about 28kD. By visually comparing profile pictures we hoped to consistently observe at least one different band when comparing susceptible and resistant protein profiles. The unique band(s) could be cut out and sequenced to determine if a xylem sap protein is mediating resistance to ALS. Circled bands in the pictures are those we are now sequencing.

Figure 14. Xylem sap protein profiles determined by PAGE analysis.



S1 S2 B2 B4
February 2008

B1 B3 S3 S4
February 2008

Organic acids analysis

The organic acids analyzed for this research were kindly processed by Brent Holtz and Pete Anderson at the University of Florida. Concentrations of oxalic, citric tartaric, malic, and malonic acids were determined for the resistant and susceptible almond varieties. The graphs (**Figures 15-19**) show fluctuating concentrations in the almond varieties and across months. A good separation of resistant and susceptible varieties was only observed for oxalic acid in January 2008. Full statistical analysis of this data is incomplete at this time, however, similar analysis of grapevine xylem sap revealed that organic acids were not involved in mediating the cold-curing affect for Pierce's disease (M. Meyer unpublished data).

Figure 15.

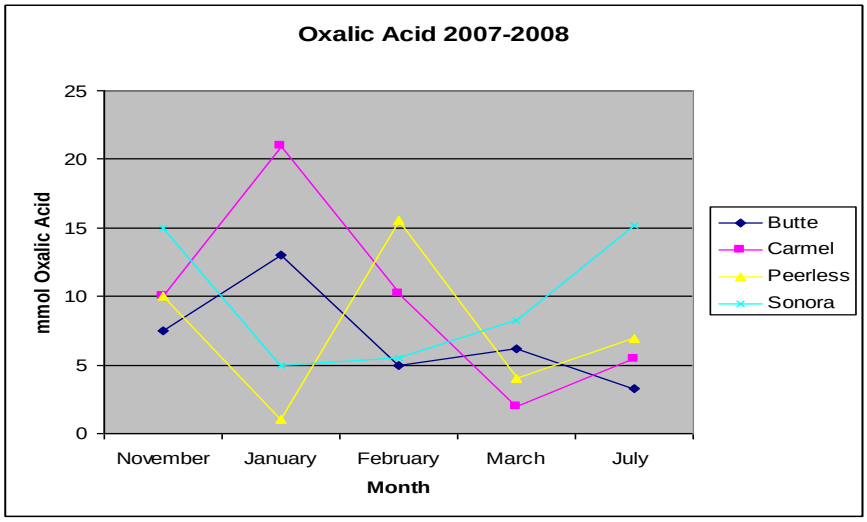
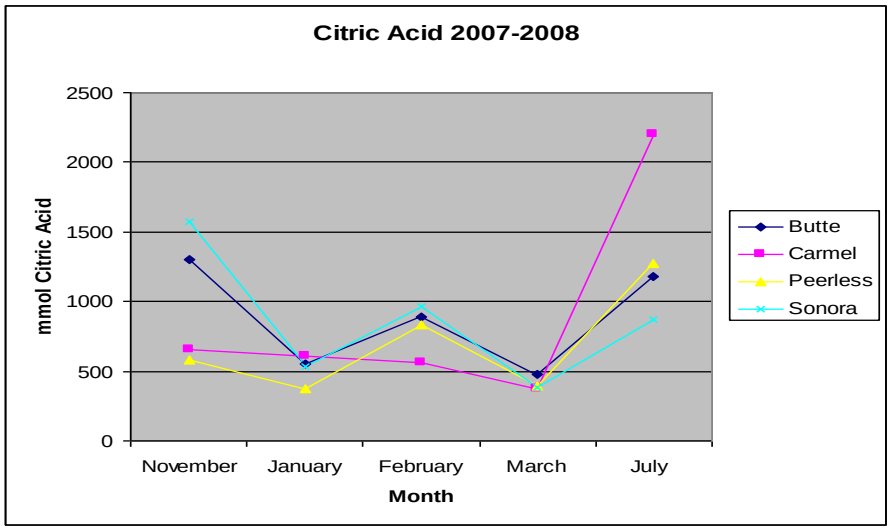


Figure 16.



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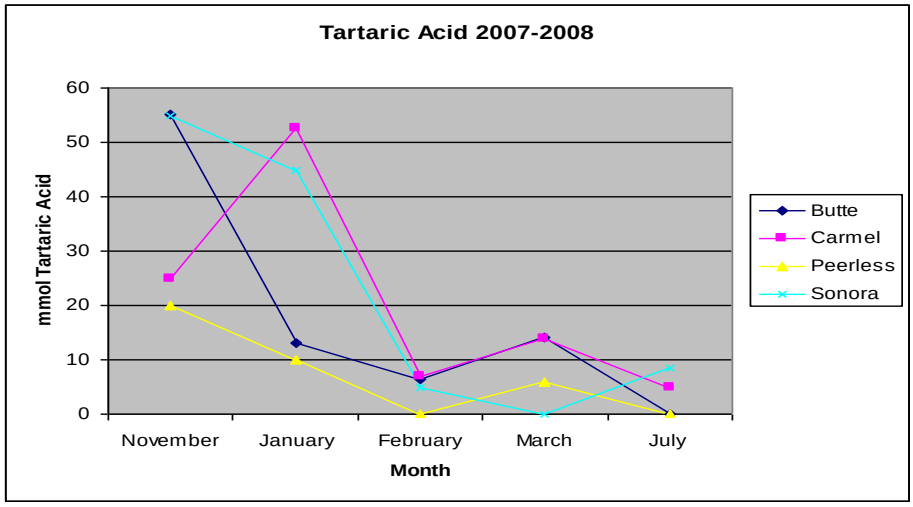

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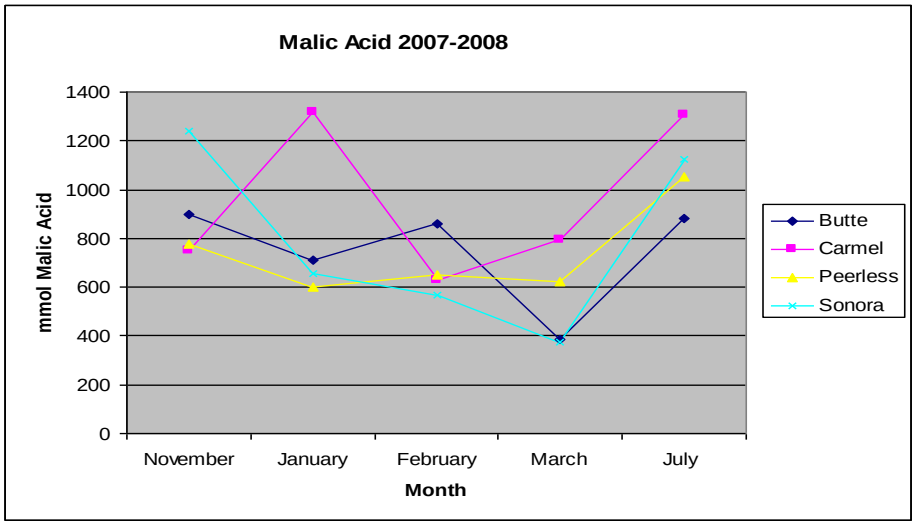
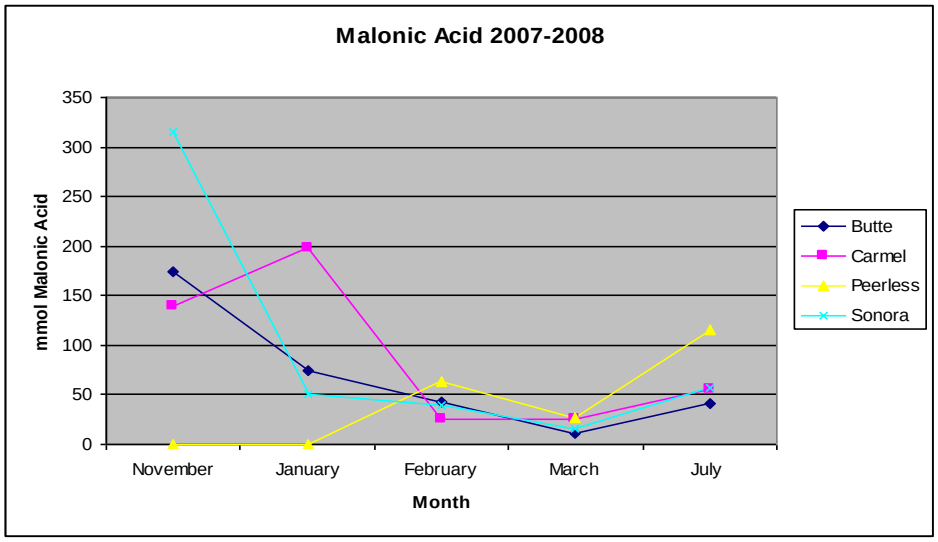


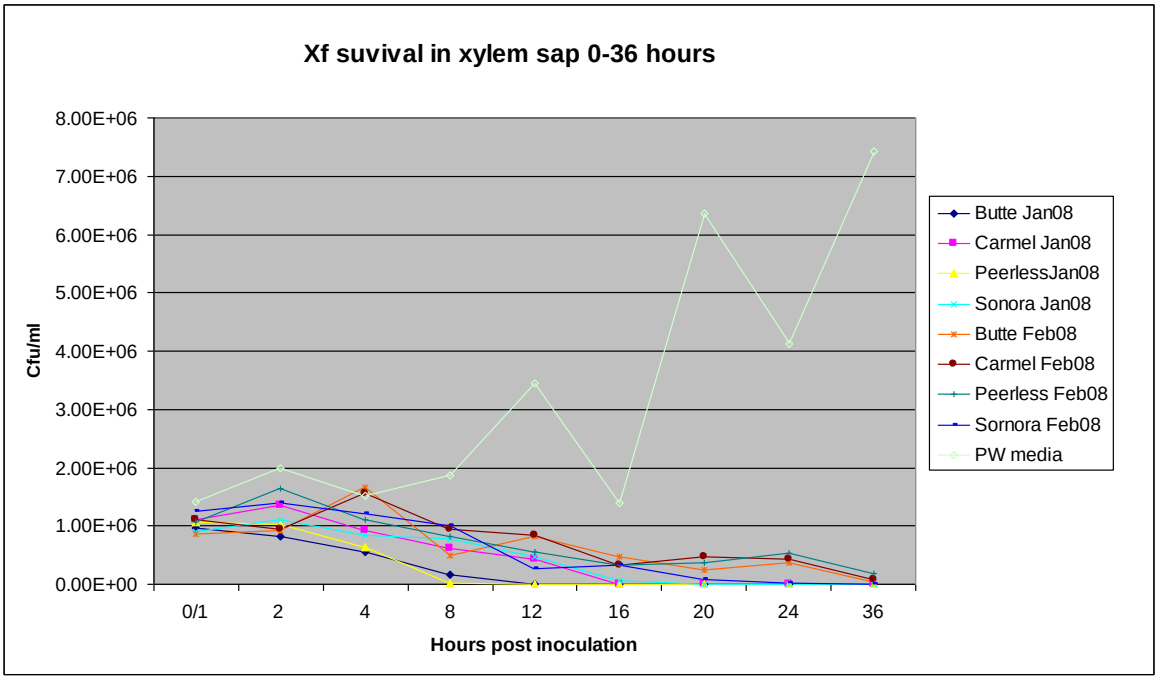
Figure 19.



Bioassay results

Using plating bioassays we hoped to show that populations of *Xf* bacteria would die more quickly in xylem sap from ALS resistant trees and would survive longer in xylem sap from ALS susceptible trees. We tested xylem sap collected in January and February 2008. Higher polyphenolic levels in resistant cultivars might have influenced *Xf* survival in those samples. However, we found that *Xf* survival did not correlate well with either higher polyphenolic concentration or higher sugar concentrations in xylem sap samples.

Figure 20.



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