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Potential Anatomical Methods for the Determination of Weak Wood in Apple

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Abstract

Two experiments were performed to study the anatomical traits related to the development of graft unions of relatively weak ('Honeycrisp'/'M.26 EMLA', 'Cripps Pink' cv. Maslin/'Geneva® 41', 'Scilate' (Envy™)/'Geneva® 41' and strong ('Honeycrisp'/'M.7 EMLA', 'Zestar!'/'M.26 EMLA', 'Zestar!'/'M.7 EMLA', 'Cripps Pink' cv.Maslin/'M.9 NAKB T337', 'Scilate' (Envy™)/'M.9 NIC29') scion/rootstock combinations of apple. The objective was to determine the cause of the weak unions so it may be used to develop a rapid screening tool to identify new potentially weak combinations. Fiber cell walls were thinner below and at the union in 'Honeycrisp' and 'Zestar!' when propagated on 'M.26 EMLA'. 'Honeycrisp' had significantly thicker cell walls at the union than 'Zestar!' combinations. 'Cripps Pink' and 'Scilate' combinations were thinner below and above the graft union on 'G.41' rootstocks. Trees propagated on 'M.26 EMLA' produced significantly less fiber tissues than those propagated on 'M.7' EMLA', and 'Honeycrisp' produced significantly less fiber and conductive tissues than 'Zestar!'. Laser ablation tomography (LAT) revealed weak and strong combinations both contained areas of poor xylem differentiation at the graft union. Xylem tissues at the graft union are highly variable, making it difficult to determine the strength of a scion/rootstock combination based off of anatomical features of the union alone.

The formation of a mechanically weak graft union in young nursery trees is a problem associated with some scion/rootstock combinations of apple. Recently, commercial nurseries have been losing large numbers of newly budded trees of 'Cripps Pink' and 'Scilate' on 'G.41' (N. Manly, personal communication). Other combinations are prone to weakness in the nursery and throughout their life in the orchard, including 'Honeycrisp'/'M.26 EMLA' (Privé et al., 2011), and 'Gala/'G.30' (Robinson et al., 2003).

Graft failure may be caused by many factors, including poor environmental conditions, poor propagation practices, or by an incompatibility between the rootstock and scion (Andrews and Serrano Marquez, 1993). Fiber cells of apple xylem provide much of the mechanical strength to the tree (Winandy and Rowell, 2013), as their secondary cell walls are heavily lignified (Déjardin et al., 2010). This suggests differences in the anatomical characteristics of the fiber cells may lead to the structural weaknesses of the union.

Strong, mechanically resistant wood is characterized by having dense, thick-walled fiber cells. The secondary cell walls of fiber cells are heavily lignified, and the lignified layer provides tensile strength to the wood. Apples propagated to a dwarfing interstem produced thinner fiber cell walls (Doley, 1974). Trees with thin-walled fiber cells may bend more easily under high winds (Déjardin et al., 2010). If the stems bend while being attached to a rigid stake or support post, the tree may be more likely to break.

In addition to fiber cells, the secondary xylem of apple wood consists of ray parenchyma, axial parenchyma, fiber-tracheids, and vessel elements (Pratt, 1990). The relative proportions of these cell types vary between

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rootstock cultivars in both the roots (Beakbane and Thomsen, 1947) and in the trunks below the graft union (Komarofski, 1947). The relative proportions of each cell type is partially related to the vigor of the rootstock, as more vigorous rootstocks tend to produce more fiber cells and less parenchyma cells than dwarfing rootstocks.

While fewer fibers are generally found in dwarfing rootstocks, an underproduction of fiber cells has been observed in scion/rootstock combinations exhibiting incompatibility at the union, and incompatibility may play a role in the formation of some weak graft combinations (Simons, 1987). Incompatibility has been defined by Andrews and Serrano Marquez (1993) as "the failure of a graft combination to form a strong union and to remain healthy due to cellular, physiological intolerance resulting from metabolic, developmental, and/or anatomical differences." Rather than differentiating into fiber cells, the callus tissues produced at the graft union differentiate into irregularly oriented ray parenchyma cells (Mosse, 1962). Unions of the combination 'Jonagold/Mark' had regions of poorly differentiated parenchyma, and some of these trees broke along a line of this parenchyma tissue (Warmund et al., 1993). A decreased proportion of fiber cells at the union may lead to weaknesses of young nursery trees.

Visualizing a large portion of the union may allow for further understanding of the causes of structural weaknesses between scion/rootstock combinations. Anatomical work to visualize the entire graft union has been performed on apple (Warmund et al. 1993) and grape (Milien et al., 2013) using magnetic resonance imaging (MRI) and X-ray computed tomography (CT-Scan) respectively. In laser ablation tomography, a laser beam ablates samples while images are simultaneously captured. These images are then layered back together to form a three-dimensional model of the sample (Chimungu et al., 2015). Laser ablation tomography is a method that may also allow for the imaging of a large section of the union, and may help to determine the cause of weakness in young trees.

The purpose of this study was to investigate the cause of weak unions in three scion/rootstock combinations that are known to be prone to graft failure ('Honeycrisp'/'M.26 EMLA', 'Cripps Pink'/'G.41', and 'Scilate'/'G.41') and to evaluate anatomical methods for determining union strength that may be employed to identify weak combinations in the future.

Materials and Methods

Sample Preparation. In Feb. 2014, finished chip-budded apple trees were received from Willow Drive Nursery, Ephrata, WA. These were budded in 2012, and included six trees each of 'Cripps Pink' on the rootstocks 'G.41' and 'M.9 NAKB T337' and 'Scilate' on the rootstocks 'G.41' and 'M.9 NIC29'. In Apr. 2014, additional chip-budded trees were received from Adams County Nursery, Aspers, PA. These included ten trees each of the cultivars 'Honeycrisp' and 'Zestar!' on the rootstocks 'M.26 EMLA' and 'M.7 EMLA'. All trees were kept at 6 ° C until sampling. Weak combinations consisted of 'Cripps Pink' and 'Scilate' on the 'G.41' rootstocks, and 'Honeycrisp' on the 'M.26 EMLA' rootstock. Strong trees included 'Cripps Pink' and 'Scilate' on the 'M.9' rootstocks, 'Honeycrisp' on 'M.7 EMLA', and 'Zestar!' on both the 'M.26 EMLA' and 'M.7 EMLA' rootstocks.

Beginning in May 2014, trees were cut using a circular saw to 10.0cm in length from 7.0cm below to 3.0cm above the union, and then sectioned to 3.0-4.0mm thick longitudinal sections using a band saw. Two longitudinal sections from the center of the tree were kept for use in the following studies (Figure 1).

Fiber Cell Walls. Six trees of each combination were utilized in the experiments. Following the initial sample preparation, sections were placed in water for three to seven days to soften the wood tissue for hand sec-

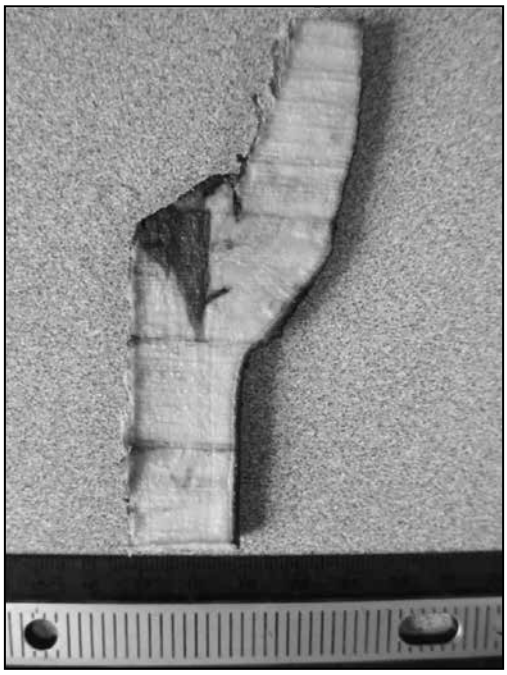

Fig. 1: Initial cuts of nursery trees produced 10cm long, 4mm thick longitudinal sections from 3cm above the top of the union to 7cm below the union. The longitudinal sections closest to the center of the tree were kept for the experiments. Sections were then cut transversely, and hand sectioned from 7cm below, at, and 3cm above the top of the union for microscopy studies.

tioning. Two replicates from the Pennsylvania nursery were kept in 70% ethanol for 38 and 27 days before being moved into water for five and six days, respectively.

After softening, the longitudinal sections were hand sectioned transversely to 12.0mm^2 from three different areas of the section: 7.0cm below the union, at the union, and 3.0cm above the union. The phloem tissue was removed from the outer edge of these blocks to facilitate hand sectioning of the xylem. Sections were placed in two drops of distilled water on glass microscope slides. Sections were then stained with 1% toluidine blue for one minute and rinsed with distilled water before cover slips were applied.

Sections were examined at 400x magnification with an Olympus® CX-41 compound microscope (Olympus Inc., Tokyo, Japan). Photomicrographs were taken using an Olympus® DP-72 digital camera connected to the microscope and Olympus® Cellsens Standard software was used for image capture and data gathering. Fifty radial fiber cell walls were measured from the middle lamella to the lumen of the cell using a measuring tool in Cellsens. Cell walls were measured from each area of the tree section (below, at, and above the union) and were subsequently averaged.

Statistical analysis was performed using the aov command in R (R Foundation for Statistical Computing, Vienna, Austria). Data from the different nurseries were considered different experiments and were analyzed separately. Each experiment was analyzed as a 2 x 2 factorial in a completely randomized design, with two cultivars and two rootstocks. A two-way ANOVA was performed, to test main effects and the interaction. For

cell wall thickness above the graft union of the Washington nursery trees, the interaction was significant. In this case the testInteractions function from the R package "phia" (Martinez, 2015) was used to compare rootstocks within each cultivar and to compare cultivars within each rootstock.

Xylem Cell Proportions. Six replications of the 'Honeycrisp' and 'Zestar!' combinations were utilized in this experiment. Samples were sectioned, stained, and imaged at 200x magnification using the same microscope/camera/software system previously described. Xylem cells were divided into three tissue types based on their function within the wood: fibrous tissue, parenchymatous tissue, and conductive tissue. Percentages of the three types of tissue were determined using ImageJ image analysis software (National Institutes of Health, Bethesda, Maryland) (Rasband, 2014). The parenchymatous and conductive cells were traced manually, while fibrous tissues were estimated by subtracting the two former measurements from the total area of the photomicrograph. Statistical analysis was performed using the aov command in R as previously described.

Laser Ablation Tomography. Four replications of each of the 'Honeycrisp' and 'Zestar!' combinations were used. After the initial sample preparation procedure, sections were cut to a width of 2.5cm to fit within the field of the laser beam. Sections were stored in 70% ethanol for at least one week, and were ablated using an AVIA 7000 355mm pulsed laser (Coherent Inc., Santa Clara, CA). Images were taken at 100.0µm intervals to either 2.5cm or 3.0cm in length from top to bottom. Images were captured using a Canon® T3i camera (Canon Inc., Tokyo, Japan) with a Canon MP-E 65mm 5x micro lens, reduced to 1x zoom to capture a greater field of view.

Images were stacked to create 3D models of the sections using Avizo™ imaging software, (FEI Company, Hillsboro, OR). Samples were visually inspected for the development of callus parenchyma tissue, irregularly oriented xylem, and areas of necrosis.

Results and Discussion

Fiber Cell Walls. In the Pennsylvania trees, the type of rootstock and cultivar had a significant effect on cell wall thickness in different regions of the tree, and the interactions were not significant (Table 1). Tree combinations on 'M.26 EMLA' had thinner cell walls than those on 'M.7 EMLA' below and at the graft union (Table 2). 'Honeycrisp' combinations had thicker cell walls than 'Zestar!' at the union.

For the Washington nursery trees, the type of rootstock significantly affected cell wall thickness (Table 1). Trees grafted to 'G.41' had thinner cell walls below and above the graft union. There were no significant differences at the graft union. Cell wall thickness differed significantly between cultivar treatments above the graft union, as trees of the 'Scilate' cultivar produced thinner fiber cell

Table 1. *P*-values from analysis of variance for rootstock (R) and cultivar (C) effects on fiber cell wall thickness 7cm below, at, and 3cm above the graft union in tree combinations from Pennsylvania and Washington nurseries.

Nursery	Treatments and Interactions	Below the Union	At the Union	3cm Above the Union
Pennsylvania	R	0.004**[z]	<0.001***	0.938
	C	0.412	0.029*	0.110
	R*C	0.186	0.422	0.875
Washington	R	<0.001***	0.163	0.017*
	C	0.158	0.324	0.021*
	R*C	0.911	0.569	0.021*

[z] Significant statistical differences are indicated by asterisks: *p<0.05, **p<0.01, ***P<0.001.

Table 2. Mean fiber cell wall thicknesses (µm) 7.0cm below, at, and 3.0cm above the unions of Pennsylvania nursery graft combinations by rootstock and cultivar.

	7cm Below	At Union	3cm Above
Rootstock			
'M.7 EMLA'	3.81a[z]	3.97a	3.88
'M.26 EMLA'	3.50b	3.66b	3.87
Cultivar			
'Zestar!'	3.61	3.72b	3.79
'Honeycrisp'	3.69	3.91a	3.96

[z] Means followed by different letters within a column indicate significant differences as determined by the ANOVA F-value at p=0.05.

Table 3. Mean fiber cell wall thicknesses (µm) 7.0cm below, at, and 3.0cm above the unions of Washington nursery graft combinations by rootstock and cultivar.

	7cm Below	At Union	3cm Above
Rootstock			
'M.9'	3.81a[z]	3.58	3.69a
'G.41'	3.31b	3.34	3.33b
Cultivar			
'Cripps Pink'	3.47	3.54	3.68a
'Scilate'	3.65	3.38	3.33b

[z] Means followed by different letters within a column indicate significant differences as determined by the ANOVA F-value at p=0.05.

Table 4. Analysis of interaction means for rootstock and cultivar effects on mean fiber cell wall thickness (µm) 3cm above the graft unions of Washington nursery trees. *P*-values are from ANOVA tests of each rootstock within each cultivar, and each cultivar within each rootstock.

Rootstock	'Cripps Pink'	'Scilate'	P-value
M.9	4.04	3.34	0.004*
G.41	3.33	3.33	0.992
P-value	0.003*	0.946	

[z] Significant statistical differences are indicated by asterisks: *p<0.01.

walls than 'Cripps Pink' (Tables 1 and 3). There was an interaction between rootstock and cultivar in the cell wall thickness above the graft union (Table 4). The fiber cell walls in the scion wood of 'Cripps Pink were thinner when grafted on 'G.41' compared to 'M.9', while the fiber walls of 'Scilate' did not differ when propagated on different rootstocks.

In a previous study (Doley 1974), the wall thickness of fiber cells within the scions of the combination 'Cox's Orange Pippin'/'MM.104' were significantly thinner when trees were grafted to the very dwarfing interstock 'M.20'. Our results support the findings that rootstock differences could lead to anatomical changes within other regions of the tree, as fiber cell wall thickness varied above the unions of 'Cripps Pink' when propagated on differing rootstocks.

'M.26 EMLA' produces a more dwarfing tree than 'M.7 EMLA', and is consistent with Doley's findings that dwarfing rootstocks may produce thinner fiber cell walls.

However, 'G.41' produced thinner cell walls than 'M.9', even though these rootstocks are in a similar size category (Marini et al., 2014).

While differences in wall thickness existed above and below the unions, there were few clear trends in the data between cell wall thickness and the combinations that have been reported weak in the field. Combinations on the weaker rootstock 'M.26 EMLA' had thinner cell walls below and at the union, and combinations on 'G.41' had thinner walls below and above the union, but combinations of 'Honeycrisp' had thicker cell walls than 'Zestar!' at the union, even though 'Honeycrisp' is considered the weaker cultivar. These findings suggest cell wall thickness may not be an appropriate measure of union strength in young trees.

Xylem Cell Proportions. Significant differences in the distribution of fiber and parenchyma tissues were observed between rootstock treatments (Table 5). 'M.26 EMLA' combinations contained significantly less fiber and more parenchyma tissue than 'M.7 EMLA' combinations (Table 6). Previous studies have found that more dwarfing rootstocks tend to have higher proportions of parenchyma and fewer fiber cells within their wood (Beakbane and Thompson, 1947), and our results with new cultivars agree with these findings.

Cultivar significantly affected the percentages of wood tissues (Tables 5 and 6). 'Honeycrisp' combinations contained significantly more parenchyma tissue and less fiber and conductive tissues than 'Zestar!' combinations. Like dwarfing rootstocks, the 'Honeycrisp' cultivar is considered a weak growing cultivar (Robinson et al., 2011), and may help to explain its decreased production of fiber cells at the union compared to trees of the 'Zestar!' cultivar.

The combination of 'Honeycrisp'/'M.26 EMLA' had the most parenchyma tissue and the least fiber (47.11 and 46.08 percent respectively), whereas the combination of 'Zestar!'/'M.7 EMLA' had the least parenchyma and most fiber (22.29 and 65.65 percent, respectively). The ratio of parenchyma to fiber cells in the 'Honeycrisp'/'M.26 EMLA' combination was 1.02, while

Table 5. *P*-values from analysis of variance for rootstock (R) and cultivar (C) effects on the proportions of parenchymatous, fibrous, and conductive tissue at the unions of tree combinations from Pennsylvania nurseries.

Treatments and Interactions	Parenchymatous	Fibrous	Conductive
R	0.021*[z]	0.041*	0.362
C	0.001**	0.012*	0.017*
R*C	0.967	0.775	0.517

[z] Significant statistical differences are indicated by asterisks: *p<0.05, **p<0.01.

Table 6. Percentages of wood tissues by rootstock and cultivar in the graft unions of the Pennsylvania nursery trees.

	Parenchyma	Fiber	Conductive
Rootstock			
'M.7 EMLA'	29.78b[z]	59.61a	10.61
'M.26 EMLA'	39.79a	50.98b	9.23
Cultivar			
'Zestar!'	27.38b	60.76a	11.85a
'Honeycrisp'	42.19a	49.83b	7.98b

[z] Means followed by different letters within a column indicate significant differences as determined by the ANOVA F-value at p=0.05.

other combinations varied from 0.34 to 0.70.

An increase in the amount of parenchyma relative to fiber cells at the union may create a weak point at the union where trees are more likely to break (Warmund et al., 1993). However, since dwarfing rootstocks are prone to producing less fiber cells, this may have caused the difference we saw between our study trees. This complication suggests this method may not be useful when comparing rootstocks across different size and vigor categories. Our subsequent study also found that tissues at the union can be very variable, making this method unlikely to be useful for determining future weak scion/rootstock combinations.

Laser Ablation Tomography. Callus parenchyma tissue was present in all combinations between the rootstock and scion (Figure 2 & 3). Swirling tissue was

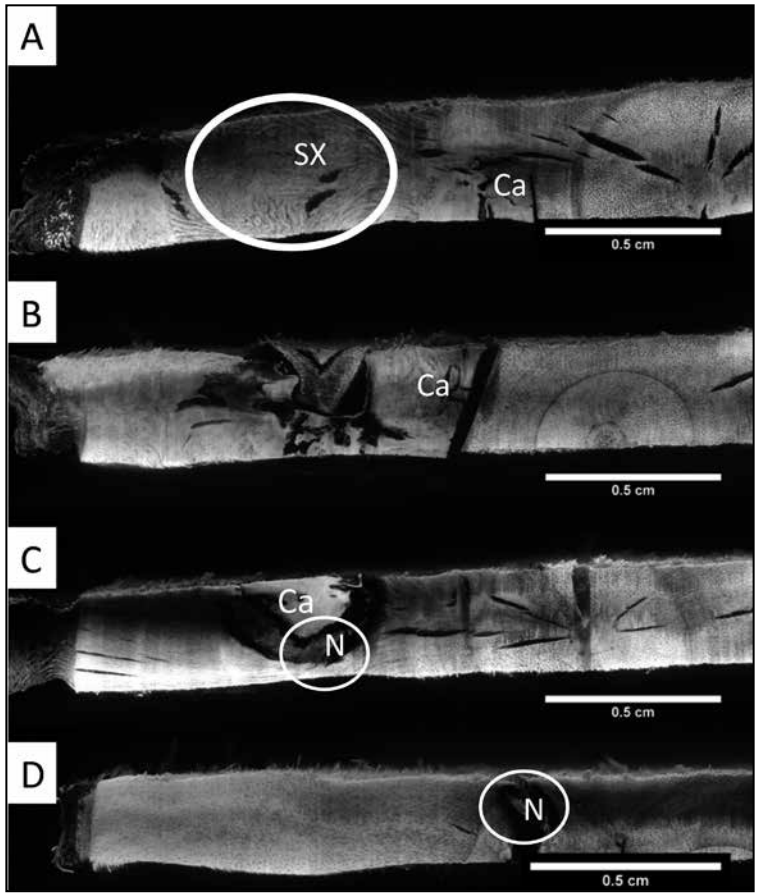

Figure 2. Transverse sections of wood from 'Honeycrisp'/'M.26 EMLA' (A) 'Honeycrisp'/'M.7 EMLA' (B) 'Zestar!'/'M.26 EMLA' (C) and 'Zestar!'/'M.7 EMLA' (D) with the scions on the left and rootstocks on the right. The wood tissue of 'Honeycrisp'/'M.26 EMLA' shows a large area of swirling xylem (SX) tissue within the subsequent year of growth. In 'Honeycrisp'/'M.7 EMLA', necrotic wood (N), callus tissue (Ca), and bark-like tissue can be seen. In 'Zestar!'/'M.26 EMLA', an area of necrosis surrounded by callus tissue can also be observed. 'Zestar!'/'M.7 EMLA' also shows a small section of bark-like necrotic tissue. Fragments of the callus tissue that initially bridged the gap between the rootstock and scion can be seen within the unions of 'Honeycrisp'/'M.26 EMLA' and 'Honeycrisp'/'M.7 EMLA'.

commonly observed in the scion adjacent to the union and in areas of callus parenchyma proliferation. A very large section of swirling xylem extended into the following season's growth in one sample of 'Honeycrisp'/'M.26 EMLA' (Figure 2A).

For 'Honeycrisp'/'M.7 EMLA', 'Zestar!'/'M.26 EMLA', and 'Zestar!'/'M.7 EMLA', one sample of each contained a large area of necrotic tissue. For 'Honeycrisp'/'M.7 EMLA', the tissue around this necrotic wood consisted mostly of callus tissue, which extended towards the outer growth of the union. 'Honeycrisp'/'M.7 EMLA' also appeared to have a few large areas of parenchyma tissue. Tissue that resembled bark was also

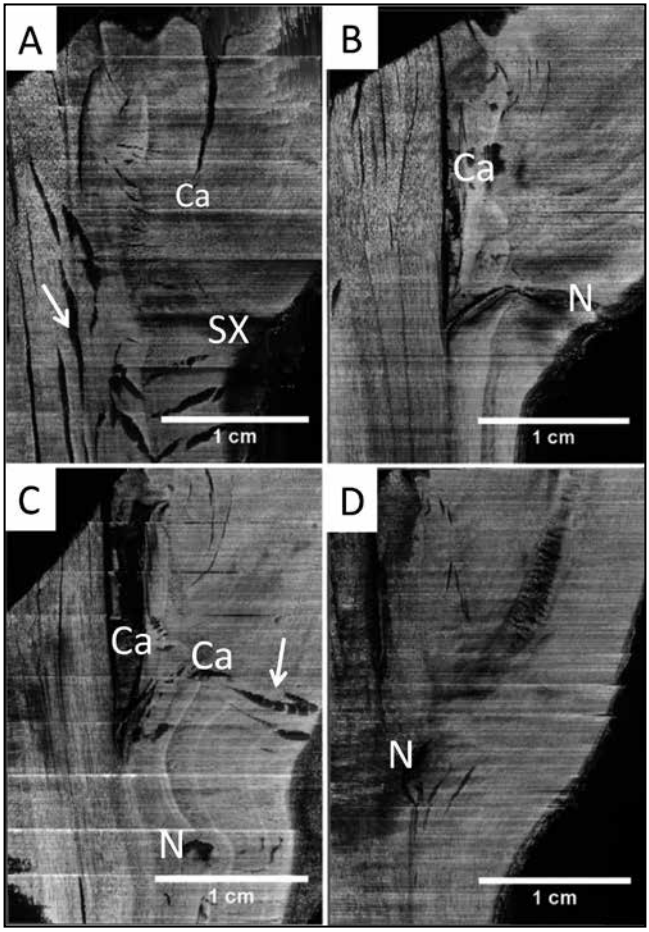

Figure 3. Unions of 'Honeycrisp'/'M.26 EMLA' (A), 'Honeycrisp'/'M.7 EMLA' (B), 'Zestar!'/'M.26 EMLA' (C) and 'Zestar!'/'M.7 EMLA' (D) in longitudinal view with the rootstock on the left and the scion portions on the upper right. Swirling xylem (SX) appears at the middle of the union extending towards the bark in 'Honeycrisp'/'M.26 EMLA'. 'Honeycrisp'/'M.7 EMLA', 'Zestar!'/'M.26 EMLA', and 'Zestar!'/'M.7 EMLA' appear to have isolated areas of necrosis (N). Callus tissues (Ca) and empty spaces surrounding them between the rootstock and scion can be easily distinguished in 'Honeycrisp'/'M.7 EMLA' and 'Zestar!'/'M.26 EMLA'. The wood tended to split at this callus layer during the ablation process, producing these gaps. An additional small area of callus is seen in 'Zestar!'/'M.26 EMLA'. Open spaces further down the union of 'Honeycrisp'/'M.26 EMLA' and in 'Zestar!'/'M.26 EMLA' (arrows) were very thin gaps also likely caused by the ablation process.

present (Figure 2B and Figure 3B). In one 'Zestar!'/'M.26 EMLA' sample, the vascular system had a small region of callus disrupting the xylem at the union, though normal xylem growth soon began to differentiate from it (Figure 3C). A region of necrotic tissue surrounded by wound callus was also observed further down the union as well (Figure 3C). A sample of 'Zestar!'/'M.7 EMLA' had a necrotic zone where new wood tissue was growing around what appeared to be remnant bark material (Figure 2D).

In terms of previous descriptions of incompatibility provided by Mosse (1962) and Andrews and Serrano Marquez (1993), we found a large area of swirling xylem tissue within the wood of one sample of 'Honeycrisp'/'M.26 EMLA', but also found regions of poor differentiation in the other combinations that are not prone to breaking in the field. Warmund et al. (1993) and Milien et al. (2012) found regions of vascular discontinuity within poor growing graft unions of apple and grape, but our observations suggest it may be difficult to determine union continuity and strength based on anatomical observations alone when trees are young in the nursery, as the tissues are still very variable across the scion/rootstock combinations, and irregularities in the wood can be found in weak and strong combinations.

We were unable to achieve cellular resolution using laser ablation tomography due to the size of our samples. While cellular level traits can be determined on small samples, such as maize roots (Chimungu et al., 2015), the size of the unions and the woody tissue made samples difficult to ablate and image to achieve cellular resolution.

Conclusions

The anatomical features of weak wood in three commercially important scion/rootstock combinations were investigated using light microscopy, laser ablation tomography, and imaging software. This is the first such report for a Geneva rootstock and for three new cultivars.

Fiber cell wall thickness varied between rootstocks below, at, and above the graft unions, and varied between cultivars at the union. Trees on 'M.26 EMLA' had thinner fiber cell walls below and at the union, and trees on 'G.41' rootstocks had thinner fiber cell walls below and above the union. However, the weak cultivar 'Honeycrisp' had significantly thicker fiber cell walls at the union than the strong variety 'Zestar!', suggesting that fiber cell wall thickness may not be useful for determining weaknesses in young nursery trees.

Scion/rootstock combinations tended to have less fiber cells at the graft union when propagated on 'M.26 EMLA' rootstocks and when 'Honeycrisp' was the cultivar. However, since we did not have a strong graft combination on a dwarfing rootstock to compare against, it is difficult to determine if strong, more dwarfing combinations would have more or less fiber cells. Additionally, as our laser ablation study suggests, tissues at the graft union can be extremely variable at a young age, making this method an unlikely candidate for determining graft strength of future scion/rootstock combinations.

Laser ablation tomography provided a larger view of the union, and showed that characteristics commonly described as features of weak combinations could be observed in some combinations not prone to graft failure in the field. Laser ablation tomography appears to be an unsuitable method for observing the cellular level anatomy of large samples of woody tissue.

The proceeding experiments suggest that while many anatomical variables have been associated with the development of weak unions, these factors may be difficult to interpret due to the variability of the tissues at the graft union in young nursery trees.

Acknowledgment

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About The Cover:

Rubus parvus Buch and *Rubus* Hybrid 'Triple Crown' Blackberry

Rubus parvus Buchanan fruit and leaves in the center. Rubus hybrid 'Triple Crown' blackberry on left and right. Both are tetraploid (2n = 4x = 28) chromosomes, though *R. parvus* has a much smaller genome.

Photo by Kim Hummer.