Proc. Fla. State Hort. Soc. 127:33–36. 2014.

Enzyme Digestion of HLB-infected Tissues: A Better Approach to Study Phloem Anatomy

Naveen Kumar, Craig Brodersen, Cody Narciso, Ed Etxeberria*

University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred, FL 33850

Additional index words. Citrus phloem, pit fields, sieve elements, sieve plates, sieve pores

At anatomical level citrus greening (HLB; Huanglongbing) is characterized by excessive deposition of starch, p-proteins, and callose in phloem tissue of various plant parts. The presence of these polysaccharides and proteins often obstruct observations of key anatomical details of phloem tissue. In the present investigation, we evaluated the effect of carbohydrate and protein metabolizing enzymes on leaf tissue aimed at studying ultra-structural details of phloem that remain hidden in HLB-infected phloem tissue. The HLB-affected 'Valencia' orange leaf samples were collected from the groves of CREC, Lake Alfred, Florida. Leaf petioles were detached, immediately stored in liquid nitrogen and transported to the laboratory. The petioles were kept in superchilled ethanol (100%) and stored in –20°C freezer for two days. Petiole samples were warmed to room temperature for two hours in distilled water and thereafter thoroughly washed. Samples were thin sectioned using a razor blade and again washed with distilled water. Petiole sections were incubated in combination and individually with 0.1% Proteinase K and 0.1% Amylase at various time intervals. After enzymatic treatments sections were washed with 100% ethanol, vacuum dried and prepared for scanning electron microscopy. Enzymatic treatments of samples revealed clear images of phloem tissue with visible pit fields on side walls, sieve tubes, and sieve plates. However, none of these structures were clearly visible in untreated HLB-affected petiole samples. The data fails to explain any possible lateral transmission of *Candidatus* Liberibacter *asiaticus* (*C*Las).

Huanglongbing (HLB; *syn*. citrus greening) is the most devastating disease of citrus. Its presumed pathological agent, *Candidatus* Liberibacter spp. (*C*Las), is a fastidious Gram-negative, obligate parasite, phloem limited α-protobacterium (Wulff et al., 2014). The disease is initially characterized by the accumulation of starch, callose, and phloem proteins in sieve tube elements, followed by their complete collapse (Etxeberria and Narciso, 2012). These deposits within the sieve elements hinder the transport of photo-assimilates and ultimately causes decline in tree health (Brodersen et al., 2014).

Phloem is the least characterized tissue in citrus due to the formation of several artifacts (such as callose and phloem proteins plugs in sieve plates/tubes) during tissue sampling and processing, conditions that limit ultra-structural clarity (Esau and Cheadle, 1961). Resolution becomes further diminished in HLB-infected phloem due to abnormal levels of starch accumulation (Etxeberria and Narciso, 2012) and callose deposition on sieve plates, which has been shown as a protective mechanism against phloem feeding insects and to keep a check on loss of assimilates (Hao et al., 2008).

Observations on the development of symptoms and on the phloem-limited nature of the disease have raised several questions regarding the movement of *C*Las within citrus trees. For example: Can *C*Las move laterally between phloem cells around the vascular tissue? To better understand the relationship between the development of HLB symptoms at the tissue level and the movement of *C*Las within the tree, we proceeded to analyze the ultrastructure of mature phloem tissue. To obtain unobstructed views of phloem cells, we utilized the enzymatic approach of Mullendore et al., 2010. The procedure involved cleaning the phloem tissue of obstructing debris by treating it with starch and protein digesting enzymes. Our approach resulted in improved optical resolution, the data which suggest only longitudinal movement of *C*Las in citrus trees. Primary wall pit fields remained intact in both healthy and HLB-infected phloem tissues, ruling out the possibility of lateral movement of *C*Las in citrus.

Acknowledgments. We thank D. Stenton and P. Gonzalez for technical help in electron microscopy work. This work was financed in part by CRDF grant # 710
*Corresponding author; phone: (863) 956-8787; email: eetxeber@ufl.edu

Materials and Methods

Plant material. Petiole and bark samples of HLB-infected trees were collected from Sweet orange 'Valencia' (*Citrus sinensis* L. Osbeck) trees grown at Citrus Research and Education Center, Lake Alfred, FL. Control samples were obtained from greenhouse grown HLB-free plants. Bark samples were excised using 2mm diameter cork borer. Leaf petioles were removed using a razor blade and immediately placed in liquid nitrogen. The tissue was then transferred to chilled 100% ethanol for 2 h. Plant samples were then sectioned using razor blade and stored for 48 h in 100% ethanol. The ethanol solution was refreshed every 24 h.

After 48 h incubation in 100% ethanol, samples were thoroughly washed in distilled water and treated with 0.1% α-amylase in 10 mM Tris-HCl, pH 7.0 at 60 °C for 48 h on a Thermomixer R (Eppendorf) at 300 rpm followed by incubation in 0.1% proteinase K in 50 mM Tris-HCl, 1.5 mM calcium acetate, 8% Triton X-100, pH ≥8.0 at 60 °C for next 48 h at 300 rpm (Mullendore et al., 2010). Both enzyme solutions were refreshed at an interval of 24 h. Enzyme treated samples were finally washed in distilled water and stored in 100% ethanol until further processing for scanning electron microscopy (SEM).

Tissue preparation for SEM. Plants samples were further dehydrated using Ladd critical point dryer (Ladd Research

Industries, Burlington, VT). Dehydrated samples were mounted on a stub and coated with gold/palladium using a Ladd sputter coater (Ladd Research Industries). Coated tissue samples were then observed under a Hitachi S530 SEM (Tokyo, Japan), and photographed using Cannon Rebel T5i digital camera (Tokyo, Japan).

Results

Effect of enzyme digestion on anatomical resolution. Sections of untreated HLB-affected petioles showed excessive amounts of starch grains in sieve elements and in cortical, phloem and xylem parenchyma cells (Fig. 1A and Fig. 2A). Phloem tissue in advanced stages of HLB was completely obliterated, and impregnated with callose, cytoplasmic contents and phloem proteins, resulting in poor ultra-structural resolution (not shown; Etxeberria and Narciso, 2012). Similarly, large amount of starch granules hindered the view of the lateral walls of sieve elements and parenchym cells (Fig. 1A). Under these conditions, few subcellular characteristics could be observed. Following enzyme digestion, HLB-infected tissue showed high degree of anatomical clarity as displayed in Figs. 1B and Fig. 2B.

Longitudinal sections of leaf petiole showed both sieve elements and companion cells (Fig. 2). After enzyme treatment, wall depressions containing pit fields could be observed in lateral walls of both sieve elements and companion cells (Fig. 2B). Cortical tissue were also free from starch grains and showed large numbers of pit fields (Fig. 2B).

Ultrastructural details of sieve elements of healthy and HLB-affected petiole in longitudinal sections. Longitudinal sections of healthy (Fig. 3A) and "early" HLB-infected petioles (Fig. 3B and C) revealed the presence of abundant pit fields in the lateral walls of sieve elements. The number of pit fields was variable, with some walls containing few (Fig. 3C) while others were completely devoid of pit fields (Fig. 3A). It is worth noting that the primary wall of pit fields was intact in both healthy and HLB-infected petioles. At this intermediate stage of HLB, the walls of SE were thickened and ridged in comparison to healthy petioles.

Ultrastructural details of sieve elements in transverse sections of healthy and HLB-infected petiole. Transverse section of phloem tissue from healthy leaves corroborate earlier observations (Achor et al., 2010; Brodersen et al., 2014; Etxeberria and Narciso, 2012) that healthy phloem is not a continuous ring of sieve elements (Fig. 4A). Instead, individual bundles are separated by elongated ray parenchyma cells (Fig. 4A). In early stages of HLB-affected tissue, a part of the phloem was degenerated and crushed near the cortex (Fig. 4B). At this early stage, metaphloem tissue was still clearly distinguishable in the form of sieve cells and companion cells (Fig. 4B). Often times, sieve elements were seen side by side (Fig. 4A, B) and occasional in groups of up to four. Sieve plates were often seen in digested tissue and positioned at different angles (Fig. 4B), but rarely transversal. The pore size in sieve plates varied from 0.5-0.8 μm (Fig. 4C).

Ultrastructural details of sieve elements in longitudinal lections of HLB-infected bark. Bark tissue was obtained by plucking pieces of bark with the aid of a cork borer and observed form the inside. HLB-affected bark (Fig. 5A) showed similar characteristics to those observed in LS of HLB-affected petiole (Fig. 3B). Several SEs were arranged side by side. Sieve plates and pit fields were distinct, and primary wall appeared intact in these pit fields (Fig. 5B). The size of pit fields varied from 2–5 μm (Fig. 5C).

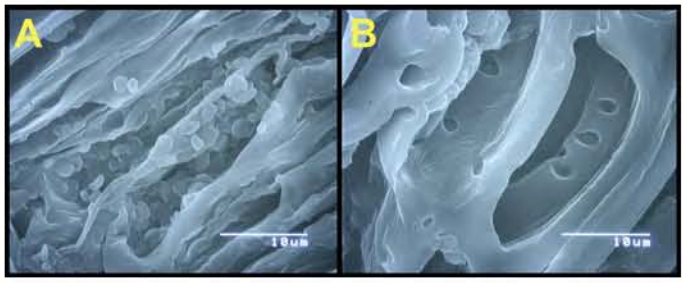

Fig. 1. (**A**) SEM of transverse section of HLB-affected petiole without enzyme digestion. (**B**) SEM of transverse section of HLB-affected petiole with enzyme digestion showing the removal of cellular debris.

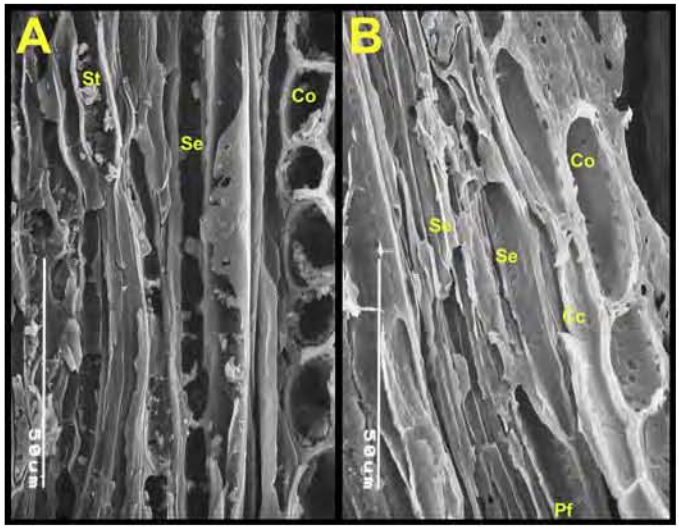

Fig.2 (**A**) SEM of longitudinal section of HLB affected petiole without enzyme digestion. St: sieve tube, Se: sieve element, St: starch grain, Co: cortex, Cc: companion cell. (**B**) SEM of longitudinal section of HLB-affected petiole with enzyme digestion. Pf: pit field.

Discussion

Citrus greening is characterized by abnormally high levels of starch accumulation in cortical, phloem and xylem parenchymatous tissues (Fig. 1A and 2A; Etxeberria et al., 2009). In addition,

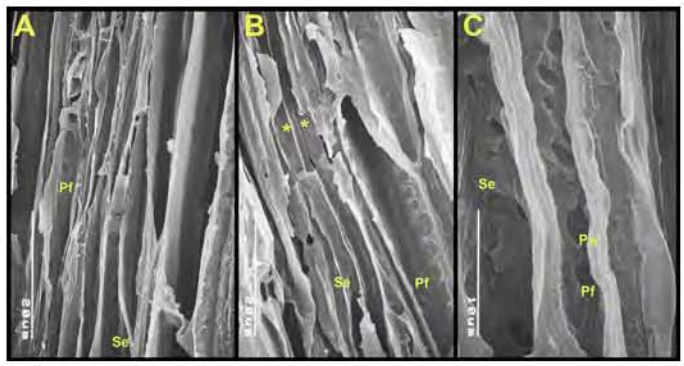

Fig. 3. (**A**) SEM of longitudinal section of healthy petiole with enzyme digestion. (**B**) SEM of longitudinal section of HLB-affected petiole with enzyme digestion. * no pit fields. (**C**) SEM of longitudinal section of HLB-affected petiole with enzyme digestion. Se: sieve element, Pf: pit field, Pw: primary wall.

psyllid (*Diaphornia citri*)-induced phloem damage further causes deposition of callose and phloem protein in sieve elements (Fig. 2A; Achor et al., 2010) as a mechanism to prevent sugar loss (Mullendore et al., 2010). All these inclusions obstruct a clear view of the ultrastructural properties of phloem cells needed for a detailed examination of potential pathways for *C*Las movement within the citrus tree. In order to achieve sufficient clarity in HLB-affected tissues, we applied a modified tissue enzymatic digestion procedure described by Mullendore et al (2010). Tissue samples were treated with 0.1% α amylase and 0.1% proteinase K after sectioning, with enzyme solutions refreshed every day. This modification reduced the sample processing time by 8 d in comparison to original method (Mullendore et al., 2010). The enzymatic treatment made the tissue free of starch, cell wall proteins, cytoplasmic components and phloem proteins, and thus facilitated higher levels of anatomical resolution during electron microscopy examination of tissue samples (Fig. 1B and 2B). Following enzymatic digestion, constituents of phloem tissue could be differentiated into sieve elements and companion cells (Fig. 2B, 3B, 4 A,B). Cortical cells with numerous pit fields were also visible in these sections (Fig. 2B, 3A). In petioles, xylem and phloem tissues were separated by a discontinuous ring of cambium cells (Fig. 3A). Similarly, vascular bundles were also separated by elongated cell of ray parenchyma that also contained abundant pit fields. Large number of pit fields in cortical, companion and ray parenchyma cells in addition to SE supports the possible alternate route of assimilate transport in HLB-affected plants that partly explains the bearing and development of fruits in phloem blocked HLB-affected trees (Etxeberria and Narciso, 2012).

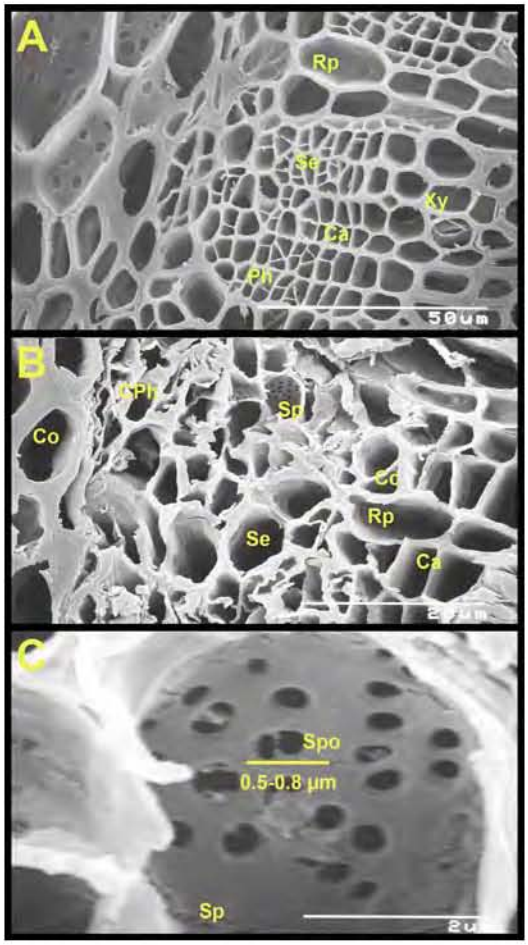

Fig. 4. (**A**) SEM of transverse section of healthy petiole with enzyme digestion. Co: cortex, Ph: phloem, Rp: ray parenchyma, Se: sieve element, Ca: cambium, Xy: Xylem. (**B**) SEM of transverse section of HLB-affected petiole with enzyme digestion. Co: cortex, CPh: crushed phloem, Rp: ray parenchyma, Se: sieve element, Sp: sieve plate, Cc: companion cell, Ca: cambium. (**C**) SEM of transverse section of HLB-affected petiole with enzyme digestion showing sieve plate. Spo: sieve pore.

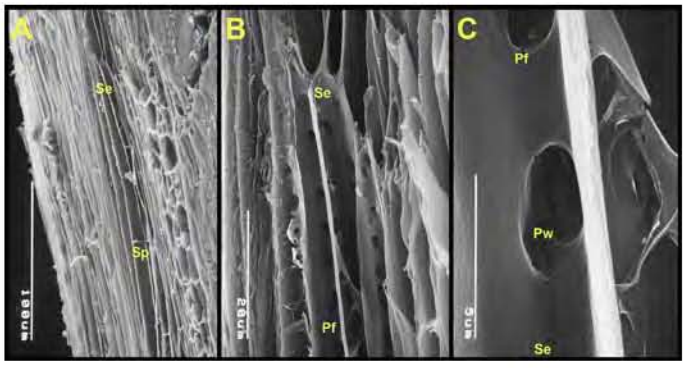

Fig. 5. (**A,B,C**). SEM of longitudinal section of HLB-bark with enzyme digestion. Se: sieve element, Sp: sieve plate, Pf: pit field, Pw: primary wall.

Patterns of cytoplasmic connections between sieve elements and adjacent cells were consistent throughout our study. The number of pit fields in lateral walls of SE sieve was variable (Fig. 4A, B, C) with some walls completely devoid of pit fields, a condition likely dependent on the functionality of neighboring cells. Most of the plasmodesmatal connections in SE exist between SE and CC (Van Bel, 2003), while there is no symplastic continuity at the interface of SE and phloem parenchyma (Kempers et al., 1998).

A leading observation of this study was that the primary wall of lateral pit fields in sieve elements from HLB-affected SEs remained intact. This observation, the first of its kind, carries significant implications to the study of HLB spread within a tree. Considering that HLB-affected cells retain the integrity of pit fields, these observations imply that, even if present, *C*Las does not move laterally from sieve element to sieve element. This also implies that collapse of phloem tissue is not directly associated with the presence of the bacteria within the cell, but with a signal yet to be identified that initiates the obliteration of the phloem tissue.

Several sieve plates were also visible in transverse and longitudinal section of petiole and bark respectively (Fig. 3B). The morphology and anatomy of sieve plate is vital for plants (Thompson, 2006) because sieve plates chiefly govern the longitudinal movement of mass within sieve tubes. In our samples, the size of sieve pores was fairly homogeneous ranging from 0.5–0.8 μm (Fig. 3C) and appeared randomly arranged. In some crop species, (Phaseolus and Cucubita) sieve pore are arranged in a definite pattern; larger pores are in the center and smaller at the periphery (Mullendore et al., 2010) to facilitate laminar flow. We did not find any such arrangement of sieve pores in sieve plates of sweet orange 'Valencia'.

Recently, Su (2008) showed the occurrence of various forms of *C*las in lateral veins of HLB-affected Ponkan (*Citrus reticulata* Blanco) leaves, ranging from spherical to elongated/rod shaped. The size of the elongated forms varied from of 2 to 4 μm (Fujikawa et al., 2013), which is much larger than the average sieve pores presented here (Fig. 3C). Of the several forms, only the rod-elongated shaped can pass through sieve pores via a 'squeeze' mechanism yet to be defined (Sue, 2008). This

mechanism likely involves the reconfiguration of cytoplasm to reduce the size of bacterium.

Conclusions

In summary, enzymatic digestion enhanced the optical resolution of HLB-affected tissues and allowed clear views of ultrastructural details. Our anatomical evidences support longitudinal movement of *CLas* in phloem through sieve pores. However, we found no evidence in support of a lateral movement of *C*Las between sieve elements. Several anatomical and physiological characteristics support a restricted lateral movement of *C*Las: 1) Primary walls of pit fields remain intact in HLB-affected sieve elements and CC (Fig. 5B and 5C); 2) the *C*las lacks cell wall modifying enzymes like cellulase, pectinase, xylanase, and endogluconase (Wulff et al., 2014); 3) the size of plasmodesmatal connections (Terry and Robards, 1987) is magnitudes smaller than the minimum size of *C*Las; and 4) there is no symplastic continuity between SE and phloem parenchymatous cells (Kempers et al., 1998) as they form tangential barriers. Based on the above data: 1) *C*las can move longitudinally in phloem tissue, although movement of *C*Las through sieve plates is highly impaired; and 2) *C*Las appears not to be able to move laterally from between phloem elements. Lateral movement of the disease is likely transported by other transmissable elements.

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