Plant Pathology Journal

ISSN 1812-5387

science alert

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Plant Pathology Journal 9 (2): 56-64, 2010
ISSN 1812-5387

Sequence of Anatomical Symptom Observations in Citrus Affected with Huanglongbing Disease

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Abstract: This study was undertaken to develop a better understanding of the relationship between symptom development and the nature of the citrus disease, huanglongbing. The most characteristic symptom of huanglongbing (HLB) is the non-symmetrical mottled chlorosis of leaf blades. Starch accumulation and phloem collapse have been associated with symptom development in this disease presumed to be caused by *Candidatus* Liberibacter asiaticus. Several hypotheses regarding phloem disruption to starch accumulation to chlorosis evolved concerning symptom development. These were tested using light and Transmission Electron Microscopy (TEM). Samples collected and fixed for TEM with various stages of HLB symptoms, revealed the following: starch accumulation occurred after phloem plugging and cell collapse and therefore, localized carbohydrate deficiency may be a factor. Starch packing of chloroplasts did not rupture the outer membranes, but the inner grana structure was disrupted thus, leading to chlorosis. This occurred only in parts of the leaf where phloem plugging occurred. Sieve elements were obstructed by both amorphous and filamentous materials and both occurred in readily observed amounts, while bacteria were insufficient to directly cause plugging. The amorphous material was positively identified as callose by immunoassay with gold labeling. Phloem protein 2 was identified in the filamentous plugging material using immunoassay with gold labeling. This information supports the development of HLB symptoms in the following sequence: phloem plugging and necrosis with cell wall swelling of sieve elements and companion cells followed by some phloem cell collapse, presumed sugar backup in localized leaf blade areas leading to starch accumulation until chloroplast structure is disrupted with resulting chlorosis.

Key words: Sieve element plugging, phloem necrosis, starch accumulation, chlorosis, callose, phloem protein, *Candidatus* Liberibacter asiaticus

INTRODUCTION

Huanglongbing (HLB) is a highly destructive citrus disease recently found in Florida. The disease probably originated in Southeastern Asia and was commonly found in China by 1925 (Lin, 1956). Linked to a fastidious, gram-negative, phloem-limited bacterium (*Candidatus* Liberibacter sp.) that recently may have been cultured to meet Koch's postulates (Schaad *et al*., 2009), the disease is devastating and seriously affecting citrus production in Asia and Africa and most recently in Brazil, Cuba and the US. There are three known strains of the bacteria associated with HLB: i.e., *Candidatus* Liberibacter asiaticus, *Ca.* Liberibacter africanus and *Ca.* Liberibacter americanus (Bove, 2006). Recently, a phytoplasma also has been associated with citrus trees showing HLB-like symptoms in Southern China (Chen *et al*., 2009). However, a comprehensive study of bacterial diversity associated with HLB-diseased citrus trees indicated *Ca.* Liberibacter asiaticus as the putative pathogen responsible for HLB disease in Florida (Sagaram *et al*., 2009).

When trees are presumably infected with HLB, some leaves develop asymmetric chlorotic mottling (blotchy mottle) over all or part of the leaf blade. On chronically infected trees, secondary growth leaves are small and often totally yellow, or show green or corky veins with chlorotic interveinal areas (http://www.crec.ifas.ufl.edu/extension/greening/symptoms.htm). Fruit on infected trees are frequently small, misshapen and poorly colored, hence, the origin of the alternate name greening. The juice of symptomatic fruit is low in quality and abnormally bitter (http://www.crec.ifas.ufl.edu/extension/greening/symptoms.htm).

HLB can be tentatively identified in the field by foliage and fruit symptoms. Until 1992, TEM visualization of the bacterium in the sieve elements of blotchy mottle leaves was the most reliable method for diagnosis of the

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disease (Garnier and Bove, 1983). However, the bacteria were difficult to find in citrus samples by microscopy methods. Since the bacteria were found to grow in larger numbers in periwinkle, this came to be the most efficient source of the bacteria, as culturing the bacteria had not been achieved. Jagoueix *et al.* (1994) showed through hybridization and Polymerase Chain Reaction (PCR) experiments that the HLB bacteria could be identified most efficiently through PCR methods (Bove, 2006). Currently, more precise diagnosis is performed by real time PCR and by finding the bacterium in the leaf phloem tissue using TEM (transmission election microscopy).

In a study of symptom ontology in South Africa using Light Microscope (LM), the presence of large amounts of starch accumulation and phloem collapse were observed in leaves of sweet orange with typical HLB symptoms (Schneider, 1968), although presence of the HLB or citrus greening pathogen in the phloem was not confirmed. These reports need to be verified because new information has come to light indicating that *Phytoplasma* sp., can cause similar symptoms to HLB in citrus in Brazil and China (Teixeira *et al*., 2008; Chen *et al*., 2009).

The HLB at an advanced stage can be detected by several iodine tests for starch accumulation. For example, the underside of the test leaf can be scratched with cloth back sandpaper and the sand paper placed in the iodine solution (Tetsuya *et al*., 2007), or the cut edge of the leaf can be submerged in the iodine solution for 30 sec to 1 min (Etxeberria *et al*., 2007) to reveal a dark iodine-starch reaction.

In Indonesia, the disease was named vein phloem degeneration (Tirtawidjaja *et al*., 1965). The HLB-associated phloem blockage apparently results from plugged sieve pores rather than the HLB bacterial aggregates, since, *Ca*. Liberibacter asiaticus does not occur in large numbers or form aggregates in citrus phloem sieve elements (Garnier and Bove, 1983; Kim *et al*., 2009). Kim *et al.* (2009) showed the presence of callose plugs (aniline blue staining under light microscopy view) in the phloem sieve elements of HLB infected plants. In addition, a related genomic study revealed that the p-protein 2 (PP2) gene was highly up-regulated in HLB-infected leaves. P-protein 1 and 2 and callose are involved in the plugging of sieve tube pores during wounding (Dinant *et al*., 2003; Knoblach and van Bel, 1998). The PP2 lectins have been reported to plug sieve elements in Cucurbita species (Read and Northcote, 1983) in response to wounding.

Schneider (1968) proposed that the collapse of the phloem tissue observed in HLB-associated aerial plant samples came secondarily from the necrosis in the phloem elements caused by the disease organism. According to his analysis, the collapse was due primarily to increased phloem development from the cambium compressing the necrotic phloem cells. He also, stated that the accumulation of starch in surrounding cells was due to the necrosis in the phloem impeding the transport of sugars out of the areas of the plant that the necrotic phloem served. A recent study by Kim *et al.* (2009) has shown that phloem disruption, sucrose accumulation and plugged sieve pores were associated with HLB diseased sweet orange concomitant with the presence of *Ca.* Liberibacter asiaticus. Those anatomical data were associated with up-regulation of four key starch biosynthetic genes including ADP-glucose pyrophosphorylase, starch synthase, granule-bound starch synthase and starch debranching enzyme which likely contribute to the hyper accumulation of starch in HLB affected leaves, yet genes associated with photosynthesis were not affected. The PP2 gene which encodes the phloem protein 2 was also reported to be up-regulated parallel to callose formation in phloem sieve tubes as indicated from aniline blue staining.

The objectives of this study were to evaluate the relationship between HLB-associated symptom severity with anatomical changes such as leaf starch accumulation and phloem disruption. At the start of this study and at later stages one or more authors hypothesized that: (1) Starch accumulation in leaves resulted from the inability to transport sucrose or other sugars across cell membranes or resulted from disruption of phloem transport, (2) starch accumulation leads to chloroplast disintegration and thus produces chlorosis, (3) phloem necrosis results from bacterial toxins or signals, or from sieve element plugging and carbohydrate deficiency and (4) sieve element plugging results from bacteria accumulation, callose production or from accumulation of gels from up-regulated phloem proteins.

MATERIAL AND METHODS

Plant tissue preparation: For the basic study, samples of leaves and petioles were taken from two sweet orange (*Citrus sinensis* L. Osbeck) cultivars (Valencia, Madam vinus) and grapefruit (*Citrus paradisi* Macfadyen) (Duncan) from grove sites or quarantine green house from early 2008 through most of 2009. The quarantine samples were PCR positive for HLB infection. The field samples showed blotchy mottle symptoms. Controls of the above varieties were either PCR negative or from trees showing no symptoms. To determine, the sequence of chlorosis and sieve element plugging samples were

carefully taken from the transition areas between chlorotic and green areas on blotchy mottled leaves, from totally chlorotic areas and from asymptomatic leaves that were PCR positive as well as control leaves. The samples were prepared for Light Microscopy (LM) and Transmission Electron Microscopy (TEM) using routine preparation techniques. The samples were fixed with 3% glutaraldehyde, post fixed in 2% osmium tetroxide, dehydrated in an acetone series and embedded in Spurr's resin (Spurr, 1969). For LM, one micrometer sections were mounted on a slide and stained with methylene blue-azure A and basic fuchsin (Schneider, 1981). For TEM, 100 nm sections were collected on grids and stained with uranyl acetate and lead citrate. Observations were made using a Leitz Laborlux S LM (Leitz, Germany) and a Morgagni 268 TEM (FEI Company, The Netherlands).

Gold labeling: For identification of the callose plugging, a mouse IgG monoclonal antibody to (1-3)-β-glucan (Biosupplies, Australia Pty Ltd.) was used as the primary antibody. The secondary antibody for EM labeling was a 10 nm gold-labeled Goat Anti-Mouse (GAM) antibody (Sigma-Aldrich). The freeze dried primary antibody was reconstituted in 120 mM NaCl which made the antibody concentration 1 mg mL^{-1} in 10 mM phosphate buffer (pH 7.2), 0.8% sodium chloride and 0.02% sodium azide. The reconstituted antibody was diluted 1:50 in 0.1 M KPO_4 buffer pH 7.2, 0.5 M NaCl, 0.1% BSA, 0.05% Tween 20 and 5% fetal bovine serum. The gold labeled GAM antibody was diluted 1:10 in the same buffer combination.

For the labeling process, samples were collected from PCR positive Rohde Red Valencia, Ruby Red Grapefruit and Eureka Lemon (*Citrus limon* L. Burm. F) grown in a quarantine greenhouse. Valencia samples were collected from trees showing HLB symptoms in a commercial grove. The samples were fixed for 4 h in either Karnovsky's fixative or 3% glutaraldehyde in phosphate buffer only. They were subsequently dehydrated in ethanol, embedded in LR White resin and 100 nm sections were mounted on Formvar coated nickel grids. The grids were first incubated for 30 min at RT on the buffer solution used above to dilute the antibodies. They were then blotted and transferred to (1-3)-β-glucan antibody for 60 min at room temperature, washed 3 X in dilution buffer, blotted, then incubated on gold labeled GAM antibody for 60 min at room temperature. After incubation, the grids were rinsed 3X with dilution buffer and 2X with distilled water before staining with uranyl acetate for 15 min, followed by 5 min in lead citrate. Fresh solutions were made each time.

For the PP2 protein, the EST sequence of PP2 CF831073 was acquired from NCBI. The protein sequence encoding by CF831073 was used for antigen design using Antigen Profiler (Open Biosystems, Inc. (Huntsville, AL)). Antigen sequence LPEDCFAHILSYTSPRDA was synthesized and used for antibody production using a 90 day protocol in rabbit (Open Biosystems, Inc.). The same citrus tissue as described above was sectioned and placed on Formvar coated nickel grids. The grids were pre-incubated for 15 min on 0.1 M potassium phosphate buffer, pH 7.2, .1 M Bovine Serum Albumin (BSA) or for 30 min on phosphate buffered saline with 0.1% acetylated BSA (Aurion BSA-c, Electron Microscopy Sciences). The primary antibody was diluted 20:100 μL in the same buffers. The grids were incubated 2 h in the solution to overnight at 4°C in the respective buffers at this dilution then washed 3X in the above buffer. Gold labeled (10 nm particles) Goat Antirabbit Antibody (GAR) was also diluted 20:100 μL in the above buffers. The samples were incubated on the GAR for 2 h at room temperature then washed 3X in buffer, then 2X in distilled water. The grids were stained as above for TEM.

RESULTS AND DISCUSSION

After much observation of HLB affected Valencia, Madam vinus and grapefruit trees it was determined that the light and TEM results were similar enough to use the results from these varieties interchangeably in the following presentation.

Leaves from healthy trees had little starch accumulated in either the palisade, spongy or epidermal cells (Fig. 1a), whereas accumulation of starch in transition areas of blotchy mottled HLB affected leaves was consistently seen in the spongy mesophyll tissue of the lower leaf area (Fig. 1b). Advanced symptoms were associated with starch accumulation in all of the parenchyma and epidermal cells of the affected leaf (Fig. 1c). The observed starch accumulation first in spongy mesophyll (Fig. 1b) and the extensive loading of cells with starch in severely symptomatic leaves is likely an indication of disease progression and may be the result of better gas exchange near stomata and possibly higher photosynthesis in that area of the leaf.

The starch packing of the chloroplasts (Fig. 2a) did not disrupt the outer membranes (Fig. 2b, d), but the internal structure was compressed and visibly disrupted (Fig. 2d) to the point that the normal grana (Fig. 2c) structure was no longer present. In citrus, visual observation of chlorosis is usually associated with the lack of essential internal thylakoid structure prompted by grana structure breakdown as in Fe, Zn or Mg deficiency

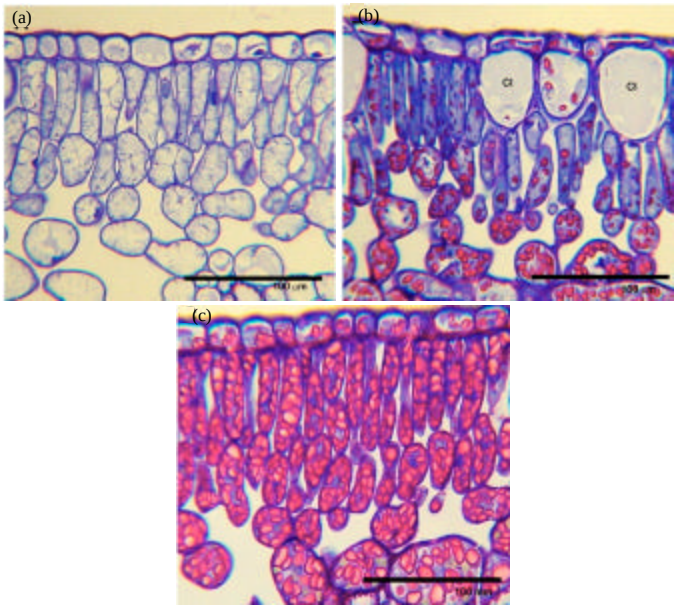

Fig. 1: Progress of starch accumulation in HLB affected leaf, light microscopy. (a) Cross section of healthy leaf showing lack of starch in the palisade layer, (b) cross section of green area of blotchy mottle leaf showing that the starch accumulates in the spongy mesophyll layer first. CI-crystal idioblasts and (c) cross section of chlorotic area of HLB leaf showing starch (red granules) accumulated in palisade layer and adaxial epidermis

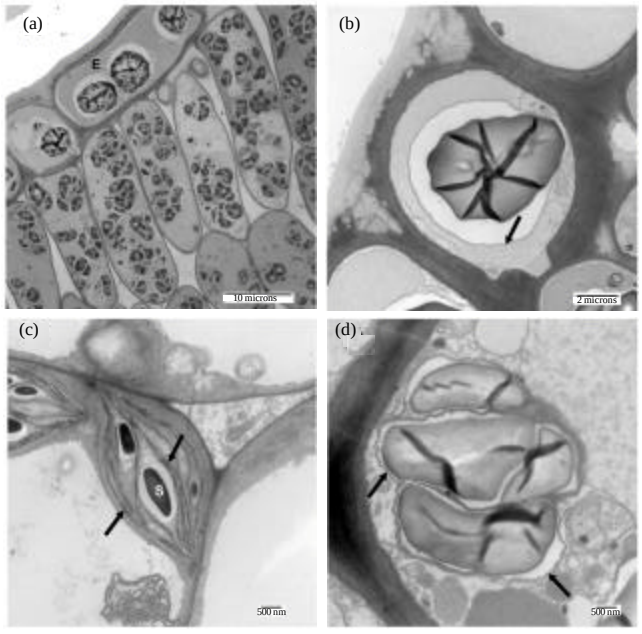

Fig. 2: Relationship of starch accumulation to chloroplast structure: TEM. (a) TEM of palisade layer showing accumulation of starch granules in palisade layer of leaf, cross section. Note large starch granules in the adaxial epidermis (E), (b) large starch granules in the adaxial epidermis showing continuity of outer double membrane (arrow) of the plastid, (c) control chloroplast showing granal stacks and small starch grains and (d) large starch granules in plastid located in spongy mesophyll. Arrows show continuity of double outer membrane of plastid. Note lack of inner membranes

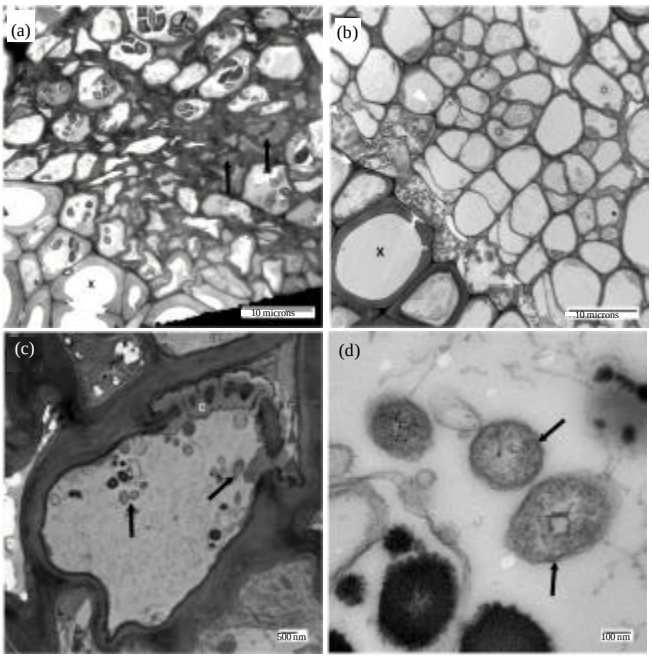

Fig. 3: Collapsed phloem and bacteria presence. (a) Collapsed phloem in HLB affected midvein. Arrows-plugged sieve elements, X = xylem, (b) normal phloem in healthy midvein. X = xylem, (c) sieve element in HLB affected petiole containing what is presumed to be Liberibacter (arrows) Note sieve plate plugged with smooth material (c) and (d) high mag of Liberibacter. Note double outer cell wall (arrows) characteristic of gram negative bacterium

(Hellin *et al*., 1995; Rufner and Barker, 1984; Possingham *et al*., 1964; Thomson and Weier, 1962) or when starch accumulates as the result of branch girdling (Schaffer *et al*., 1986).

No starch accumulation and accompanying chlorosis were seen until there was evidence of the sieve element plugging, phloem necrosis, some cell wall swelling and cell collapse (Fig. 3a), none of which are normally seen in healthy phloem tissue (Fig. 3b). The presumed causal agent of HLB, *Ca*. Liberibacter bacteria, was never seen in high enough numbers (Fig. 3c, d, arrows) to account for the restriction of phloem sap flow, as earlier suggested by Garnier and Bove (1983).

In the present study, we were able to identify two different types of plugging materials obstructing the sieve elements in the phloem of HLB -affected citrus (Fig. 4a, c). One of these plugging materials was amorphous, resembling callose (c) whereas, the other was filamentous (F) in appearance. Amorphous plugging occasionally occurs in healthy tissue (Fig. 4b) but in smaller amounts. This material in healthy tissue will stain with a gold labeled callose specific antibody (Biosupplies, Australia Pty Ltd., original antibody development by Meikle *et al*. (1991). The amorphous plugging associated with HLB and seen in phloem sieve elements also was gold labeled with the callose specific antibody (Fig. 4c), while the filamentous material did not stain under the same circumstances (Fig. 4d).

To the contrary, when tissue samples containing the two types of plugs (Fig 5a and b) were subjected to the gold labeled PP2 antibody, only the filamentous material labeled with gold (Fig. 5c) but not the amorphous, callose, plugs (Fig 5d). Figure 5b shows a sieve element completely filled with the filamentous material. Phloem protein lectin plugs of *Cucurbita maxima* were localized in the phloem by immunochemical methods similar to those used here (Smith *et al*., 1987).

Callose plugging in plant tissues commonly occurs particularly in response to infections such as from fusarium or phytophthora (Benhamou, 1995; Beckman *et al*., 1982; Hinch and Clarke, 1982). The PP2 lectin formation as a form of plugging has been reported in Cucurbita (Read and Northcote, 1983) as part of a disease defense mechanism. They suggest that this lectin immobilizes bacteria and fungi to the cross-linked filaments, which seal wounded phloem sieve-tubes and thus maintains sterility. In our observations, we did not see any evidence of bacteria embedded in either the callose or PP2 plugging materials.

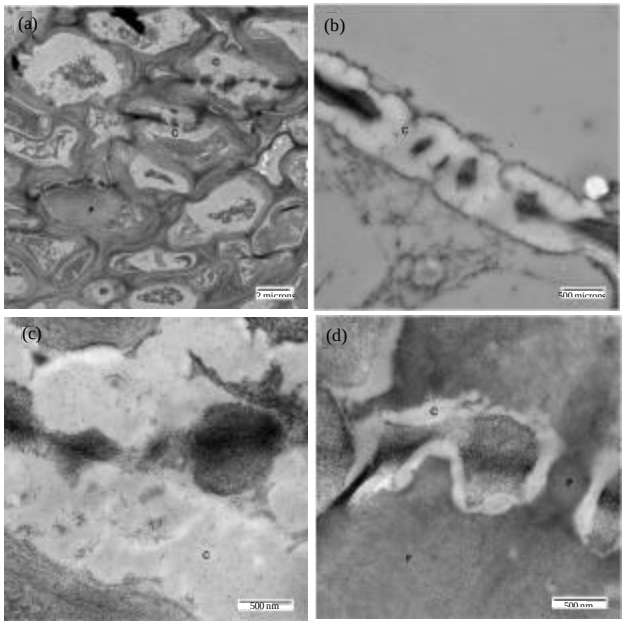

Fig. 4: Presence of callose and fibrillar (filamentous) plugging of sieve elements. (a) Three plugged sieve elements in HLB affected petiole. Two of the elements are primarily plugged with smooth (C) and one with filamentous plugging (F), (b) sieve element plugged with a small amount of callose that is normally seen in healthy phloem. Note the gold labeling from 10 nm gold labeled callose antibody, (c) higher magnification of gold labeled callose plugging as shown in A and (d) higher magnification of gold labeled sieve element showing that the filamentous plugging material (F) does not label with antibody for callose but the lighter, amorphous areas (C) around the sieve pore walls do label. The pore itself (P) is filled with filamentous material

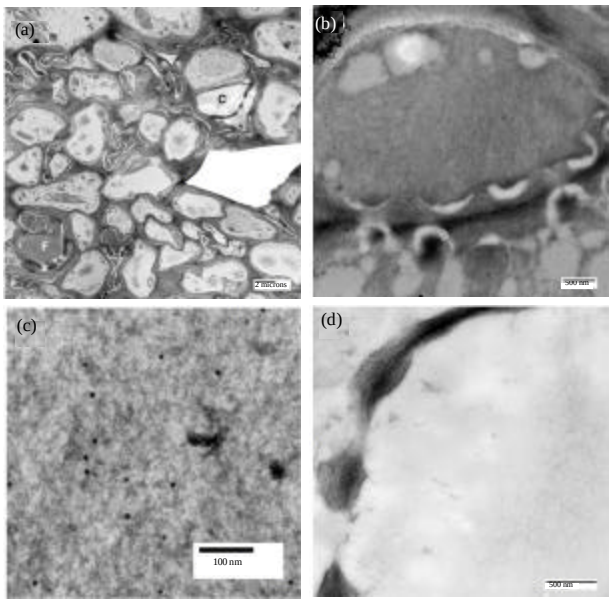

Fig. 5: Phloem protein plugging. (a) Two plugged sieve elements in HLB affected petiole. One element is plugged with filamentous plugging (F) and one with callose (C), (b) filamentous plugging filling sieve element, (c) higher magnification of B showing 10 nm gold labeling attached to PP2 antibody and (d) higher magnification of callose plugging showing no binding to PP2 gold labeled antibody

Our observations agree with earlier study (Garnier and Bove, 1983) and strongly suggest that insufficient bacteria were present to directly cause plugging that would result in the subsequent development of disease symptoms, therefore, some other mechanism of plugging must be operative. One likely mechanism of sieve pore plugging involves PP1, PP2 and callose (Dinant *et al*., 2003; Knoblach and van Bel, 1998). The PP2, a dimeric poly-GlcNAc-binding lectin, covalently cross-links with PP1 via disulphide bonds forming polymers that close sieve pores (Read and Northcote, 1983) and is normally accompanied by the synthesis of the (1,3)β-glucan callose (McNairn and Currier, 1968). Accordingly, microarray analysis comparing HLB infected and healthy sweet oranges (*Citrus sinensis*) indicated that the PP2 gene was highly up-regulated in HLB-infected leaves (Kim *et al*., 2009) and its protein product likely could participate in phloem blockage together with callose. We observed sieve plates plugged with filamentous material (Fig. 4d) and callose plugging of sieve plates also were commonly observed (Fig. 4) substantiating this likely mechanism. Both plugging types were found occuring separately.

Callose deposition is thought to reinforce the cell wall and is regarded as a defense response. It was reported that callose is deposited in cell wall appositions (papillae) beneath fungal infection sites and is thought to provide a physical barrier to penetration (Aist and Bushnell, 1991). However, this assumption is somewhat contradicted since a recent finding indicated that a plant mutation with a deletion of callose synthase, resulting in a loss of the induced callose response, became resistant to powdery mildew rather than more susceptible (Nishimura *et al*., 2003). This could be an example of over expression of callose being responsible for a large part of the disease syndrome. It has been suggested that callose or callose synthase negatively regulates the Salicylic Acid (SA) defense signaling pathway (Nishimura *et al*., 2003). Additionally, callose deposition due to aluminum toxicity has been reported to be responsible for the inhibition of cell-to-cell trafficking of molecules through plasmodesmata (Sivaguru *et al*., 2000).

Overall, the data collected from samples at different stages of visual symptomatology indicate that phloem plugging is a key primary response to HLB infection which culminates in leaf chlorosis. As a consequence of the photoassimilate blockage, leaf chlorosis results as grana are disrupted by the enlarged starch grains. Retention of carbon photoassimilates in aerial parts generates localized tissue starvation of lower structural plant organs, especially roots (Etxeberria *et al*., 2009). Tree collapse from low starch reserves in roots has been demonstrated in Murcott citrus (Smith, 1976). Confirmation of the plugging materials suggests that their over-expression and deposition in the phloem sieve elements is crucial to the mechanism for disease symptom development from the HLB disease. Schneider's (1968), earlier study identified phloem collapse in response to HLB but was not done with modern equipment that could easily show the phloem transport disruption such as the sieve element plugging shown here that accompanies phloem necrosis.

Since the number of observed bacteria was small, why does the plant apparently over-react to the bacteria and produce large amounts of sieve element plugging materials? A chemical signal or toxin produced by the bacteria may stimulate production of beta-glucan and PP2. In bacteria, there are at least six independent secretion systems for various products including toxins, adhesions, hydrolytic enzymes and effectors delivered into the hosts to serve the bacteria (Alfano and Collmer, 1996; Bender *et al*., 1999; Economou, 1999; Filloux *et al*., 2008). Earlier study of phloem-limited phytoplasma bacterium, indicated that multiple proteins were secreted into the host to target developing tissues of plants (Hogenhout *et al*., 2008). It is unknown what virulence factors *Ca*. Liberibacter sp., are using, but knowing these factors seems crucial to further elucidation of the HLB disease process.

Although, this study did not determine if sugar membrane transport was altered, it did show that phloem transport was disrupted by plugging and concurrent phloem collapse. This probably leads to the sugar and starch accumulation. The starch in chloroplasts did not disrupt the outer membranes but did significantly alter internal structure causing chlorosis. The phloem disruption is at least partially the result of callose (smooth) and phloem protein 2 (filamentous) plugging of the sieve elements. The causes of the phloem cell wall swelling and phloem cell necrosis were not determined by this study. Cell starvation below plugged areas may be the primary cause of cell necrosis (Albrigo *et al*., 1981), but bacterial produced toxins could also be causing cell death. This possibility is now under study.

CONCLUSION

Based on the data presented in this communication, several conclusions can be drawn with high degree of certainty on the nature and sequence of HLB symptom development in citrus trees. The distinctive initial blockage of the phloem elements observed in HLB-affected leaves (Fig. 4a, c, f) results from the deposition of two distinctive plugging substances. Our data demonstrate that these plugs are made up of callose

(Fig. 4c) and p-protein (Fig. 5c) material. Simultaneous with the obstruction of phloem transport functions, a structural collapse of this tissue is observed (Fig. 3a). With the disruption of photoassimilate flow, massive starch accumulation takes place in the leaves from an abaxial to adaxial direction (Fig. 1b-c). At its maximum expression, the large size of accumulated starch grains damages the chloroplast grana system resulting in localized chlorosis. As a consequence of carbon sequestration in the leaves, roots starvation occurs resulting in tree decline (Etxeberria *et al*., 2009).

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