Anatomical distribution of abnormally high levels of starch in HLB-affected Valencia orange trees

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The citrus disease Huanglongbing (HLB or citrus greening) is characterized, among other symptoms, by extraordinary levels of starch accumulation in leaves. This condition denotes imbalances in carbohydrate source sink relationship which in turn may have direct implications in the overall health of HLB-trees and in future strategies to manage the disease. Using light, scanning, and transmission electron microscopy we investigated the extent of carbohydrate partitioning imbalances throughout the tree. In all aerial tissues, starch accumulation in HLB-affected trees far exceeded that of HLB-negative control trees. Starch accumulated extensively in photosynthetic cells as well as phloem elements and vascular parenchyma in leaves and petioles. In stems, starch was commonly observed in xylem parenchyma and in the phelloderm of HLB-affected trees but absent from control samples. In contrast, roots from HLB-affected trees were depleted of starch whereas roots from control trees contain substantial starch deposits. The data supports the notion that the substantial changes in carbohydrate partitioning observed throughout the citrus tree may not only be a result of HLB infection, but in itself, a cause for the rapid decline and death of infected trees.

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1. Introduction

Citrus huanglongbing (HLB, or citrus greening) is a highly destructive, fast spreading disease of citrus. The disease is linked to a fastidious, gram-negative, phloem-limited bacterium (Candidatus Liberibacter spp.) [9,13] not yet culturable, although recent attempts have succeeded in partially culturing the organism [6]. Two types of HLB were commonly known: heat-sensitive African form caused by (Candidatus Liberibacter africanus) and heat-tolerant Asian form by (Candidatus Liberibacter asiaticus). A third type (Candidatus Liberibacter americanus) was recently identified in Brazil [27]. In Florida, only Ca. L. asiaticus has been detected [15] and is transmitted by Diaphorina citri, a psyllid vector also found in Louisiana, Texas and in Southern California. The HLB associated bacteria can infect most citrus cultivars, species and hybrids [12] with most sweet oranges, mandarins, and mandarin hybrids severely affected, whereas lime and lemons show less severe symptoms. The bacterium is hosted by a variety of other common ornamental plants [12] making its eradication even a more difficult task and management of the disease more crucial.

Although long established in Eastern Asia and South Africa [2,5], recent findings of HLB in Brazil [4,27] and Florida [11,1] have brought renewed interest on this disease given its devastating potential to their citrus industry. Citrus juice production between Florida and Brazil accounts for over one-third of the world’s output [15]. In Florida, the disease has spread quickly and is now established in all citrus growing counties. In some grove blocks located in the southern part of the state, as much as 80% of trees have been infected with HLB (Mark Colbert, personal communication). Under the present circumstances and pathogen distribution, the disease threatens to decimate all 640,000 remaining acres of the Florida Citrus Industry.

In citrus trees, specific HLB symptoms are difficult to characterize, but leaf blotchy mottle is very specific for the disease. Symptoms such as yellow shoots, leaf blotchy mottle, and lopsided fruits with color inversion and aborted seeds, are all characteristic, but they do not always occur together in the same tree. They can be distorted or masked by symptoms of other diseases, or in some cases, induced by conditions unrelated to HLB [2].

Amongst other HLB-induced characteristics, Schneider [20] noted massive starch accumulation in citrus leaves presumably the result of necrotic phloem pockets scattered throughout the vascular system in the leaf petioles. In his analysis, Schneider [20] theorized that these phloem blockages create a photoassimilate back-log resulting in starch levels ubiquitously observed in leaves of HLB-affected trees. In fact, the excessive starch build-up causing
disintegration of the chloroplast thylakoid system is believed to produce the yellowing leaf mottle symptom. Leaf yellowing resulting from above normal starch accumulation and thylakoid destruction can be artificially induced by branch girdling [19].

Starch content of HLB-affected leaves can be 20 times higher than leaves from control trees [26]. Based on the ability of iodine to bind starch (resulting in a blue/purple-colored solution; [16]), the contrasting levels of starch accumulation in citrus leaves have been used as visual indicator of HLB presence in many locations [17,26] including Florida [8]. The enormous discrepancies in starch content between control and HLB-affected leaves reflect significant variations in the natural balance of carbon source/sink relations. Whether the imbalance in carbon metabolism brought about by HLB infection is restricted to leaf blades or is widespread throughout the plant, may have direct implications in the overall health decline observed in HLB-affected trees and in future strategies to manage the disease. In this report, we analyze in greater detail the tissue distribution of starch in leaves from trees in advance stage of HLB infection. In addition, we further determine starch distribution in other plant parts compared to control trees.

2. Materials and methods

2.1. Plant material

Leaves, young (green) stems, bark and roots of HLB-confirmed 10 year old Valencia orange (Citrus sinensis) trees were collected from groves near Dover, FL. Overall, sampled trees showed foliar HLB symptoms in approximately 70% of the canopy indicating an advanced stage of disease infection (PCR = 19.7, 20.9 and 22.3 cycles). Corresponding samples from HLB-negative Valencia trees obtained from trees grown at the Citrus Research and Education Center in Lake Alfred FL, were used as control.

2.2. Iodine staining

For starch staining, leaves, petioles, stems, bark and roots were cut perpendicular to the long-axis with a sharp razor blade and immersed for 2 min in a solution of 2% iodine [16] at room temperature. Tissue samples were rinsed in water and immediately observed under a 40/14 Wild Heerbrugg stereoscope. Images were captured with a Canon PowerShot S3 IS equipped with MM99 adapter (Martin Microscope Co.).

2.3. Light microscopy (LM) and transmission electron microscopy (TEM)

Leaves, petioles, stems, bark and roots were sampled from both HLB-affected and control Valencia trees. The samples were fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.2, for 4 h at room temperature to overnight in the refrigerator. They were then washed in the same buffer and postfixed 4 h at room temperature in 2% osmium tetroxide in the above buffer. The samples were then dehydrated in an acetone series and embedded.
in Spurr's resin [24]. For light microscopy 1 μm sections were cut with glass knives and stained with methylene blue/azure A, poststained in basic fuchsin [21]. Light micrographs were taken on a Leitz Laborlux S compound microscope (Germany) with a Canon Powershot S31S digital camera (Tokyo, Japan). For TEM, the same blocks were thin sectioned (90–100 nm) with a diamond knife, collected on 200 mesh copper grids and stained with 2% aq. uranyl acetate and poststained with lead citrate [21]. Micrographs were made with an AMT (Advanced Microscopy Techniques Corp., Danvers, MA) digital camera on a Morgagni 268 (FEI Company, Hillsboro, OR) transmission electron microscope.

2.4. Scanning electron microscopy (SEM)

Leaf tissue from HLB-affected and control Valencia was taken and fixed using the above procedure. The samples were then dehydrated in acetone and critical point dried using a Ladd critical point dryer (Ladd Research Industries, Burlington, VT). The samples were then carefully re-cut with a razor blade down the long-axis of the leaf, mounted on stubs and coated with gold/palladium using a Ladd sputter coater (Ladd Research Industries, Burlington, VT). The samples were viewed on a Hitachi S530 scanning electron microscope (Tokyo, Japan) and photographed using a Canon EOS Rebel XT digital camera (Tokyo, Japan).

3. Results

3.1. Foliar tissues

To determine the extent by which HLB-induced imbalances in carbohydrate metabolism (reflected as starch accumulation) are distributed throughout the leaf, we sampled autotrophic as well as heterotrophic foliar tissues. Micrographs of cross sectioned leaves from HLB-affected compared to controls trees confirmed early observations by Schneider [20] that HLB infection results in abnormally high levels of starch accumulation (Fig. 1A versus 1B). In light micrographs, starch grains are visible throughout both palisade and spongy mesophyll cells (Fig. 1A). Furthermore, sizable starch grains are also evident in epidermal cells (Fig. 1A), phloem elements, phloem parenchyma as well as the xylem parenchyma (Fig. 1C). Leaves from nonsymptomatic HLB-negative trees were almost completely devoid of starch grains (Fig. 1B, D), although occasional grains were observed in palisade and/or spongy cells but never within the vascular tissue as in HLB leaves. In fact, the disparity in starch content between no symptomatic HLB-negative and HLB-affected leaves were substantial enough to be visible at the lowest magnification when leaves are stained with 1.2% iodine (Fig. 1B). In addition to the starch accumulation, there was evidence of phloem collapse in Fig. 1C. This can be seen as non-oval cell outlines (crushed) and opaque inner cell areas (arrows).

The intensity of starch accumulation and details of grain size and intracellular distribution within leaf cells are better appreciated in electron micrographs (Figs. 3 and 4). Multiple starch grains per chloroplast are visible in HLB-affected leaf palisade cells (Fig. 3A, C), whereas control-leaf chloroplasts contained only a small number of lipid inclusions and infrequent smaller starch grains (Fig. 3B, D). In addition, lipid inclusions in HLB-affected leaf cells (Fig. 3C, arrowheads) were more numerous and visibly larger than in controls (Fig. 3D, arrowheads). Starch grains in epidermal cells were less numerous than in mesophyll and palisade cells, however their sizes were considerably larger and in combination occupied larger portion of the cell's volume compared to palisade cells of affected leaves (Fig. 3A). Higher magnification electron micrographs of spongy palisade cells revealed distinctive cytological differences between control and HLB-affected leaf cells. In cells from HLB-affected leaves, starch granules-containing chloroplasts comprise a larger portion of the cell's volume (Fig. 4A, B), the cytosol contained a larger diversity of membranous organelles, and mitochondria, aside from being more numerous, tend to congregate around the starch-filled chloroplasts (Fig. 4C, arrowheads). The larger number of mitochondria and their close proximity to the chloroplasts in HLB-cells was evident in all preparations made and indicate a higher rate of metabolic activity associated with starch accumulation. Another prominent characteristic was the more granular and denser appearance of the control-leaf vacuole (Fig. 4B, D). It is noteworthy that control leaves often contained starch grains, however, these were usually considerably smaller (Fig. 4D, arrows) than those in leaf cells of HLB-affected trees (Fig. 4C, arrow). The differential starch content and vacuolar texture were not only evident in transmission electron micrographs, but scanning electron views confirmed these observations (Fig. 5). Clusters of starch grains were evident in HLB-affected leaf cells likely originating from separate chloroplasts (Fig. 5A) whereas these were absent from control cells (Fig. 5B). The granular texture of the vacuole in control leaves was seen as a filamentous network forming a web within the cell (Fig. 5B). This filamentous network was absent from the cellular space likely occupied by the vacuole in HLB-infected leaves (Fig. 5A).

Chloroplasts from HLB-affected leaf cells became virtually unrecognizable (Fig. 6A) as the result of the enormous starch grains.
The thylakoid system, with no distinctive granna, was visible only between the large starch granules or pressed against the chloroplast double membrane envelope (Fig. 6A). In contrast, chloroplasts from control leaves showed the typical oblong morphology with well developed granna and containing small starch grains (Fig. 6B, arrows). Another distinguishing characteristic of photosynthetic cells from HLB-affected tree was the visibly thicker cell walls (Figs. 4 and 6), which give the leaves their cardboard, corky texture.

Following a similar pattern of starch distribution, petioles of HLB-affected trees contained large amounts of starch grains in parenchyma cells both within the vascular bundle (Fig. 7A) and surrounding parenchymatous tissue (Fig. 7C) compared to petioles from control leaves (Fig. 7B, D, respectively). In all cases, grains were visible in the xylem vascular rays and in phloem elements (Fig. 7A). The latter are often concealed by the collapsed phloem cells (Fig. 7A, arrowhead), an intrinsic characteristic of greening disease [20]. In contrast, petioles from control trees showed a normal, active phloem with little or no starch accumulation (Fig. 7B). Scanning-EM micrographs confirmed the disparity in starch accumulation in petiole parenchyma (Fig. 7C, D). Whereas petiole cells from control leaves contained an occasional starch grain (Fig. 7D), HLB-affected petiole parenchyma contained substantial amounts of starch grains all throughout (Fig. 7C).

### 3.2. Stem and bark

The distribution of starch in stem and bark tissue was analogous to leaves in that accumulation of starch in HLB-affected tissues far exceeded control ones (Fig. 8). In stems of HLB-affected trees, xylem parenchyma contained copious amounts of starch. Whereas starch accumulated conspicuously in xylem parenchyma cells, phloem collapse allowed only scattered accumulation in the phloem tissue. In addition, the cambial zone and phloem appear obliterated in HLB-affected stems with no distinctive cellular organization as the result of phloem collapse. Xylem tissue in HLB-affected stems was also distinctive in that all cell types had thinner cell walls when compared to healthy controls.

Starch grains were also highly abundant in the exterior phellem of HLB-affected trees (Fig. 9A), in agreement with the remaining observations of aerial parts. However, our observations were also consistent in that starch also accumulates in the bark of control trees, but to a lesser extent (Fig. 9B).

### 3.3. Roots

Whereas starch accumulated disproportionally in cells of all aerial parts in HLB-affected trees (Figs. 1–9), starch in root cells...
presented a contrasting situation. Iodine staining of HLB-affected tree roots revealed a complete absence of starch from root cells (Fig. 10A, C). A similar size root from a control tree appeared to contain starch in virtually all living cells (Fig. 10B, D). The complete depletion of root starch may have severe implications to the survival potential of HLB-infected trees compared to control-tree roots.

4. Discussion

Starch is a natural product of photosynthetic CO2 fixation in green tissues. Formed by α-1,4 glucose linkages, starch exists in 2 forms, the soluble, small linear chain amylose and the highly branched insoluble amlopectin [22,28]. In green cells, starch accumulates during light hours and mobilized at night (and other times of low photosynthetic activity) to maintain a constant carbon supply to heterotrophic tissues. Citrus leaves, however, normally accumulate very low levels of starch at any time [29] and considerable amounts are accumulated only as a result of zinc deficiency [23] or girdling [19]. Once accumulated, starch in citrus leaves is not degraded [10] even during the night cycles and remains in the leaves indefinitely.

In the current study we demonstrate that the accumulation of abnormally high levels of starch resulting from HLB infection is not restricted to photosynthetic leaf cells as widely documented

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Fig. 4. Electron micrographs of single palisade cells from HLB-affected (A) and control (B) citrus leaves. Chloroplasts in HLB-affected leaves are not easily recognizable due to the large size of starch grains which obliterate chloroplast morphology compared to control-leaf chloroplasts. C and D are close-ups of chloroplasts from A and B respectively. Note the large number of mitochondria at the periphery of the chloroplast from the HLB-affected leaf (C). V = vacuole. Arrowheads = mitochondria. Arrows = starch grains.

Fig. 5. Scanning electron micrographs of leaf palisade cells from (A) HLB-affected and (B) control citrus leaves. Note the starch grains in the cell of HLB-infected leaf and the fibrous nature of the control cell.
[17,20,25,26], but in fact, this characteristic is extended to virtually all aerial tissues. In our samples, starch grains were commonly observed throughout all photosynthetic cells of the leaf blade and petiole, in parenchyma cells of the pith and vascular tissue and even in the phloem elements (Figs. 1–9). That carbohydrate metabolism is significantly altered by HLB beyond leaf tissue is unequivocally supported by additional data presented here. Starch accumulation was sharply different between control and HLB-affected trees in all tissues investigated. Whereas starch in stem vascular parenchyma and phelloderm of HLB trees also accumulated at levels much higher than in control trees, roots were practically depleted of any starch reserves. This observation contrasts with control trees where accumulation of starch in leaves and other aerial parts was minimal (Figs. 1–9), yet roots were starch laden (Fig. 10). The absence of starch reserves in roots of HLB-affected trees (Fig. 10A, C) is likely the result of the diminished transport of photoassimilates to the roots and the ensuing usage of starch reserves to sustain their own metabolic activities. Final starch depletion likely leads to starvation and to lowering the survival potential when compared to control trees (Fig. 10B, D).

Given that our observations were made from mature trees at a relatively advanced stage of HLB decline (estimated by the widespread symptoms), it is difficult to reconcile a chronological progression of events that lead to starch accumulation in leaves and other plant parts. Nevertheless, several conclusions can be drawn with a high degree of certainty. First, according to...

Fig. 6. Transmission electron micrographs of chloroplasts from (A) HLB-affected and (B) control citrus leaves. Note the thylakoid membrane pressed between starch grains and against the chloroplast membrane in samples from HLB-affected leaves (A). Thylakoid and granna stacks are evident in samples from control leaves (B).

Fig. 7. Cross section light micrographs of petiole midveins from HLB-affected (A) and control (B) citrus leaves. (A) Vascular bundle showing the abundance of starch grains both in the pith and vascular parenchyma. C and D are scanning electron micrographs of cortex parenchyma of HLB-affected (C) and control (D) Valencia orange trees. X = xylem tissue; P = phloem tissue; * = phloem collapse.
Fig. 8. Light micrographs of stem sections from (A) HLB-affected and (B) control Valencia orange trees. Starch grains and phloem collapse (bluish inner area of phloem) are evident in the stem sample from HLB-affected trees (A) and absent in the control tree sample (B). X = xylem tissue; P = phloem tissue; * = phloem collapse.

Fig. 9. Light micrographs of stem bark collected from (A) HLB-affected and (B) control Valencia orange trees. Bark was collected at a height of 30 cm above the graft line. Starch grains are present on both samples, although more abundant in HLB-infected trees (A).

Fig. 10. Hand cut section from root tissue from HLB-affected (A) and control (B) trees. Root tissue in A and B were stained with 2% iodine for 2 min and observed under 10X stereomicroscope. C and D show sections of corresponding root tissue under light microscopy showing the lack of starch reserves in roots from HLB-affected trees (C) and the abundance of starch in roots from control trees (D). X = xylem tissue; P = phloem tissue.
Schneider [20], starch accumulation in foliar tissues results from a photoassimilate transport blockade prompted by HLB-induced phloem necrosis. However, our observations of high starch content in stems and bark tissues further down the photoassimilate pathway argue against this hypothesis. If photoassimilate transport were to be blocked at the petiole level, no starch would be expected to accumulate in stems and bark below the leaf canopy. It is noteworthy that the bark samples collected just above the graft line from HLB-affected trees contained visibly higher levels of starch than controls (Fig. 9) without any sign of phloem blockage (data not shown). Furthermore, we often collected asymptomatic leaves located amongst highly symptomatic, starch-loaded leaves, a condition that argues against any type of phloem blockade. Alternatively, element plugging may not be entirely uniform allowing some passage of sugars and starch accumulation before phloem becomes completely necrotic. In this form, some sugars reach the lower stem but not the roots where little or no sugar is available for starch synthesis.

Second, the difficulties encountered in finding secondary roots from HLB-affected trees, and the total depletion of starch observed in samples when found (Fig. 10 A, C) are good indication of carbohydrate starvation in the root system resulting from a physiological re-distribution of reserve carbon. Under normal circumstances, the root system accumulates substantial amounts of starch presumably utilized during vegetative and reproductive flushes [10]. However, with carbohydrates sequestered in the aerial parts of the tree triggered by HLB, it is likely that the roots utilize these reserves to sustain metabolic activity until depletion results in root death or degradation.

Since starch accumulation in pepper [14] and potato [18], and phloem disintegration in barley [7], and plugging in cucurbits [3] have been observed in response to a variety of phloem-limited pathological conditions, we also examined the possibility of starch hyper-accumulation in citrus leaves from conditions such as blight, tristeza, zinc deficiency and branch fracture (girdling). Only under Zn deficiency and girdled branches did we observe above average levels of starch accumulation in leaves. However, in neither case were starch levels comparable to those observed in HLB-infected trees.

Although anatomical in nature, the data shown in this communication represent clear evidence of the systemic and far reaching influence of HLB on citrus tree carbohydrate metabolism. The data supports the notion that the substantial changes in carbohydrate partitioning observed throughout the citrus tree may not only be a result of HLB infection, but in itself, a cause for the rapid decline and death of infected trees as carbohydrates are channeled towards the synthesis and accumulation of starch in the aerial parts resulting in root starvation. Starch accumulation was observed in practically all living cells of the aerial parts of the tree denoting a clear redirection of carbohydrate transport. Whether the widespread starch accumulation in aerial parts resulted at the expense of root starch mobilization or whether the capacity of carbon fixation is increased as a consequence of HLB infection is yet to be demonstrated. However, upward movement of reserve starch carbon is highly unlikely with a plugged phloem system. Most likely, root starch is consumed to sustain root metabolic activities when little sugar is translocated down from the leaves resulting in root death and eventually tree decline.

References