Title: **Citrus photosynthesis and morphology acclimate to phloem-affecting huanglongbing disease at the leaf and shoot levels**

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Citrus photosynthesis acclimates to huanglongbing

**Abstract**

Huanglongbing (HLB) is a phloem-affecting disease of citrus that reduces growth and has impacted citrus production in most global production regions. HLB is caused by a phloem-limited bacterium (*Candidatus* Liberibacter asiaticus; *C*Las). By inhibiting phloem function, HLB stunts sink growth, including reducing production of new shoots and leaves, and induces hyperaccumulation of foliar starch. Despite evidence that HLB induces feedback inhibition of photosynthesis by reducing foliar carbohydrate export, its effects on net $CO_2$ assimilation ($A_{net}$) have not been reported. In this work we assessed the relationship of bacterial distribution within the foliage, foliar starch accumulation, and $A_{net}$. Because HLB impacts canopy morphology, we developed a chamber to measure whole shoot $A_{net}$, and tested the effects of HLB at both leaf and shoot levels. Starch accumulation was correlated with bacterial population, and starch was negatively correlated with $A_{net}$ at the leaf but not at the shoot level. Starch increased between the uninfected group and the shortest duration of infection, then decreased progressively with increasing length of infection. HLB infection reduced $A_{net}$ at the leaf level, but increased it at the whole shoot level. We attribute this enhancement of whole shoot $A_{net}$ to the increased contribution of stem photosynthesis due to the altered shoot morphology induced by the disease. Despite the increased photosynthetic efficiency, total carbon fixation per shoot decreased because shoot size and leaf area were reduced. Overall, our results indicate a localized relationship of *C*Las distribution with negative impacts on foliar carbohydrate export and photosynthesis. Additionally, starch accumulation and $A_{net}$ acclimate over time at the shoot level to the localized impacts of the disease. Stems contribute important proportions of whole shoot $A_{net}$, and these contributions are likely increased by the morphological acclimation induced by

HLB. This study highlights the importance of temporal and spatial scale in assessing photosynthesis.

## Introduction

Huanglongbing (HLB) is a disease of citrus causing economic loss to the citrus industry around the world (Bové, 2006; Singerman and Useche, 2016). Its causal agent, *Candidatus* Liberibacter asiaticus (*C*Las), vectored by the Asian citrus psyllid (ACP; *Diaphorina citri* Kuwayama), is a phloem-limited bacterium which affects phloem dysfunction leading to starch accumulation in leaves and loss of sink growth (Achor et al., 2020; Bové, 2006), as well as eventual development of thin canopies (Tang et al., 2019).

Because of the reduction in phloem function, HLB is hypothesized to lead to feedback inhibition of photosynthesis (Cimò et al. 2013). Assessing chlorophyll fluorescence, a reduction in maximal PSII quantum yield was found in *C*Las-infected citrus leaves compared to uninfected leaves (Cen et al., 2017; Sagaram & Burns, 2009). On the one hand, starch accumulation in the leaves has been noted as a symptom of HLB, which indicates a surplus of fixation relative to export (Etxeberria et al., 2009; Folimonova & Achor, 2010; Schneider, 1968). On the other hand, starch accumulation can disintegrate the chloroplast membrane (Nebauer et al., 2011; Snyder-Leiby & Wang, 2008), which may contribute to the reduction in PSII yield noted above. However, no measures of the impacts of HLB on net $CO_2$ assimilation ($A_{net}$) have been reported.

Measures of $A_{net}$ in citrus have previously been taken at the leaf level (Kriedemann, 1968; Mesquita et al., 2016; Tsagarakis et al., 2012; Vu and Yelenosky, 1988; Vu et al., 1986; Zambrosi et al., 2011) and at the field level (Contreras et al. 2017; Er-Raki et al., 2009; Maestre-

Valero et al., 2017; Penddinti and Kambhammettu, 2019). Due to the specificity of the leaf level measurements (light levels, angle of radiation incidence, leaf position within the canopy), leaf-level data is difficult to extrapolate to the whole canopy. Because, HLB induces reductions in canopy density (Tang et al. 2019), larger scale measures may be more indicative of changes in carbon assimilation than leaf-level measures.

In this study, our objective was to compare relationships of $A_{net}$, *C*Las infection, and foliar symptoms at the leaf and shoot level. To achieve our objective, we first developed and validated the use of a gas exchange chamber capable of measuring whole shoots. We assessed the value of such measures by comparing leaf-level and shoot-level light response curves. In our subsequent HLB experiment, we hypothesized that: 1) greater time of *C*Las inoculation exposure would increase starch content; 2) higher starch content, as an indicator of reduced leaf carbohydrate export, would correlate with downregulation of $A_{net}$; and 3) because HLB causes morphological changes at the canopy level, mean leaf area, leaf number, and stem surface area and length were expected to affect $A_{net}$ at the shoot level.

## Materials and Methods

### *Study 1: Whole shoot gas exchange chamber development and testing*

#### *Plant Material*

The trees utilized were greenhouse-raised, four-year-old sweet orange 'Valencia' (*Citrus x sinensis*) on 'Kuharske' (*C. sinensis* x *Poncirus trifoliata* (L.) Raf.) rootstock in 20-L pots. Plants were maintained with recommended rates of slow-release fertilizer and 3x weekly irrigation with sufficient volume to exceed field capacity of the pots.

*Chamber construction*

The chamber was built of acrylic tubing, acrylic glue, and a 3-D printed adapter designed and provided by Jason Hupp, LI-COR Bioscience, Inc. Lincoln, NE, USA. The chamber was fit to an infrared gas analyzer (LI-6800, LI-COR Bioscience, Inc. Lincoln, NE, USA) using a custom chamber adapter (LI-6800-19, LI-COR Bioscience, Inc. Lincoln, NE, USA). To seal the opening, a large conical stopper was step-drilled and cut to provide an easy-to-fit cap around the stem of the branch (Appendix S1). The chamber could then be made airtight using plumbers' putty.

Leaves and shoots were measured on trees recently acclimated to the relative darkness of the laboratory environment (2-4 μmol photons $m^{-2}$ $s^{-1}$). Leaf-level measurements were recorded for 15 minutes on three leaves at photosynthetic photon flux density (PPFD) of 1000 μmols $m^{-2}$ $s^{-1}$. Environmental settings for the leaf level were set at flow 300 μmol $s^{-1}$, 0.2 kPa pressure, 60% relative humidity, reference $CO_2$ at 400 μmol $CO_2$ $mol^{-1}$ , and a fan speed of 10,000 RPM. Chamber measurements were logged every 15 seconds for five minutes after a 15-minute stabilization period under controlled conditions at PPFD of 1000 μmol $m^{-2}$ $s^{-1}$. Environmental settings for the whole-shoot chamber were set based on volume, 1000 cubic centimeters, at flow 1000 μmol $s^{-1}$, 0.2 kPa pressure, 60% relative humidity, reference $CO_2$ of 400 μmol $CO_2$ $mol^{-1}$, and a fan speed of 14,000 RPM to maximize air mixture. Increases in air flow rate from 300 μmol $s^{-1}$ in leaf measurements to 1000 μmol $s^{-1}$ in whole -shoot chamber compensated for the increase in photosynthetic material and increased volume present in the custom chamber. The fan speed was increased to 14,000 RPM to help mix air in the chamber as recommended by the IRGA manufacturer. Using the red-light capability of the red, far red, and blue light adjustable fluorometer head unit of the LI-6800F, light intensity was controlled for the leaf-level

measurements. An LED Grow Light of similar spectral composition (ZXMean 1500W Full Spectrum LED, BliGli, Wuhan HuBei, China) was manipulated by distance and a dimming switch (Credenza Plug-In Lamp CFL-LED Dimmer, Lutron Electronics Co., Coopersburg, PA, USA) to achieve desired PPFD for the whole-shoot chamber. PPFD for whole-shoot measurements was measured using the quantum sensor of the LI-6800 at the mid-point of the shoot adjacent to the whole-shoot chamber.

*Stem assimilation*

To determine stem contribution to $A_{net}$ of the shoot (the leaf and stem material together), the stems were covered with fitted black drinking straws following the method of Nilsen and Bao (1990) testing stem contributions to photosynthesis in soybean (*Glycine max* L.). Shoots were exposed to PPFD 1400 μmol $m^{-2}$ $s^{-1}$ as described above. The shoot was then removed from the chamber and black straws were measured to fit the space between petioles on the stem, sliced lengthwise and wrapped around the stem (Appendix S2). Gas exchange measurements then rerecorded with the darkened stems. The LED Grow Light was then turned off and the custom chamber was covered by a black cloth to achieve 0 μmol $m^{-2}$ $s^{-1}$ PPFD to determine respiration of the shoot, again following the same stabilization and logging duration. Stem (shoot $A_{net}$), without stem ($A_{net}$ with darkened stem), and at 0 μmol $m^{-2}$ $s^{-1}$ PPFD (shoot respiration) were used for analysis. Measurements of $A$ under the different measurement conditions were used to calculate gross assimilation ($A_{gross}$) contributions of the stem and leaves as follows:

- Shoot $A_{net}$: shoot net photosynthesis, measured as $A$ of shoot at 1400 μmol $m^{-2}$ $s^{-1}$ PPFD; inherently the sum of stem and leaf $A_{gross}$ minus respiration (shoot $R$).

- Stem $A_{gross}$: stem gross $A$; measured as the difference between shoot $A_{gross}$ under full illumination and $A_{gross}$ with darkened stem.
- Shoot $R$: whole shoot respiration, measured as $|A|$ at 0 µmol $m^{-2}$ $s^{-1}$ PPFD.
- Shoot $A_{gross}$: shoot gross assimilation, estimated as shoot $A_{net}$ – shoot $R$.
- Leaf $A_{gross}$: leaf gross assimilation of all leaves on the shoot, estimated as Shoot $A_{gross}$ – stem $A_{gross}$

Immediately following gas exchange measurements, shoots were excised and defoliated for leaf area analysis using the LI-3100 (LI-COR Biosciences, Inc. Lincoln, NE, USA). Stems were measured for length and diameter (Appendix S3) to calculate surface area using an adjusted formula for the lateral area of a truncated cone (Equation 1). Due to the irregular shape of citrus stems, two diameter measurements were taken at the widest and narrowest widths at the base, middle, and tip of the stem. The circumference of an ellipse is approximately equal to that of a circle with a radius of the average of the widest and narrowest point of the ellipse.

$$Stem\ Surface\ Area\ =\ \pi(r_1 + r_2)\sqrt{(r_1 - r_2)^2 + {h_1}^2} + \pi(r_2 + r_3)\sqrt{(r_2 - r_3)^2 + {h_2}^2} \quad \text{(Eq. 1)}$$

Where:

$r_1$= radius of stem base

$r_2$= radius of stem middle

$r_3$= radius of stem tip

$h_1$ =length between measurement point of $r_1$ and $r_2$

$h_2$ =length between measurement point of $r_2$ and $r_3$

Leaves and stems were dried at 60 °C for 72 hours and weighed separately. Leaf and stem surface area and dry weight were used to calculate $A_{gross}$ rates per meter squared and per gram dry weight.

*Leaf and shoot light response curves*

Nine fully mature young shoots, three from each of the same trees were measured in the laboratory. Shoots were placed in the shoot gas exchange chamber and carbon assimilation was measured at PPFD of 0, 25, 50, 100, 150, 200, 400, 600, 800, 1000, 1400, and 1800 μmols $m^{-2}$ $s^{-1}$. Environmental settings, stability criteria, and logging were the same as previous whole-shoot measurements (Appendix S4). The largest, healthiest leaf (assessed visually) on each of the nine measured shoots was selected to measure a leaf-level comparison (Appendix S5) measurement at PPFD of 0, 25, 50, 100, 150, 200, 400, 600, 800, 1000, 1400, and 1800 μmols $m^{-2}$ $s^{-1}$. Environmental settings and stability criteria were set as in the previously described measurements.

*Study 2: HLB impacts on leaf and shoot net $CO_2$ assimilation*

*Plant material selection*

Trees were selected based on length of exposure to citrus greening infection. The trees utilized were greenhouse-raised, four to five-year-old sweet orange ‘Valencia’ on ‘Kuharske’ rootstock in 20-L pots. These trees had been exposed to ACP inoculation of *C*Las by growing plants in infested screenhouses for varying lengths of time. Exposure categories were defined as follows: the High category of exposure had been exposed to infected ACP feeding and breeding for 2.5 years; the Mid category of exposure had been exposed to infected ACP feeding and

breeding for 1.5 years; and the Low category of exposure had been exposed to infected ACP feeding and breeding for 1 year. The uninfected trees had not been exposed to infected ACP. Three trees of each category were arranged in a greenhouse in a randomized complete block design, totaling 12 trees for the study.

Shoots and leaves were selected on each tree for measurements. Three shoots per tree were selected from the exterior of the canopy. Shoots were chosen based on accessibility of the instrument using the custom chamber, and only those with at least three leaves suitable for leaf measurement using the standard leaf chamber were selected. Thirty-six shoots were measured in this study (3 shoots x 3 trees x 4 exposure groups).Three leaves were selected per shoot. These were distributed along the length of the shoot, always using one apical leaf. To be selected, leaves had to be large enough to completely cover the leaf chamber (6-$cm^2$ circle). A total of 108 leaves were measured for this study (36 shoots x 3 leaves).

*Gas exchange measurements*

$A_{net}$ was measured in using the LI-6800 (LI-COR Bioscience, Inc. Lincoln, NE, USA). Leaf chamber environmental settings were set at a PPFD of 1000 µmol $m^{-2}$ $s^{-1}$ and shoot chamber was placed at PPFD of 1400 µmol $m^{-2}$ $s^{-1}$ with all other settings according to those described above for Study 1 for leaf and shoot gas exchange measurements.

*CLas quantification*

Midrib and petiole tissues excised from selected leaves were chopped using a razor blade and bead-beaten using Vortex Genie 2 (Scientific Industries Bohemia, NY, USA) to a fine homogenized powder in liquid nitrogen. Total genomic DNA was extracted and purified from

100 mg of homogenized material using GeneJET Plant Genomic DNA Purification Mini Kits (Thermo Fisher Scientific Waltham, MA, USA). Samples were processed following manufacturer instructions and stored at -20° C. Primers and TaqMan probes were obtained for *C*Las based on method developed by Coy et al. (2014). The PCR master mix was used with 10 µL TaqMan Buffer, 8.6 µL sterile deionized water, 0.4 µL CQUL primer, and 1 µL of extracted purified DNA. PCR reactions performed with standards using QuantStudio 3 Real-time PCR (Applied Biosystems-Thermo Fisher Scientific Waltham, MA, USA) yielded cycle threshold values (Ct). Bacterial copy-count estimates were generated by regression using processed Ct results from standards of known copy quantity, processed in each plate batch. At the shoot level Ct and bacterial copy number were considered to be the mean of the measurements in the three selected leaves on each shoot.

*Starch content*

Starch content was determined by extracting starch from a single 28.27 $mm^2$ hole punch on the selected leaf blade (Whitaker et al., 2014). The leaf punch was homogenized for 2.5 minutes at 6500 RPM in 500 µL of distilled water with five beads. Samples were boiled for 10 minutes at 200° C and agitated prior to a two-minute centrifuge at 2500 rpm, after which 300 µL of the supernatant was added to 900 µL pure ethanol and agitated for three seconds to precipitate starch. Prepared samples were centrifuged at 14000 RPM for 10 minutes and the supernatant was discarded and replaced by one mL of deionized water. Samples were then shaken for four-minutes to dissolve the starch pellet and two 250 µL of prepared sample were each placed in individual wells of a 96 well microplate, and 50 µL of 2% iodine was added to each well. Absorbance at 595nm was read using SpectraMax 250 microplate reader (Molecular Devices San

Jose, CA, USA). Linear functions based on standard curve concentrations included in each plate were generated with the absorbance of standards to estimate starch content. As for *C*Las variables, at the shoot level starch values were considered to be the mean of the measurements of the three selected leaves on each shoot.

*Visual rating*

A rating of the visual symptoms of *C*Las infection was assessed on a scale of 0-10, where 0 = no symptom, 10= full symptom expression, on each leaf. Prior to assessment a scale of leaf symptoms was generated using photos of sampled *C*Las infected leaves to ensure consistency in ratings. All disease ratings were performed by the same individual. Visual symptom ratings used in the leaf level measurements were recorded for every leaf on the measured shoot; an average of the assessment of all leaves was used for the shoot value.

*Shoot morphology*

Total leaf area per shoot was measured using a LI-3100 (LI-COR Bioscience, Inc. Lincoln, NE, USA) immediately following the assimilation measurements and visual ratings, prior to sampling for qPCR and starch content. Stem length and diameter in two directions was measured using a micrometer to generate a stem surface area estimates using Eq. 3. Average internode length was calculated by dividing the stem length by the total number of leaves per stem.

*Analysis*

Analysis of variance was performed using the anova() command in base R, after assessing appropriateness of parametric tests by testing for normal distribution, to assess the effect of treatment groups (R Core Team, 2019, Version 3.6.1). Where exposure group was significant, Fisher's LSD was used as a post hoc test for least significant difference between the four tree categories used for all variables using the {agricolae} package (de Mendiburu, 2020). Correlation analysis was generated among the variables at the shoot and leaf-level and correlation matrices were tested for significance of Pearson's correlations using the {Hmisc}package (Harrell, 2020). For correlation analyses, because some variables might co-vary in the case of disease, tests were performed with and without the HLB- plants. For variables with significant correlations, linear regression models were generated at both shoot and leaf-level using the lm() command, similarly comparing relationships with and without HLB- plants. The models were tested for significant relationships using anova(). All linear models nested shoot within tree, and leaf-level data was nested leaf within shoot. A natural log transformation of bacterial copy count and starch content facilitated a linear relationship between the transformed data. Light response curves for leaf and shoot were fit using an nonlinear least squares regression using the self-starting asymptotic function in R (Pinheiro et al., 2014) with a function supplied by Tomeo (2017). This function fits an asymptotic function and calculates the parameters of fully saturated photosynthesis ($A_{sat}$), quantum yield of photosynthesis ($\Phi$), dark respiration ($R_d$), light compensation point (LCP), and PPFD of 75% saturation ($Q_{sat75}$). These parameters were then subjected to analysis of variance to test the effects of shoot vs. leaf light responses.

## Results

*Stem contributes significantly to whole-shoot photosynthesis*

Vegetative stem on young shoots contributed 5.7% to total shoot surface area, 17.0% to total shoot mass, and 10.7% to shoot $A_{gross}$ (μmol $CO_2$ $s^{-1}$; Table 1). Mean stem $A_{gross}$ $m^{-2}$ was nearly double leaf $A_{gross}$ $m^{-2}$ (9.62 μmol $CO_2$ $m^{-2}$ $s^{-1}$ and 5.13 μmol $CO_2$ $m^{-2}$ $s^{-1}$, respectively; Figure 1).

*Light response curves differ between shoots and leaves*

Leaves and shoots had distinct light response curves with differences in all parameters tested, except for quantum yield (Table 2). Leaf level $R_d$ was more than 3x that of the shoot level $R_d$, resulting in a correspondingly higher LCP, despite the less acute rise in $A_{net}$ response to PPFD (Figure 2). Leaf level 75% saturation was achieved at approximately 528 μmol photons $m^{-2}$ $s^{-1}$, while that of shoots was estimated at 1532 μmol $m^{-2}$ $s^{-1}$. The corresponding $A_{sat}$ was also approximately 20% higher in shoots than in leaves.

*Photosynthesis and starch are affected by CLas exposure*

Uninfected leaves had a higher $A_{net}$ (mean of 3.97 μmol $CO_2$ $m^{-2}$ $s^{-1}$) than infected leaves; however, as *C*Las exposure increased from low, to medium, to high so did $A_{net}$ (means of 1.91, 2.61, 2.70 μmol $CO_2$ $m^{-2}$ $s^{-1}$, respectively; Table 3). Uninfected leaves had a lower starch content (mean of 1.08 μg $mm^{-2}$) than infected leaves; however, as *C*Las exposure increased from low to high starch content decreased (means of 11.31, 5.69, 3.55 μg $mm^{-2}$, respectively). Uninfected leaves had the lowest visual rating score, which increased as *C*Las exposure increased from a mean of 0.22 in the uninfected to a mean of 2.22 in the highest exposure group.

$A_{net}$ had a positive relationship with *C*Las exposure at the shoot-level. High exposure shoots had the highest $A_{net}$ at a mean of 3.37 μmol $m^{-2}$ $s^{-1}$, which decreased with a shortened

exposure down to a mean of 1.91 μmol $m^{-2}$ $s^{-1}$ in the uninfected shoots (Table 4). Reductions in mean leaf area (17.59 $cm^2$ in the uninfected to 11.38 $cm^2$ in the highest exposed group) and total shoot surface area (133.5 $cm^2$ in the uninfected to 68.3 $cm^2$ in the highest exposed group) were found as *C*Las exposure increased. Exposure did not affect internode length.

*Infection, starch, photosynthesis, and morphological variables were correlated*

At the leaf level, when including uninfected trees, positive correlations were found between bacterial copy count and starch content ($r$=0.609, $P$<0.00001), *C*Las copy number and visual rating (r=0.459, $P$<0.00001), and starch content and visual rating ($r$ =0.276, $P$=0.005; Appendix S6). Negative correlations were found between $A_{net}$ and *C*Las copy number ($r$ =-0.322, $P$=0.0009) as well as starch content ($r$= -0.243, $P$=0.013). No correlations were found with leaf position or between $A_{net}$ and visual rating. No additional correlations were found when excluding uninfected trees.

At the shoot level, when including uninfected trees, positive correlations were found between *C*Las copy number and starch content ($r$=0.716, $P$<0.00001), visual rating ($r$ =0.539, $P$=0.001), and $A_{net}$ ($r$=0.413, $P$=0.015); between shoot surface area and mean leaf area ($r$=0.812, $P$<0.00001) and shoot surface area and shoot length (r=0.58, $P$=0.0003); and between internode length and shoot length (r=0.344, $P$=0.046; Appendix S8). When excluding uninfected trees at the shoot level, additional positive correlations were found between starch content and mean leaf area (r=0.421, $P$=0.032) and shoot surface area (r=0.455, $P$=0.019); between shoot surface area and mean leaf area (r=0.809, $P$<0.00001) and shoot length (r=0.644, $P$=0.0004); and between internode length and $A_{net}$ (r=0.558, $P$=0.003; Appendix S9).

*Linear regressions reveal additional relationships between infection and physiology*

Based on the correlations we tested linear regressions of the following variables: *C*Las copy number by starch content, internode length by $A_{net}$, and foliar starch content by shoot surface area. After a natural log transformation of both starch content and bacterial copy count, a positive linear relationship was found at both the leaf and shoot-level (Table 5). Further observation indicated a change in the slope as *C*Las gene copy # increased and assessment using breakpoint analysis indicated that the increased starch content at the leaf level increased at a higher rate with a breakpoint at approximately $10^{11.2}$ *C*Las gene copies (Figure 3A), though the shoot level data did not indicate such a change in slope (Figure 3B). A positive linear relationship ($P$=0.048) was found between foliar starch with shoot surface area when excluding uninfected shoots, though this relationship also had a low $R^2$ (0.18) (Figure 4 and Table 5). This relationship is inverse to the *C*Las exposure duration, with the highly exposed shoots having lower starch content and total shoot surface area. When comparing morphological and *A* measurements, a positive linear relationship ($P$=0.003) was found between internode length and $A_{net}$ when excluding uninfected shoots (Figure 5 and Table 5).

## Discussion

*Leaf and shoot light response dynamics differ*

The distinct shoot light response dynamic helps to explain the success of *Citrus* in high light regions, where maximum $A_{sat}$ is attained near the maximum ambient PPFD occurring in subtropical regions. The leaf-level light response curves indicating $Q_{sat}$ occurs between 600-700 μmol $m^{-2}$ $s^{-1}$ PPFD was similar to earlier studies (Habermann et al., 2003). The increased light saturation point of citrus shoots compared to individual leaves could be explained by varied

angle of incident radiation, as well as by partial shading of basal leaves by more distal leaves. However, $R_d$ was much lower at the shoot level than at the leaf level. Lower $R_d$ is an important factor in reducing the LCP. Thus, at the shoot level citrus exhibits higher $A_{net}$ at both the lower and upper ends of irradiance than is detected at the leaf level, while at PPFD ranges between 50-1000 µmol $m^{-2}$ $s^{-1}$ shoots exhibit lower $A_{net}$ than leaves. The lack of a difference in $\Phi$ is further confirmation that differences between shoots and leaves are primarily determined by factors such as morphology, not by enzymatic or photochemical factors. This might be assumed given that some of the same leaves were involved in both measurements, though the lower rate of $R_d$ in shoots merits further investigation. The fact that much of the difference between shoot and leaf irradiance responses may be attributed to the irradiance variance within the shoot indicates that morphology is likely to have a strong impact on photosynthesis at the whole shoot level.

*Stem assimilation contributes to whole shoot photosynthesis*

Citrus vegetative stems significantly contributed to shoot $A_{gross}$. The stem was also more efficient in $A_{gross}$ on a surface area basis than leaves. The high assimilation rate of citrus stems could be explained by area-related chlorophyll content where young stems contain 50–70% more chlorophyll than adjacent leaves (Kharouk et al. 1995; Pfanz et al., 2002; Pilarski 1984; Schmidt et al. 2000; Solhaug et al. 1995). Although many factors contribute to whole-shoot assimilation, the previously unquantified contribution of the photosynthetic stem to canopy wide assimilation may be an additional factor in the lack of relationship between leaf-level gas exchange measurements to canopy-wide measurements. Stem internal re-fixation of $CO_2$ in young branches of perennial woody species have been shown to compensate for 60–90% of the potential respiratory carbon loss (Pfanz et al., 2002). Although, its contributions compared to the

leaves on whole shoot assimilation are relatively small, stem contribution may vary depending on shoot morphological characteristics such as internode length, which may affect light interception in the stem.

*HLB impacts shoot morphology and photosynthesis*

As with light responses, shoots and individual leaves differed in their responses to HLB. While *C*Las infection reduced $A_{net}$ at the leaf level, it increased $A_{net}$ at the whole shoot level, while inducing whole-shoot morphological adjustments over time. These morphological impacts explain the HLB effects on whole-shoot photosynthetic rates. The decreased leaf and shoot surface area effected by *C*Las exposure reflect the symptom of canopy thinning observed in infected citrus trees in the field. This process appears to occur on a scale of years. Given that most citrus leaves senesce by 17 months (Wallace et al. 1954), the changes in shoot morphology appear to occur after a complete turnover of old to new tissues.

We have not found any report in which a plant produced both longer internodes and smaller leaves in response to a disease or environmental condition. Typically, an increase in leaf size has coincided with increased internode length to reduce self-shading, such as in acclimation to low light conditions (Kavanagh, 2014; Pieters et al., 1998; Poorter and Rozendaal, 2008; Robertson, 1994), thus the particular morphological changes induced by HLB may be responses to the specific challenges imposed by the disease.

Shoot morphological changes were associated with the shoot-level gas exchange response to HLB. Light penetration and absorption are the primary drivers of photosynthesis, and these are heavily influenced by canopy structure (Niinemets, 2007). Canopy thinning by *C*Las-induced defoliation, as described by de Graça (1991), within the high *C*Las-exposed category (fewest and

smallest leaves and longest internode length) further facilitate light penetration into the canopy. With increased light penetration and internode length, typically shaded leaves, further from the shoot apex, and the photosynthetic stem contribute more to shoot-level assimilation, which help explain the increased assimilation efficiency found in shoots of the high *C*Las exposure group. Considering the contribution of stems to shoot $A_{gross}$, the relative increase in stem surface area, could also contribute to the enhanced shoot $A_{net}$. The increased light exposure of all parts of the shoot may also lead to compensation for the localized disease effects discussed below in reference to leaves. Although there are likely other factors that contribute to the increased assimilation efficiency of HLB-affected shoots, the correlation of these morphological variables with $A_{net}$ indicates that whole-shoot morphology plays a sufficiently important role in citrus shoot photosynthesis as to be a defining feature of shoot acclimation that leads to an increase in $A_{net}$.

The increased efficiency of $A_{net}$ should not be confused with increased total photosynthesis. While the highly *C*Las-exposed shoots had higher $A_{net}$ than the shoots from the low exposure shoots, these measures are surface-area based and do not offset carbon fixation lost in the reduction in shoot surface area with increased *C*Las exposure. Total net shoot assimilation was 40% higher for the low *C*Las-exposed shoots (0.032 μmol $CO_2$ $s^{-1}$) than high *C*Las-exposed shoots (0.023 μmol $CO_2$ $s^{-1}$). Although, growth is necessarily dependent on photosynthesis, numerous complexities have led to a low degree of correlation between net assimilation and growth. For example, Poorter and Remkes (1990) did not find net assimilation rate to be correlated with relative growth rate in a comparison of numerous species, though other multi-species comparisons have found effects of net assimilation rate on relative growth rate (Li et al. 2016). Measurements of $A_{net}$ using gas exchange are further complicated in that they are limited

in both spatial (canopy dimensions) and time scales, and neither growth-analysis-based net assimilation rate nor gas-exchange-based $A_{net}$ include the complexities of allocation and their subsequent impacts on growth (Pereira 1995). In most studies, however, total photosynthetic area has been found to affect growth more than photosynthetic activity on a leaf area basis (Pereira 1995, Sinclair et al. 2019). The present study provides an example of how morphological changes could overwhelm changes in $A_{net}$. Despite increases in photosynthetic efficiency, *C*Las infected shoots had lower total assimilation rates because the reduction in photosynthetic surface area was greater than the relative increase in $A_{net}$, leading to a reduction in assimilation per shoot. Thus citrus plants mitigate losses by increasing photosynthesis per leaf area but do not increase total assimilation.

*CLas infection induces local starch and photosynthetic responses*

Because *C*Las is a phloem-limited bacterium, the effects of infections on leaf carbohydrate dynamics have been the focus of much HLB-related research. Achor et al. (2010) observed starch disruption of chloroplast inner grana in association with local phloem blockage and collapse. Further, Fan et al. (2010) found increased foliar sucrose concentrations in association with starch accumulation in infected trees. The current study suggests that these effects are localized, where *C*Las population increases induced starch accumulation at a much higher rate after reaching a population threshold at the leaf level.

Given these earlier findings regarding the impact of HLB on citrus carbohydrate relations, Cimò et al. (2013) simulated the foliar response of HLB by girdling citrus trunks, resulting in starch accumulation and a reduction in leaf level $A_{net}$. This reduction had been hypothesized because of the source-sink hypothesis (Ainsworth and Bush 2011), which observes

that plants downregulate $A_{net}$ in response to reduced leaf carbohydrate export, a condition in which various carbohydrates may accumulate. In the case of citrus, this hypothesis was demonstrated by Syvertsen (1994), and later by Iglesias (2002) and Nebauer et al. (2011), who found $A_{net}$ responded to changes in soluble sugars, which in turn also resulted in starch accumulation or depletion. Although none of the above studies addressed the condition of HLB, their interpretation of reduced photosynthesis as a response to leaf export limitation has been applied to studies of HLB, in which gene expression of photosynthetic pathways has been found to be down regulated (Balan et al. 2018). The present study found the expected reduction of $A_{net}$ at the leaf level, but not at the shoot level.

The comparison of local versus systemic effects has not been addressed explicitly by studies of HLB. However, the authors of some studies have implicitly interpreted systemic effects on leaves (all studies used in Balan et al. 2018) while others have hypothesized that HLB primarily affects roots (Johnson et al. 2014). The indication in the present study of a localized impact of *C*Las on leaf carbohydrate export and photosynthesis supports the interpretation of Koh et al. (2012), who observed callose blockage of leaf phloem as well as reduced export of $^{14}$C-labeled photosynthates in infected trees, while also agreeing with those of Fan et al. (2010), Rao et al. (2018), and Balan et al. (2018), all of which found alterations in carbohydrate metabolism and photosynthesis in leaves. A unique contribution of the present study is to indicate that these effects are at least partly local effects of presence of the bacterial population in the leaf, rather than strictly systemic responses to infection.

Starch accumulation has been associated with *C*Las infection and has been attributed as the cause of the blotchy mottle and chlorosis symptoms seen in infected citrus leaf tissue (Etxeberria et al., 2009; Gonzalez et al., 2012; and Schaffer et al., 1986). Interestingly, we found

high starch accumulation in the most recently exposed group and a relative reduction in starch content with longer exposure durations. Meanwhile, visual estimates of symptom severity increased across exposure groups, indicating that more severe symptoms were not the result of greater starch accumulation.

*Conclusion*

This study provides the first direct comparison of $CO_2$ assimilation between leaf and shoot levels, as well as the first comparison of assimilation in HLB-affected and healthy citrus. Shoot- and leaf-level responses were distinct. Shoot light response curves were much more gradual than those of leaves with $A_{max}$ at approximately 2x the irradiance of the $A_{max}$ of leaves. This was explained by the combination of leaf angles and photosynthetic stems, which contributed a mean of 10% of the total shoot $A_{gross}$. Likewise shoot and leaf responses to *C*Las infection differed. Where $A_{net}$ was reduced at the leaf level, shoot morphological acclimation partially compensated for this downregulation in leaf-level photosynthetic activity. These morphological changes further confirm the importance of canopy density as an important measure of disease severity. This study is an example of the value of assessing physiological processes along temporal and spatial scales, with whole-shoot photosynthesis providing one tool for such efforts.

**Supplemental materials**

Appendix S1. Cap design of whole shoot assimilation chamber. A) bottom view – stepped drilled hole to limit low leaf interference, B) top view – straight cut to allow for stem placement, C) fit of cap into chamber, D) showing fit into chamber to prevent leakage.

Appendix S2. Straw pieces fit to cover stem of ‘Valencia’ sweet orange (*Citrus x sinensis* [L.] Osbeck) shoot to measure whole shoot gas exchange excluding stem photosynthesis.

Appendix S3. Diagram describing the measurements taken to estimate stem surface area.

Appendix S4. Standard deviation of $A_{net}$ at photosynthetic photon flux density of 1000 μmols $m^{-2}$ $s^{-1}$ demonstrating measurement stabilization for ‘Valencia’ sweet orange (*Citrus x sinensis* [L.] Osbeck) in leaf chamber of whole shoot chamber. Dashed vertical line indicates stabilization point for leaf; dotted line indicates stabilization point for shoots.

Appendix S5. Illustration of ‘Valencia’ sweet orange (*Citrus x sinensis* [L.] Osbeck) shoots and leaves selected for to compare assimilation in leaves vs. shoots.

Appendix S6. Correlation of leaf disease and photosynthetic variables of healthy and *Candidatus* Liberibacter asiaticus-infected ‘Valencia’ sweet orange (*Citrus x sinensis* [L.] Osbeck) exposed to 0, 1, 1.5, and 2.5 years of inoculation via Asian citrus psyllid (*Diaphorina citri* Kuwayama) feeding and reproduction. Copy # - log transformed bacterial copy count, Starch – log transformed starch concentration (μg $mm^{-2}$), Visual – visual rating of citrus greening leaf chlorosis and mottling symptoms, *A* – net $CO_2$ assimilation (μmol $m^{-2}$ $s^{-1}$), and Position – leaf position on stem (1-n starting at apical leaf ). Darkened correlation boxes denote correlations where $P \leq 0.05$

Appendix S7. Correlation of leaf disease and photosynthetic variables of *Candidatus* Liberibacter asiaticus-infected 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck) exposed to 1, 1.5, and 2.5 years of inoculation via Asian citrus psyllid (*Diaphorina citri* Kuwayama) feeding and reproduction. Copy # - log transformed bacterial copy count, Starch – log transformed starch concentration (μg $mm^{-2}$), Visual – visual rating of citrus greening leaf chlorosis and mottling symptoms, *A* – net $CO_2$ assimilation (μmol $m^{-2}$ $s^{-1}$), and Position – leaf position on stem (1-n starting at apical leaf ). Darkened correlation boxes denote correlations where P≤0.05.assimilation (μmol $CO_2$ $m^{-2}$ $s^{-1}$), and Position – leaf position on stem (1-n starting at apical leaf ). Darkened correlation boxes denote correlations where P≤0.05.

Appendix S8. Correlation of shoot disease and photosynthetic variables of healthy and *Candidatus* Liberibacter asiaticus-infected 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck) exposed to 0, 1, 1.5, and 2.5 years of inoculation via Asian citrus psyllid (*Diaphorina citri* Kuwayama) feeding and reproduction. Copy # - log transformed bacterial copy count, Starch – log transformed starch concentration (μg $mm^{-2}$), Visual – visual rating of citrus greening leaf chlorosis and mottling symptoms, *A* – net $CO_2$ assimilation (μmol $m^{-2}$ $s^{-1}$), LA – mean leaf surface area ($cm^2$), SA – total shoot surface area ($cm^2$), Length – stem length (mm), and Inter – average internode length (mm). Darkened correlation boxes denote correlations where P≤0.05.

Appendix S9. Correlation of shoot disease and photosynthetic variables of healthy and *Candidatus* Liberibacter asiaticus-infected 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck) exposed to 1, 1.5, and 2.5 years of inoculation via Asian citrus psyllid (*Diaphorina citri*

Kuwayama) feeding and reproduction. Copy # - log transformed bacterial copy count, Starch – log transformed starch concentration (μg $mm^{-2}$), Visual – visual rating of citrus greening leaf chlorosis and mottling symptoms, *A* – net $CO_2$ assimilation (μmol $m^{-2}$ $s^{-1}$), LA – mean leaf surface area ($cm^2$), SA – total shoot surface area ($cm^2$), Length – stem length (mm), and Inter – average internode length (mm). Darkened correlation boxes denote correlations where $P \leq 0.05$.

*Conflict of Interest*

The authors declare they have not conflict of interest with this study, including no stake in any product described in the manuscript.

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*Authors' contributions*

MK, CV, and DR conceived experiments. MK developed whole-shoot chamber and conducted experiments. MK analyzed data and wrote manuscript. CV supervised experiments. CV and DR supervised analyses and manuscript writing, and revised manuscript.

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**List of figures**

Figure 1. Gross assimilation for ‘Valencia’ sweet orange (*Citrus x sinensis* [L.] Osbeck) leaf, stem and shoot. Letters within each plot denote means that are significantly different at $P \leq 0.05$ according to Fisher’s least significant differences.

Figure 2. Distinct carbon assimilation light response curves of ‘Valencia’ sweet orange (*Citrus x sinensis* [L.] Osbeck) leaf (dashed line) and shoot (dotted line).

Figure 3. Relationship of log transformed bacterial copy count and log transformed starch content of *Candidatus* Liberibacter asiaticus-infected and uninfected ‘Valencia’ sweet orange (*Citrus x sinensis* [L.] Osbeck) A) leaves and B) shoots.

Figure 4. Relationship of starch and shoot surface area of *Candidatus* Liberibacter asiaticus-infected and uninfected 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck). Data point shape and color denote *C*Las-infection exposure category: Low – 1 year *C*Las-infected Asian citrus psyllid feeding and breeding, Mid – 1.5 years *C*Las-infected Asian citrus psyllid feeding and breeding, High – 2.5 years *C*Las-infected Asian citrus psyllid feeding and breeding.

Figure 5. Relationship of average internode length and net $CO_2$ assimilation (*A*) of *Candidatus* Liberibacter asiaticus-infected and uninfected 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck).

## Tables

Table 1. Percent contribution of leaves and stems to the shoot surface area and net $CO_2$ assimilation ($A_{net}$) in 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck).

| Measurement basis | Leaf | Stem |
|---|---|---|
| Surface area | 94.3 ± 1.6 % | 5.6 ± 1.6 % |
| Mass | 83.0 ± 4.8% | 17.0 ± 4.8% |
| Per shoot $A_{net}$ | 89.2 ± 8.5 % | 10.7 ± 8.5% |

Table 2. Analysis of variance and means comparison of parameters of a light response curve function fit to leaf and whole shoot light response curves of 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck). Values are means ± standard error. Values followed by different letters are significantly different at P<0.05. $A_{sat}$: light-saturated net $CO_2$ assimilation (µmol $CO_2$ $m^{-2}$ $s^{-1}$); Φ:quantum yield (mols $CO_2$ fixed:mol photons absorbed) ; $R_d$: dark respiration (µmol $CO_2$ $m^{-2}$ $s^{-1}$); LCP: light compensation point (µmol photosynthetically active radiation $m^{-2}$ $s^{-1}$); Qsat75: photosynthetic flux density at 75% saturation (µmol photosynthetically active radiation $m^{-2}$ $s^{-1}$).

| Function parameter | Leaf | Shoot | F-value | *P*-value |
|---|---|---|---|---|
| $A_{sat}$ | 7.7 ± 0.46 b | 10.4 ± 0.74 a | 8.7 | 0.01 |
| Φ | 0.044 ± 0.002 | 0.053 ± 0.02 | 0.2 | 0.65 |
| $R_d$ | 1.2 ± 0.12 a | 0.35 ± 0.04 b | 42.4 | <0.0001 |
| LCP | 28.0 ± 8.4 a | 14.7 ± 10.8 b | 7.9 | 0.01 |
| $Q_{sat75}$ | 528 ± 80 b | 1532 ± 226 a | 17.3 | 0.0016 |

Table 3. Effect of huanglongbing symptom category on leaf-level net $CO_2$ assimilation ($A_{net}$), cycle threshold (Ct) and Copy # count of *Candidatus* Liberibacter asiaticus, foliar starch content, and visual disease rating in 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck). *P*-value of analysis of variance with infection categories as the fixed effect. Means (± standard error) followed by different letters are significantly different at $P<0.05$ according to Fisher's LSD.

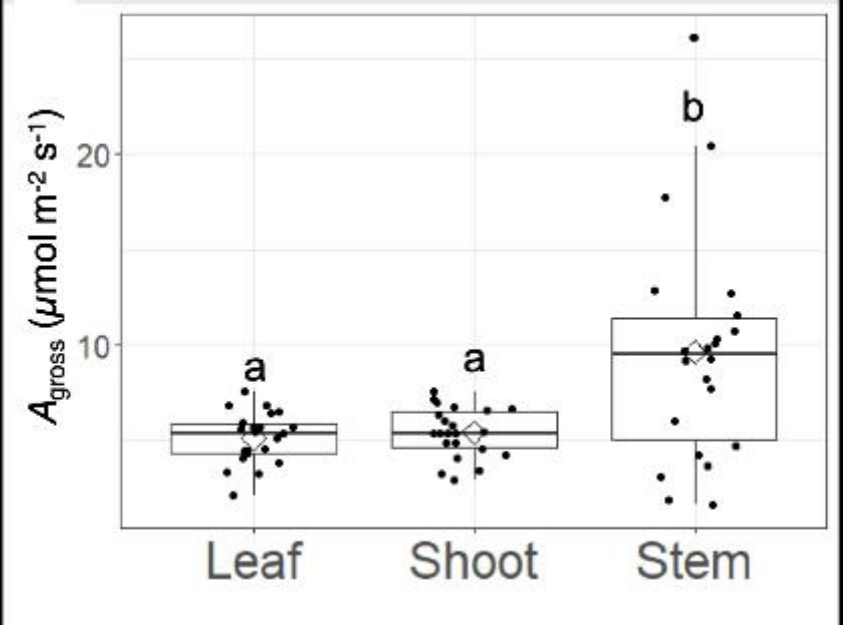
| | Uninfected | Low | Medium | High | *P*-value |
|---|---|---|---|---|---|
| $A_{net}$ (μmol $m^{-2}$ $s^{-1}$) | 3.97 ± 1.73 a | 1.91 ± 1.24 b | 2.61 ± 1.62 b | 2.70 ± 1.75 b | 0.00027 |
| qPCR Ct | 37.87 ± 2.35 a | 24.69 ± 4.14 b | 24.16 ± 3.13 b | 24.10 ± 2.52 b | <0.00001 |
| qPCR Copy # (log) | 2.60 ± 2.65 b | 11.78 ± 2.90 a | 12.27 ± 1.93 a | 12.30 ± 1.66 a | <0.00001 |
| Starch (μg $mm^{-2}$) | 1.08 ± 1.27 d | 11.31 ± 4.30 a | 5.69 ± 4.39 b | 3.55 ± 3.17 c | <0.00001 |
| Visual Rating (0-10) | 0.22 ± 0.60 c | 1.26 ± 0.98 b | 2.11 ± 1.53 a | 2.22 ± 1.42 a | <0.00001 |

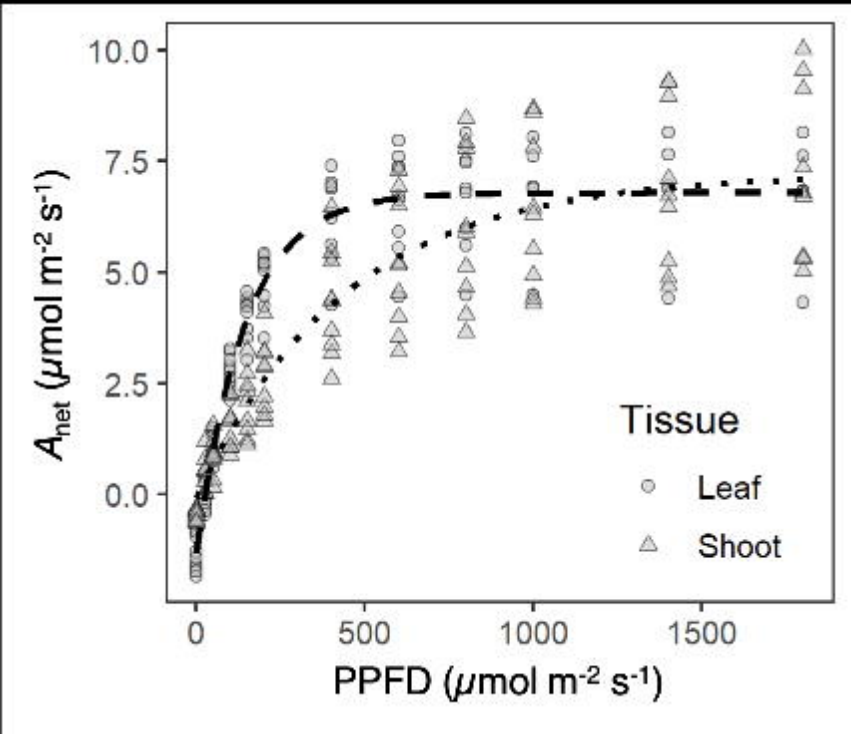
Table 4. Effect of huanglongbing symptom category on shoot-level net $CO_2$ assimilation ($A_{net}$) and shoot morphological variables. *P*-value of analysis of variance with infection categories as the fixed effect 'Valencia' sweet orange (*Citrus x sinensis* [L.] Osbeck). Means (± standard error) followed by different letters are significantly different at $P<0.05$ according to Fisher's LSD.

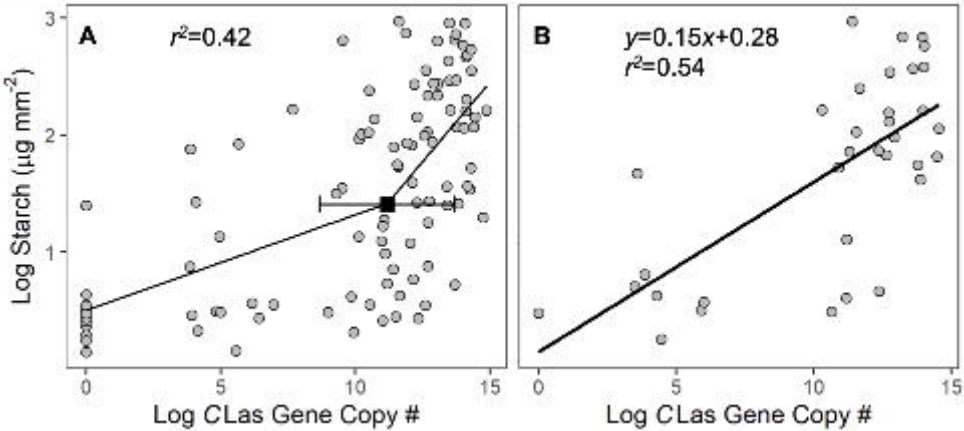
| | Uninfected | Low | Medium | High | P-value |
|---|---|---|---|---|---|
| $A_{net}$ (μmol $m^{-2}$ $s^{-1}$) | 1.91 ± 1.07 b | 2.42 ± 0.88 b | 2.44 ± 0.52 ab | 3.37 ± 1.34 a | 0.03167 |
| Stem length (mm) | 112.50 ± 22.5 | 112.30 ± 19.7 | 91.20 ± 26.1 | 90.30 ± 30.6 | 0.12900 |
| Mean leaf area ($cm^2$) | 15.79 ± 3.39 ab | 17.59 ± 5.86 a | 15.40 ± 6.33 ab | 11.38 ± 2.08 b | 0.05469 |
| Shoot surface area ($cm^2$) | 117.40 ± 42.0 a | 133.50 ± 42.8 a | 100.40 ± 48.8 ab | 68.30 ± 27.1 b | 0.01398 |
| Leaves per shoot | 6.75 ± 1.49 | 7.22 ± 1.48 | 6.25 ± 2.25 | 5.33 ± 1.58 | 0.14980 |
| Internode length (mm) | 17.41 ± 5.49 | 15.99 ± 3.92 | 15.33 ± 3.99 | 16.99 ± 2.67 | 0.73790 |

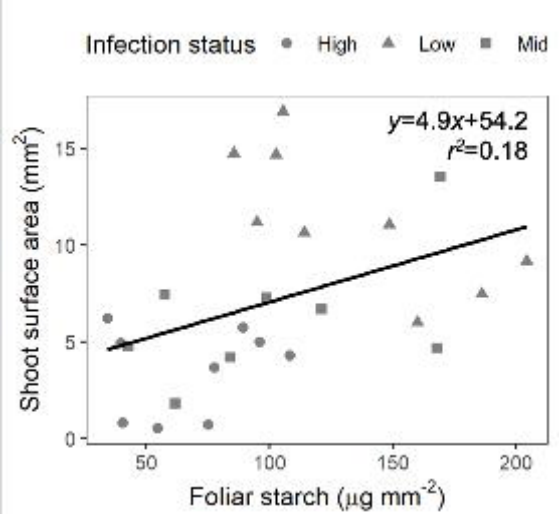
Table 5. Linear regressions of effects of gene copy number of *Candidatus* Liberibacter asiaticus on foliar starch content, of internode length on net $CO_2$ assimilation, and of foliar starch content on shoot surface area each assessed across three huanglongbing symptom categories including or not including a fourth category of uninfected trees, and measured at leaf or whole shoot levels. *P*-values below 0.05 are labeled with asterisks. "/" denotes nested variables.

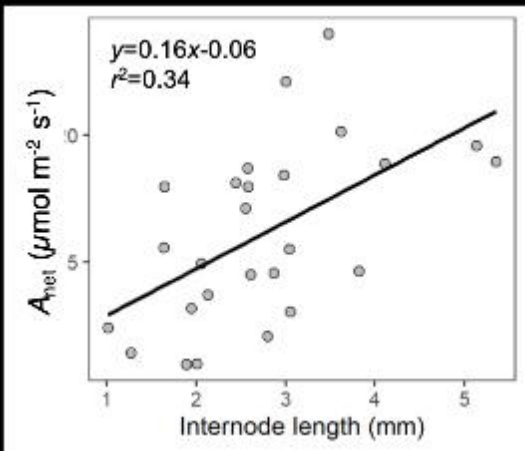
| Dependent variable | Including uninfected | Tissue | Effect | F-value | *P*-value |
|---|---|---|---|---|---|
| Starch content | yes | leaf | Bacterial copy number | 52.2130 | <0.00001* |
| | | | Shoot/tree | 8.1480 | 0.0053* |
| | | | Leaf/shoot/tree | 0.4403 | 0.5085 |
| | | shoot | Bacterial copy number | 33.1526 | <0.00001* |
| | | | Shoot/tree | 3.7404 | 0.0623 |
| Net $CO_2$ assimilation | yes | shoot | Internode length | 1.3619 | 0.2521 |
| | | | Shoot/tree | 3.9077 | 0.0570 |
| | no | shoot | Internode length | 10.7940 | 0.0032* |
| | | | Shoot/tree | 0.8399 | 0.3689 |
| Shoot surface area | yes | shoot | Starch content | 1.7849 | 0.1913 |
| | | | Shoot/tree | 0.3502 | 0.5583 |
| | no | shoot | Starch content | 4.3675 | 0.0479* |
| | | | Shoot/tree | 0.7133 | 0.4071 |


$A_{gross}$ ($\mu$mol m$^{-2}$ s$^{-1}$)
20
10
a
a
b
Leaf
Shoot
Stem


10.0
7.5
5.0
2.5
0.0
$A_{net}$ ($\mu$mol m$^{-2}$ s$^{-1}$)
0
500
1000
1500
PPFD ($\mu$mol m$^{-2}$ s$^{-1}$)
Tissue
Leaf
Shoot


A
$r^2$=0.42
B
$y$=0.15$x$+0.28
$r^2$=0.54
Log Starch (µg mm$^{-2}$)
Log *C*Las Gene Copy #
Log *C*Las Gene Copy #
0
5
10
15
1
2
3


Infection status
High
Low
Mid
y=4.9x+54.2
r²=0.18
Shoot surface area (mm²)
0
5
10
15
50
100
150
200
Foliar starch (μg mm⁻²)


y=0.16x-0.06
r²=0.34
A_net (μmol m⁻² s⁻¹)
10
5
1
2
3
4
5
Internode length (mm)