



Leaf Starch and Nutrient Responses to Stem Girdling and Drought Stress with Respect to Understanding HLB (Greening) Symptoms in Citrus

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The most important problem in world citrus production is the bacterial disease Huanglongbing (HLB; greening) presumably caused by a phloem-limited bacterium that is vectored by a phloem-feeding psyllid. The earliest visible symptoms of HLB in leaves are an asymmetrical chlorosis referred to as “blotchy mottle,” thought to be from starch accumulation from a phloem dysfunction and a decline in root health. We tested the hypothesis that such a visible symptom is not unique to HLB by stem-girdling two-year-old seedling trees of Cleopatra mandarin and Swingle citrumelo rootstocks in the greenhouse. Girdling induced a 4-fold greater starch concentration in leaves on well-watered trees after 32 days while starch in woody roots of girdled trees decreased up to 19-fold relative to non-girdled trees. Drought stress cycles induced some starch accumulation in non-girdled roots but there were no effects of drought stress on root starch in girdled trees. Girdling reduced leaf transpiration in well-watered trees. Leaves on girdled trees clearly had HLB-like visible blotchy mottle symptoms but no visible symptoms developed on non-girdled trees. The up to 40% increase in leaf starch increased leaf dr wt per leaf area (DW/LA) and consequently reduced many leaf nutrients on a leaf DW basis. Most of these differences disappeared when expressed on a LA basis except for girdling-induced decreases of leaf phosphorous and sulfur. Leaf boron (B) was inversely related to leaf starch when both were expressed on a LA basis. In the absence of HLB, girdling increased leaf starch, decreased root starch, and duplicated the asymmetric blotchy mottled visual leaf symptoms that have been associated with HLB-infected trees.

Currently, the most important problem in citrus production worldwide is the bacterial disease HLB (Huanglongbing; syn. citrus greening). HLB is presumably caused by the bacterium *Candidatus Liberibacter asiaticus* (Garnier and Bove, 1983) which when vectored into a citrus tree by phloem-feeding psyllids, triggers a cascade of events (Achor et al., 2010; Folimonova and Achor, 2010) causing phloem dysfunction, cellular collapse, and over-accumulation of carbohydrates in leaves (Bove, 2006). The earliest visible symptoms of HLB in leaves are vein yellowing and an asymmetrical chlorosis referred to as “blotchy mottle,” thought to be the result of starch accumulation (Etxeberria et al., 2009) and disintegration of chloroplast lamellae (Bondada and Syvertsen, 2005). Leaves can also develop a variety of chlorotic patterns that often resemble mineral deficiencies such as those of zinc, iron, and manganese deficiency. Such symptoms, however, are likely secondary symptoms resulting from root dysfunction (Johnson et al., 2014) and carbohydrate starvation (Jagoueix et al., 1994). Root loss or root growth restriction can lead to drought stress and changes in mineral nutrition in the shoot (Spann and Schumann, 2009). Thus, secondary symptoms resulting from mineral deficiency can complicate the diagnosis of HLB symptoms (Etxeberria et al., 2009).

Stem girdling, the removal of a strip of bark which includes phloem tissue, has been widely used in many fruit tree crops to increase carbohydrate accumulation in shoots, increase flower-

ing, fruit set, and fruit size (Goren et al., 2004). Gonzalez et al. (2012) showed that girdled ‘Valencia’ citrus trees accumulated large amounts of starch, producing symptoms analogous to those characteristic of HLB-affected tissues. This similarity prompted us to investigate the increase in starch concentration through time using girdled citrus trees of Cleopatra and Swingle rootstocks also exposed to water deficit. We hypothesized that both girdling and drought stress should lead to measurable changes in tree growth, starch allocation, and mineral nutrient composition that are similar to those associated with HLB. Understanding these responses will contribute to our knowledge of symptom development in HLB trees (Cimo et al., 2013).

Materials and Methods

Forty-eight uniform, well-nourished 2-year-old seedlings of Swingle citrumelo (*Citrus paradisi* Macf. x *Poncirus trifoliata* L.) with trifoliolate leaves and Cleopatra mandarin (*C. reticulata* Blanco) with entire leaves were purchased from a local nursery certified free of HLB. Seedling trees were about 180 cm tall and grown in 2.25-L pots filled with a soilless media consisting of peat/sponge-rock/vermiculite (3:1:1). Trees were grown in an unshaded greenhouse from 29 May to 29 June 2012 at the University of Florida/IFAS, CREC, Lake Alfred, FL. Maximum photosynthetically active radiation (PAR) at tree level was 1200 mmol·m⁻²·s⁻¹ (LI-170; LICOR, Inc., Lincoln, NE). Average day/night temperature was 40/30 °C and relative humidity varied diurnally from 40% to 100%.

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Each cultivar was divided into four treatment groups with six trees per treatment. The treatments were: 1) non-girdled and well-watered (= control); 2) non-girdled and drought stressed; 3) girdled and well-watered; and 4) girdled and drought-stressed. Well-watered trees were thoroughly irrigated three times a week, whereas drought stressed trees were irrigated only when wilting symptoms appeared, which averaged approximately once per week. All trees were fertilized once a week with 600 mL of complete fertilizer solution containing chelated iron (Fe; 1 mL·L⁻¹), 7N-3P-7K, and all micronutrients. No psyllids were ever observed but for preventative pest control, trees were sprayed once a week with either 1% agricultural spray oil or 1% soap solution.

On 29 May 2012, girdling was accomplished by surgically removing a 4-mm wide ring of stem bark tissue about 20 cm above the pot (Fig. 1). For leaf starch analyses, two 28-mm² diameter disks per leaf from two leaves per tree were taken at the same time each sampling using a sharp paper punch. All samples were taken from mature leaves (4–7 months old) and used to evaluate starch on the day of girdling (time zero) and then at 3, 8, 10, 13, 17, 21, 24, 28, and 31 d after girdling. Two additional disks were weighed, put in an envelope, and dried for 24 h at 60 °C to calculate leaf dr wt/leaf area (DW/LA). At harvest (Day 32), woody roots (> 2mm diameter) and fibrous roots (< 2 mm diameter) from each tree were washed and dried separately at 100 °C for 1 h to stop respiration and then at 60 °C for 48 h until completely dry. Dried root starch was quantified according to the method of Gonzalez and Etxeberria (Rosales and Burns, 2011). Starch determinations were corroborated by iodine (KI₂) staining of fresh tissue (Cimo et al., 2013).

Treatment effects were analyzed as a completely randomized 2 x 2 x 2 (rootstock, girdling, drought stress) design using analysis of variance from the SAS Institute Inc. (Cary, NC). Tukey's honestly significant difference was used to separate means when interactions among main factors were present. Regression analyses of selected variables were used to fit response lines and to investigate associations.

Results and Discussion

Two weeks after girdling, a few new shoots developed directly below the girdle in some trees but not all (Fig. 1). Regardless of rootstock, drought stress significantly decreased stem growth in non-girdled trees after 32 d but girdling had no effect on shoot growth (data not shown). In both rootstocks, there was a significant increase in DW/LA on girdled trees compared with the non-girdled trees, and Cleopatra leaves (Fig. 2A) tended to have greater DW/LA than the trifoliolate Swingle leaves (Fig. 2B). Similarly, leaf starch accounted for up to 50% of leaf DW and the increase in DW/LA over the 32 d (Fig 2 C,D). Girdling reduced woody root starch but drought stress had little effect on root starch (Fig. 3). These data were visibly corroborated by iodine staining (KI₂) of fresh tissue (Fig. 4)

In girdled trees, asymmetrical blotchy mottle patterns were evident in leaves when starch accumulated as shown in Cleopatra leaves (Fig. 5A). These patterns appear identical to those associated with HLB trees in the field (Fig. 5, inset). On non-girdled trees, which did not accumulate starch, no leaf yellowing occurred (Fig. 5B). Similar symptoms were observed in leaves on Swingle trees (data not shown).

When expressed on a leaf DW basis, leaf nitrogen levels were reduced ($P < 0.001$) by girdling from the optimum range (24–25 mg·g⁻¹) to deficient levels (18–20 mg·g⁻¹) for citrus trees (Obreza

and Morgan, 2008) in both rootstocks regardless of water status. Although other macronutrients generally were in the sufficient range, girdling also reduced leaf phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) (data not shown). Similarly, girdling reduced all the measured micronutrients, leaf B, zinc (Zn), manganese (Mn), Fe, and copper (Cu) on a leaf DW basis, but almost all remained in the sufficient range (data not shown). Since leaf DW/LA was increased by starch from girdling, levels of leaf nutrients were undoubtedly artificially decreased by leaf starch when expressed on a DW basis. Thus, almost all of the



Fig. 1. Girdled trunk with rapidly growing shoot sprout below the 4-mm wide girdle.

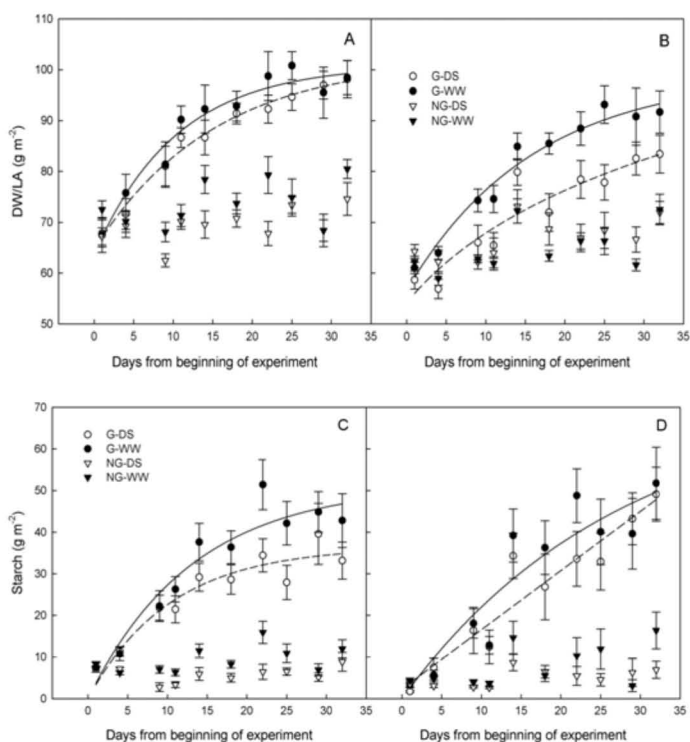


Fig. 2. The increase in leaf dr wt per leaf area (DW/LA; A, B) and leaf starch (C, D) during 32 d after girdling (G), non-girdled (NG), drought-stressed (DS) or well-watered (WW) treatments for Cleopatra (A, C) and Swingle (B, D) trees. Error bars indicate SEMs (n = 6).

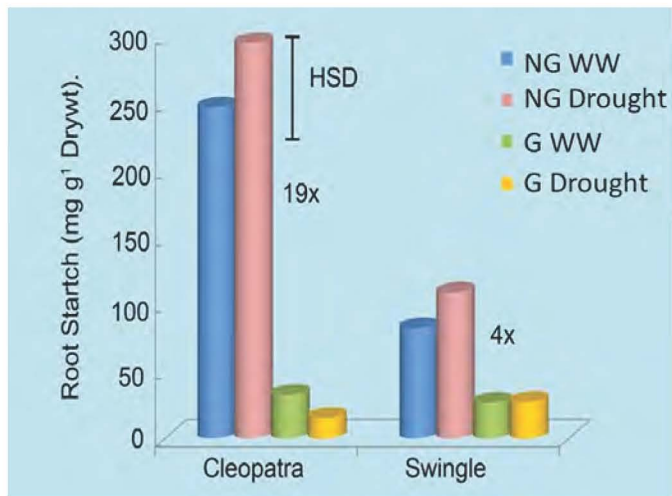


Fig. 3. The girdling treatment (G) reduced starch in woody roots 19 fold in Cleopatra and 4 fold in Swingle but drought stress cycles had no effect on root starch after 32 days. HSD = Tukey's honestly significant difference.

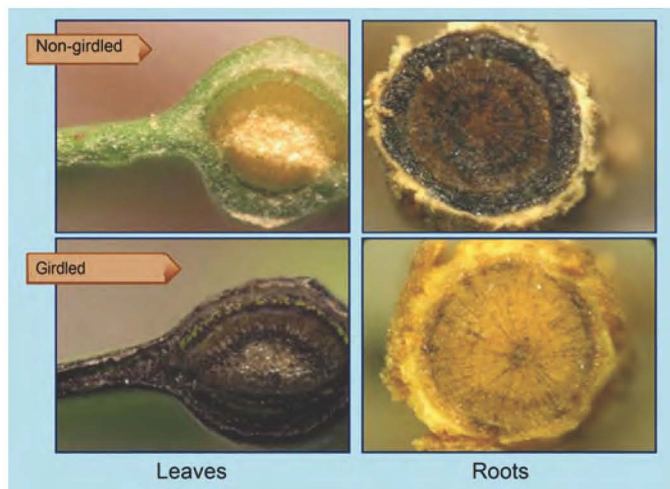


Fig. 4. Visual corroboration of leaf and root starch (KI_2 test) in well-watered non-girdled and girdled trees after 32 days.

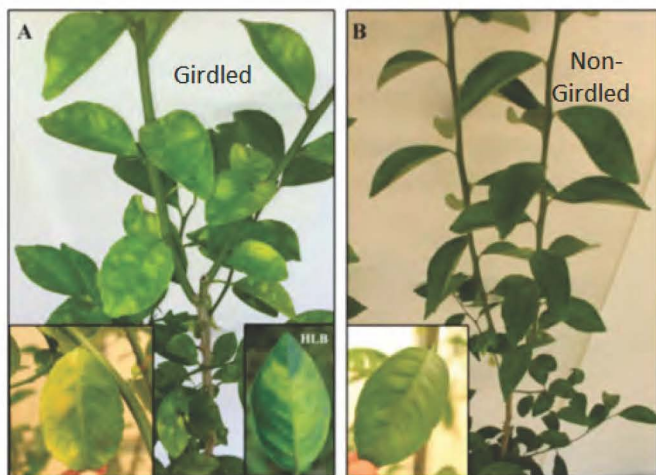


Fig. 5. Visual appearance of leaves from girdled (A and left inset) and non-girdled (B) Cleopatra trees after 32 d of treatment. For comparison, the lower right inset in A is an HLB-positive leaf from the field.

nutrient changes disappeared when expressed on a leaf area basis except for decreased P, sulfur (S; Fig. 6), and Fe below that of non-girdled leaves (not shown). The prominent increase in leaf DW resulting from starch accumulation could have led to erroneous conclusions about HLB effects on DW-based leaf nutrient changes (Spann and Schumann, 2009).

Using all Swingle leaf B and starch values across both drought and girdling treatments, there was a significant negative non-linear relationship ($P < 0.001$) between leaf B and starch content when expressed on a LA basis (Fig. 7). The relationship revealed that most of the decrease in leaf B occurred with moderate increases in leaf starch and that the rate of decrease in leaf B decreased as leaf starch increased. However, there was no effect of leaf starch on leaf B in Cleopatra leaves which may indicate a more direct link between B accumulation and carbon translocation rather than between B and starch concentration. Decreases in leaf B previously have been associated with HLB (Spann and Schumann, 2009). In addition, Hass and Klotz (1931) described high concentrations of carbohydrates in leaves and morphological symptoms associated with low B as "internal girdling" because of the breakdown of

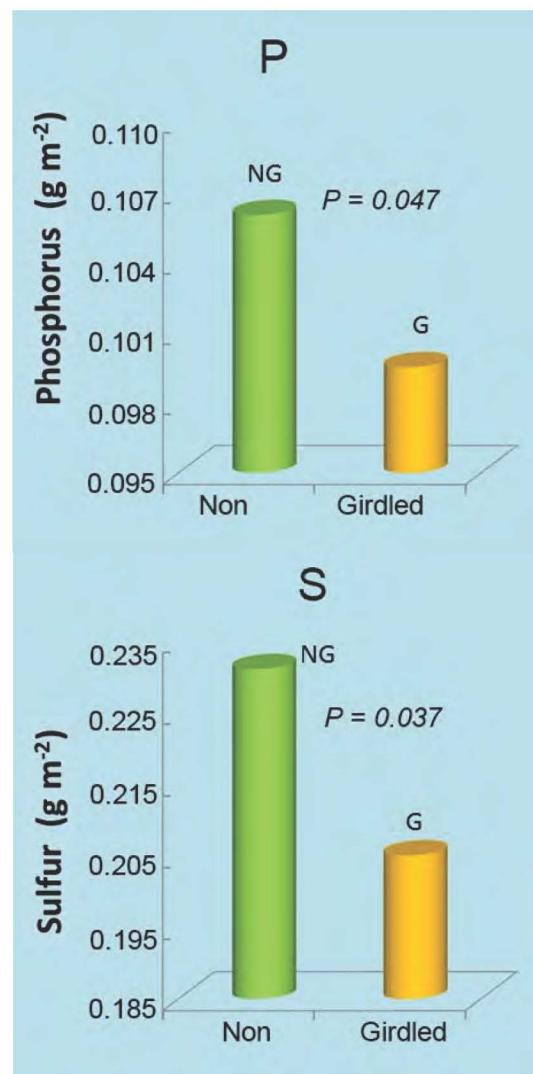


Fig. 6.. Girdling decreased leaf P and S even when expressed on a leaf area basis.

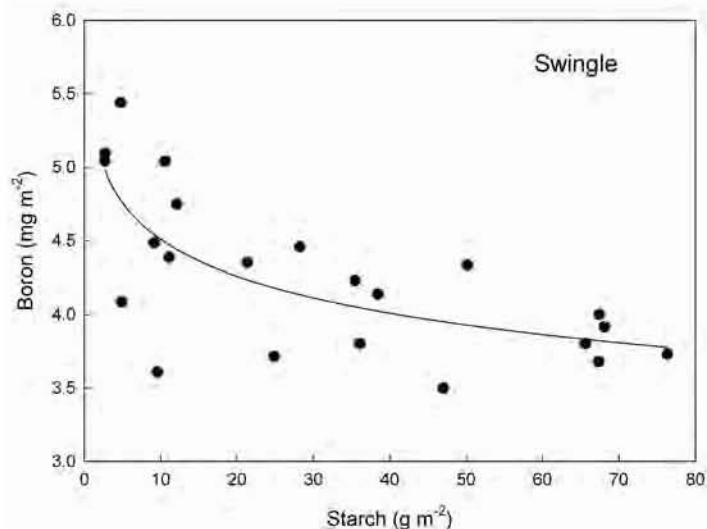


Fig. 7. Relationship between leaf boron and starch expressed on a leaf area (LA) basis in Swingle trees. Each point represents one leaf; data are from all drought and girdling treatments pooled together.

the phloem tissues and a concomitant reduction in translocation. An accumulation of starch in Swingle leaves independent of HLB, can be associated with a decrease in leaf B even when expressed on a LA basis. This is an important outcome because it describes a new relationship between leaf starch and B in the absence of HLB disease.

Conclusions

In the absence of HLB, girdled trees of both rootstocks developed asymmetrical blotchy mottle patterns in leaves when starch accumulated. These patterns appear identical to those associated with HLB trees in the field. The increase in leaf starch, reduced most leaf nutrients when expressed on a leaf DW basis but when nutrients were expressed on a LA basis, girdling only reduced leaf P, S, and Fe. Non-linear regression analysis, however, revealed a decrease in leaf B as starch increased in Swingle leaves, especially at relatively low levels of B and starch expressed on a LA basis.

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