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# Photosynthesis down-regulation precedes carbohydrate accumulation under sink limitation in *Citrus*

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*Keywords*: *Citrus sinensis* L. 'Salustiana', gene expression, girdling, leaf-to-fruit ratio, soluble sugars, starch

**Summary** Photosynthesis down-regulation due to an imbalance between sources and sinks in *Citrus* leaves could be mediated by an excessive accumulation of carbohydrates. However, there is a limited understanding of the physiological role of soluble and non-soluble sugars in photosynthesis regulation and the elements triggering the down-regulation process. In this work, the role of non-structural carbohydrates in the regulation of photosynthesis under a broad spectrum of source-sink relationships has been investigated in the Salustiana sweet orange. Soluble sugar and starch accumulation in leaves, induced by girdling experiments, did not induce down-regulation of the photosynthetic rate in the presence of sinks (fruits). The leaf-to-fruit ratio did not modulate photosynthesis but allocation of photoassimilates to the fruits. The lack of strong sink activity led to a decrease in the photosynthetic rate and starch accumulation in leaves. However, photosynthesis down-regulation due to an excess of carbohydrates was discarded because photosynthesis and stomatal conductance reduction occurred prior to any significant accumulation of carbohydrates. Gas exchange and fluorescence parameters suggested biochemical limitations to photosynthesis. In addition, the expression of carbon metabolism-related genes was altered within 24 hours when strong sinks were removed. Sucrose synthesis and export genes were inhibited, while the expression of ADP-glucose pyrophosphorylase was increased to cope with the excess of assimilates. In conclusion, changes in starch and soluble sugar turnover, but not sugar content per se, could provide the signal for photosynthesis regulation. In these conditions, non-stomatal limitations strongly inhibited the photosynthetic rate prior to any significant increase in carbohydrate levels.

## Introduction

Plants maintain a balance between carbon assimilation, storage and growth in response to developmental and environmental signals (Smith and Stitt, 2007). In subtropical regions, *Citrus* plants accumulate carbohydrates as reserves during the winter in roots and leaves. These reserves are mobilised and used during the main flush of growth and bloom in spring (Goldschmidt and Koch, 1996). Fruit set and further vegetative and fruit development in *Citrus* is mainly supported by actual photosynthetic rates because carbohydrate reserves in the tree have been depleted after the initial stages of bud sprouting and flowering (Syvertsen and Lloyd, 1994).

The improvement of crop yield through enhancing photosynthesis depends on an understanding of the nature of the control mechanisms (Paul and Foyer, 2001). It is assumed that photoassimilate production in leaves is modulated by the demand of the sinks (Jang and Sheen, 1994; Goldschmidt and Koch, 1996), although the sink effects on photosynthesis are not observable in all conditions and species. A positive effect of crop load has been reported in *Citrus* (Lenz, 1978; Syvertsen, 1994; Iglesias et al., 2002). In contrast, photosynthesis was reduced in some cases when fruits were eliminated (Bustan et al., 1992; Yamanishi, 1995; Okuda et al., 1996; Syvertsen et al., 2003). Furthermore, under regular cropping conditions, the root system seems to be a particularly strong and unsaturable sink (Goldschmidt and Koch, 1996). It is not clear to what extent sink demand controls citrus photosynthetic rates under cropping conditions.

The sink effect on photosynthesis may operate through feedback/feedforward regulatory controls mediated by several sugar sensitive systems (Paul and Pellny, 2003; Rolland et al., 2006). Soluble sugar accumulation in leaves in response to decreased sink demand has been related to the down-regulation of photosynthesis in several species (Franck et

al. 2006; Paul and Driscoll 1997; Quilot et al. 2004; Urban et al. 2004). Source-sink imbalances through girdling, defruiting, defoliation and in vivo sucrose supplementation have been carried out in *Citrus* to study the role of leaf non-structural carbohydrates in photosynthesis regulation (Iglesias et al. 2002; Syvertsen et al. 2003). A feedback inhibition of photosynthesis due to starch accumulation has been proposed, although the role of soluble sugars and starch is not well established. The up- and down-regulation of photosynthesis after defoliation and sucrose supplementation could be correlated with the sucrose and starch content when measured after 10 days (Iglesias et al. 2002). However, after 20 days, neither the rate of changes in carbohydrate content nor the absolute content of soluble sugars correlated with the photosynthetic rate. In contrast, starch content showed a correlation with photosynthesis at this time. Notably, it is difficult to compare the different techniques used to alter carbohydrate content because other physiological parameters could also be altered. Whether the regulation of photosynthesis depends on sink activity or carbohydrate content is not clear. Furthermore, the kinetics of the down-regulation has not been studied, and it is unknown whether there is a starch threshold that triggers the down-regulation process.

The mechanism of photosynthetic inhibition due to excessive starch accumulation could involve $CO_2$-restricted diffusion or chloroplast rupturing (Schaffer *et al.*, 1986). However, in the above-mentioned experiments, the relatively small starch accumulation appears to preclude its negative effects on $CO_2$ diffusion, and symptoms of chlorosis were not observed in leaves.

Diverse studies involving different species (Paul and Foyer, 2001, and references herein) have cast doubt on the existence of a simple relationship between starch

accumulation and feedback regulation. When sinks cannot use all of their assimilated carbon, sugar accumulates in leaves, having direct effects on the expression of ADP-glucose pyrophosphorylase (Müller-Rober *et al*., 1991) and other carbohydrate-responsive enzymes involved in sucrose and starch metabolism (Koch, 1996), resulting in adaptive changes in assimilate partitioning. In general, conditions of limited carbohydrate availability can enhance the expression of genes coding for proteins involved in reserve mobilisation and export processes (Koch, 1996). This starch turnover may play a role in signalling assimilates abundance and regulating photosynthetic gene expression. Some evidence suggests that hexose, derived from starch in the night hours, may provide signals for feedback regulation through the modulation of gene expression (Cheng *et al*, 1998). However, sugar signalling is not the only factor responsible, and photosynthesis responds to and is controlled by whole plant source-sink and nutrient balance, mainly by carbon to nitrogen ratio (Paul and Foyer, 2001).

In this work, a broad range of source-sink relationships have been examined in field grown sweet orange trees to investigate the following:1) The effects of soluble sugar and starch leaf contents on the photosynthetic rate in the presence of sinks. Short- and long-term responses have been studied by testing different levels of leaf-to-fruit ratios in girdled branches. 2) Photosynthetic down-regulation under sink limitation and the role of carbohydrate content. The long-term responses and the kinetics of the process during the first 48 h have been studied. 3) The expression of genes related to starch and sucrose metabolism in relation to sink activity and carbohydrate content. The responses within the first 24 h for different source-sink relationships have been determined.

## Materials and methods

*Plant material*

The experiments were performed on 40-year-old Salustiana sweet orange trees (*Citrus sinensis* L.) grafted on Troyer citrange (*C. sinensis* [L.] Osb. x *Poncirus trifoliata* Raf.) rootstock. Trees were drip irrigated, and mineral elements were supplied in the irrigation water from February to September. The fertilisation was decided based on leaf analysis performed the previous year. Trees present alternate bearing habits, and the flowering intensity depends on the fruit load of the previous year. The trees alternated between years of abundant flowering and fruit set ('on' year) and years of almost no flowering ('off' year). During each year, 'on' and 'off' trees were found in the same orchard. Experiments were performed on 'on' trees.

*Gas exchange and fluorescence measurements*

Net $CO_2$ fixation rate ($A_N$), stomatal conductance ($g_s$) and substomatal $CO_2$ concentration ($C_i$) were measured at steady-state under conditions of saturating light (1200 μmol $m^{-2}$ $s^{-1}$) and 400 ppm $CO_2$ with a LI-6400 (LI-COR, Nebraska, USA). During the experimental period (June to July), midday air temperatures ranged from 26 to 35 ºC, and water vapour pressure deficit (vpd) ranged from 1.5 to 3 kPa. To evaluate the presence of chronic photoinhibitory processes, the maximum quantum yield of PSII ($F_v/F_m$) was measured on leaves after 30 min in darkness using a portable pulse amplitude modulation fluorometer (MINI PAM, Walz, Effeltrich, Germany). The background fluorescence signal in dark adapted leaves ($F_o$) was determined with a 0.5 μmol photon $m^{-2}$ $s^{-1}$ measuring light at a frequency of 600 Hz. The application of a saturating flash of 10000 μmol photon $m^{-2}$ $s^{-1}$ allowed for estimations of the maximum fluorescence ($F_m$). The electron transport rate (ETR) and non-photochemical quenching

(NPQ) were estimated as described in Maxwell and Johnson (2000). Gas exchange and fluorescence measurements were performed from 900 h to 1200 h on clear, cloudless days. One measurement per tree was performed on a fully expanded mature leaf (third or fourth leaf from the shoot apex). Ten trees were measured for each treatment.

The maximum rate of Rubisco-mediated carboxylation ($V_{c\ max}$) and the maximum rate of electron transport ($J_{max}$) were estimated in attached leaves from $A_N/C_c$ curves based on the equations of Farqhuar et al. (1980) and modified by Harley and Sharkey (1991). Temperature was maintained at 30 ºC, irradiance at 1200 µmol photon $m^{-2}$ $s^{-1}$, and ambient $CO_2$ concentration ($C_a$) in the cuvette was controlled with a $CO_2$ mixer across the series 400, 300, 200, 100, 50, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 ppm. Measurements were recorded after equilibration to a steady state, and $CO_2$ leakage was determined at each $C_a$ value by placing a dead leaf in the cuvette (Long and Bernacchi, 2003). Five independent $A_N/C_c$ curves were produced for each treatment.

*Carbohydrate analysis*

The determination of soluble sugars and starch (as percentage per dry weight, % DW) was performed as described by McCready et al. (1950). Three independent extracts, obtained from nine trees (two leaves per tree and three trees per extract), were assayed for each treatment in all determinations. Unless otherwise stated, leaves were sampled at 1200 h.

*The effect of leaf-to-fruit ratio*

Uniform 1-year-old shoots with at least 50 leaves and a single fruit borne in a unifloral leafy inflorescence formed during the current year were girdled on June 20, 2008. A complete ring of bark (2 mm wide) was removed, leaving 10, 25 and 40 fully expanded

young leaves (from the spring flush of the current season) along the fruit above the girdle. The girdle was protected with PVC tape and maintained during the experiments. At the date of girdling, the average diameter of the fruitlet population was 23.0 ± 0.4 mm. No vegetative growth was produced in the girdled shoots during the experiments. Each treatment was replicated ten times in ten different trees. Non-girdled unifloral leafy inflorescences were used as controls. Photosynthesis was measured 5, 7, 15 and 35 days after girdling the shoots. Leaf carbohydrates were determined after 7, 15 and 30 days.

*The effect of sink availability*

Uniform 1-year-old vegetative (without fruit) shoots with at least 20 leaves were selected and girdled, leaving ten fully expanded young leaves distal to the girdle. Girdling was performed between 0930 and 1000 h. Measurements were also performed on shoots bearing one fruit and ten leaves above the girdle. The results were compared to those obtained in non-girdled vegetative and inflorescence (bearing one fruit) shoots. Each treatment was replicated ten times in ten different trees.

In a first long-term experiment (June 28, 2009), photosynthesis was measured 1, 2, 5, 7, 21 and 35 days after girdling, and carbohydrate levels were determined after 24 and 48 h. The experiment was repeated (July 3, 2009), and photosynthesis measurements were performed 1, 2, 3, 5, 8 and 24 h after girdling. The carbohydrate-related gene expression was also studied 2, 8 and 24 h after girdling in this experiment.

*Gene expression analysis*

Leaf tissue was finely ground in liquid nitrogen and total RNA was extracted using the TRIzol reagent (Invitrogen), purified using the RNEasy Mini Kit (Quiagen) and treated

with RNase-free DNase (Quiagen), according to manufacturer's instructions. RNA was quantified with a UV/VIS spectrophotometer, and first-strand cDNA was synthesised from 1.2 µg of total RNA by using the First Strand cDNA Synthesis Kit AMV (Roche) for real-time PCR (RT-PCR).

Oligonucleotide primers (Table 1) were designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA) after sequence alignments using sequence databases (Citrus HarvEST, University of California and BLAST, NCBI). *C. sinensis* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The amplification efficiency was tested for all pair of oligonucleotides (Livak and Schmittgen, 2001).

Diluted cDNA (3 µg) was used as the template for semi-quantitative RT-PCR amplification in 20 µL reactions containing 0.3 µM of each primer (0.15 µM for GAPDH) and 10 µL SYBR Green PCR master mix (Power SYBR®Green PCR Master Mix, Applied Biosystems). The PCR mixtures were preheated at 50 °C for 2 min and then at 95 °C for 10 min, followed by 40 amplification cycles (95 °C for 15 s; 60 °C for 1 min). Amplification specificity was verified by a final dissociation (95 ºC 15 s, 60 ºC 20 s and 95 ºC 15 s) of PCR products. The levels of PCR products were monitored with an ABI PRISM 7000 sequence detection system and analysed with ABI PRISM 7000 SDS software (Applied Biosystems). At least three independent biological replicates of each sample and two technical replicates of each biological replicate were used for the RT-PCR analysis. Relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen 2001).

*Statistical analysis*

Analysis of treatment comparisons was performed by ANOVA (Statgraphics Plus 5.1 for Windows, Statistical Graphics Corp.). Mean separations were performed with the Tuckey multiple range test. Linear regression analysis was used to evaluate the relationships between parameters.

## Results

*The effect of leaf-to-fruit ratio*

Net $CO_2$ fixation rate did not change ($P < 0.05$) in girdled shoots with up to 40 leaves per fruit when compared to ungirdled shoots (Fig. 1). There were no differences in $A_N$ (Figure 1) and $g_s$, $C_i$ and $F_v/F_m$ (data not shown) among shoots with 10, 25 and 40 leaves during the experiment (30 days). During the experiment, changes in the daily $A_N$ values within each treatment were due to changes in the daily environmental conditions. The photosynthetic rate was closely related to the leaf temperatures ($r = -0.85$; $P < 0.05$). Total non-structural carbohydrates and soluble sugars showed a similar trend during the course of the experiment in the non-girdled unifloral shoots (Table 2). Both levels decreased initially during the first 7 days and recovered after 35 days. Starch content in the leaves decreased with time, from an initial value of 9.7% to a value of 4.5%. Leaf area had no effect on the total sugar content after 7 days of girdling, but at the end of the experiment, the accumulation of total carbohydrates in leaves (Table 2) was closely related to the total leaf area per fruit ($r = 0.79$; $P < 0.01$). Notably, the carbohydrate content in leaves markedly differed between the control and the girdled shoots with 40 leaves (17.7 % and. 29 %, respectively), mainly due to the changes in soluble sugars (13.2 % and 20.9 %, respectively). Despite these differences, photosynthetic rates remained unchanged.

*The effect of sink availability*

The evolution of the effect of sink presence on photosynthesis is presented in Fig. 2. Net $CO_2$ fixation rates did not differ among ungirdled vegetative and unifloral shoots and girdled shoots bearing one fruit and 10 leaves during the experiment. However, twenty-four hours after girdling, $A_N$ fell significantly ($P < 0.05$) in vegetative girdled shoots with 10 leaves (Fig. 2). This decrease was maintained over the course of the experiment. In these shoots, the leaves became chlorotic with time, and almost all abscised after 35 days (data not shown).

There was also a significant decrease in stomatal conductance and the maximum quantum efficiency of PSII photochemistry (Table 3) after 24 h in vegetative girdled shoots. The substomatal $CO_2$ concentration ($C_i$) increased after 48 h in these shoots. The decrease in $F_v/F_m$ was due to a significant increase in $F_o$ (Table 3). No changes were observed in $F_m$, ETR, NPQ and leaf temperatures among treatments (data not shown). The maximum carboxylation rate of Rubisco fell ($P < 0.05$) in leaves of vegetative girdled shoots as compared to ungirdled shoots (80 and 145 μmol $m^{-2}$ $s^{-1}$, respectively). No changes were observed in the maximum rate of electron transport (102 and 119 μmol $m^{-2}$ $s^{-1}$, respectively).

The total soluble sugars and starch content did not differ among treatments 24 h after girdling (Table 4). After 48 h, vegetative girdled shoots accumulated starch and soluble sugars. A transient increase in soluble sugars was observed in unifloral girdled shoots after 48 h.

In a further experiment, photosynthesis was measured within the first 24 h after girdling. $A_N$ did not differ in vegetative and unifloral girdled shoots during the first 8 h

(Fig. 3), corresponding to the main diurnal light period (from 1000 to 1800 h). The net $CO_2$ fixation rate only fell after 24 h in vegetative girdled shoots.

Diurnal changes in the relative expression of genes implicated in carbon metabolism have been observed (Fig. 4). All analysed genes, except sucrose synthase 1, exhibited increased expression during the day, returning to initial levels the next morning. Gene expression among treatments did not differ two hours after girdling, with the exception of ADP-glucose pyrophosphorylase. The expression of this gene was strongly suppressed in vegetative girdled shoots. Compared to ungirdled shoots, the expression of sucrose synthase A, sucrose transporter 1 and sucrose phosphate synthase were reduced after 8 hours of girdling in vegetative shoots (Fig. 4). This decrease was maintained after 24 hours, with the exception of sucrose transporter 2, which showed similar expression levels. The expression of ADP-glucose pyrophosphorylase increased with time in girdled vegetative shoots. In contrast, unifloral girdled shoots showed increased levels of the expression of sucrose synthase A, sucrose transporter 1 and 2 and sucrose phosphate synthase after 8 h of girdling, compared to ungirdled vegetative shoots (Fig. 4). However, after 24 h, the expression levels of these genes did not differ from those observed in vegetative shoots. Sucrose synthase 1 expression did not change with time or treatment, with the exception of the decrease in unifloral girdled shoots observed after 24 h.

## Discussion

*Effect of leaf-to-fruit ratio*

Trunk girdling is widely used in citrus mainly to increase flowering, fruit set and fruit size (Goren et al., 2003). The downward translocation of photoassimilates and

metabolites through the phloem is blocked, creating a closed environment for carbon metabolism and transport. Carbohydrates accumulate in the leaves and shoot bark above the girdle in fruitless trees, whereas the developing fruit consumes all available carbohydrates (Li et al., 2003a). In this work, imbalances in source-sink ratios have been induced through girdling experiments.

We report an increase in starch and soluble sugars with leaf-to-fruit ratios in girdled shoots, as described previously in several species (Urban et al. 2004; Proietti et al. 2006), including citrus (García-Luis et al. 2002). At high leaf-to-fruit ratios (40), soluble sugars accumulated up to 21% DW and up to 8% starch at the end of the experiment, as compared to 13 and 5% in ungirdled controls, respectively. Approximately twenty-five leaves per fruit developed naturally in mature Salustiana trees (non published data), and at the time of the experiments, root growth did not occur in 'on' trees, and carbohydrates did not accumulate in the roots because fruits are the predominant sinks for the available carbohydrates (Goldschmidt and Koch, 1996). Despite the sink strength of the fruit, some basipetal transport of sugars cannot be ruled out in the controls because total carbohydrates in girdled shoots bearing 25 leaves per fruit are higher (23 and 18% DW, respectively). The sugar content at lower leaf-to-fruit ratios, although higher than controls, was not significantly different.

Although source-sink ratios altered the carbohydrate content in leaves, no effect of fruit load on photosynthesis was observed in Salustiana sweet orange. These results contrasted with previous reports for apple (Palmer, 1992), grapevine (Edson et al., 1995) and mango (Urban and Lechaudel, 2005), where a negative correlation was found. Iglesias et al. (2002) reported that non-structural carbohydrates accumulated up

to 26% DW after sucrose supplementation in Satsuma mandarin, and photosynthesis was inhibited. These values are close to those presented in our study, but nevertheless, the photosynthetic rate was not repressed. However, the interpretation of sugar feeding experiments can be complicated because other metabolites or pathways may also be altered (Paul and Foyer, 2001).

Our results suggest that whenever a sink (fruit) is present, the photosynthetic rate is not affected by the fruit load. Since the transport capacity of the phloem is not limiting in the Salustiana sweet orange (García-Luis et al. 2002), the higher availability of photoassimilates with leaf-to-fruit ratio allowed for a higher rate of dry matter accumulation in the fruits (García-Luis et al. 2002). Differences with other species could be explained by the export rate of photoassimilates from leaves to fruits, which could be limited at some point in the pathway linking these organs, as reported by Franck et al. (2006) for *Coffea*.

An effect of the crop load on $A_N$ in relation to starch content has been reported (Syvertsen et al., 2003). However, differences in photosynthesis were only present between fruiting and non-fruiting trees and not between full- and half-crop trees. The non-fruiting trees were very young (5-years-old), and therefore the root system could not be a strong and unsaturable sink.

Thus, it can be concluded that whenever a sink was present, the photosynthetic rate remained unchanged relative to the sink demand, and soluble sugar accumulation per se did not provoke a down-regulation of the photosynthetic rate. Furthermore, the leaf-to-fruit ratio did not modulate photosynthesis but did affect photoassimilate allocation to

the fruits. In fact, environmental conditions accounted for most of the variation in the photosynthetic rate and stomatal conductance during the experiment in Salustiana sweet oranges (Fig. 1). $A_N$ was closely related to temperature, and no chronic photoinhibition was caused by the environmental conditions or girdling.

*The effect of sink availability*

The effect of alternative sinks on photosynthesis was assessed by comparing the photosynthetic rates among girdled and ungirdled unifloral and vegetative shoots (Fig. 2). When any strong sink was available (e.g. roots and/or fruits), no differences were observed in $A_N$ or $g_s$. No effects related to leaf-fruit distance were observed in the Salustiana sweet orange, as photosynthetic rates were similar in fruiting and non-fruiting shoots. In addition, non-structural carbohydrate content did not differ among these treatments. These results could contradict previous observations of a higher photosynthetic rate in non-fruiting shoots (Syvertsen et al. 2003). However, in contrast to the present study, measurements were performed in leaves immediately adjacent to the fruit in that work. The authors reported effects that were opposite to the responses of $A_N$ to crop load in most leaves of the canopy.

A strong reduction in photosynthesis was only observed in girdled vegetative shoots when sink strength was sharply reduced. Similar observations have been noted previously (Goldschmidt and Koch, 1996; Iglesias et al. 2002), although the kinetics of the down-regulation and its relation to carbohydrate content variation have not been previously studied. In our experiments, no changes were observed during the first 12 h (Fig. 2), but $A_N$ and $g_s$ fell after the night period (Fig. 3), and low values were maintained until severe symptoms of chlorosis developed and leaves abscised (Schaffer

et al. 1986). Nevertheless, starch and soluble sugars only accumulated after 48 h, and $A_N$ down-regulation preceded any increase in leaf non-structural carbohydrates. Thus, a direct relationship between sugar accumulation and photosynthesis repression must be questioned. Furthermore, soluble sugar levels are lower than those observed in the high leaf-to-fruit ratio experiment, where $A_N$ remained unchanged. Starch accumulation in leaves has been reported to repress photosynthesis (Iglesias et al. 2002). Although $A_N$ was also inversely related to starch content in our study after 48 h, down-regulation of photosynthesis was not a direct consequence of starch accumulation.

DaMatta et al. (2008) proposed that decreased $A_N$ in defruited trees was independent of carbon metabolism and directly related to a lower $CO_2$ availability coupled with a lower $g_s$. Our results indicate that the inhibition of $A_N$ was not attributable to a $g_s$-associated decrease in $C_i$ within the first 24 h, as described by other authors (Urban et al., 2004 and Li et al. 2007), because $C_i$ did not decrease in girdled vegetative shoots. On the contrary, $C_i$ increased in parallel with the decreased $g_s$ after 48 h, suggesting non-stomatal limitations to the net $CO_2$ fixation rate (Pérez-Pérez et al. 2007).

Source-sink manipulations may also alter the fluorescence kinetics of chlorophyll a (Syvertsen et al. 2003; Urban et al. 2004; Rivas et al. 2007). In our study, fluorescence parameters were only altered in the absence of fruits. Fv/Fm declined after 24 h in the leaves of girdled vegetative shoots in response to an increase in Fo, indicating chronic photoinhibition events and a reduction in functional PSII units (Flexas et al. 2001). However, photosynthesis was not impaired through effects on the PSII reaction centres caused by higher leaf temperatures associated with a lower $g_s$ (Li et al. 2007). Furthermore, xanthophyll cycle-mediated quenching (NPQ) did not change in $A_N$ down-

regulated leaves, suggesting that another pathway for dissipating excess energy might also have been utilised (Wünsche et al. 2005). Despite the described photoinactivation of PSII, the electron transport rate remained unaltered during the first 48 h, indicating that the IPQ (inactive PSII mediated quenching) mechanism should be working in Salustiana sweet orange leaves. The inhibition of $A_N$ may also result from a change in the key components of photosynthetic capacity (Urban et al., 2004). The RuBP carboxylation capacity of Rubisco ($v_{c\ max}$) was repressed after 24 h in our study, despite $J_{max}$ remaining unchanged. A lower $v_{c\ max}$ suggested a decrease in the content or activation state of Rubisco. Araya et al. (2006) proposed that a decrease in Rubisco content is the main cause of carbohydrate repression during photosynthesis.

Sugar-regulated genes provide a means for integrating cellular responses to transport sugars, and thus information on carbohydrate status, and for coordinating changes in resource utilisation and allocation among organs (Koch, 1996). When sinks cannot use all of the assimilate generated, sugar accumulation in leaves has a direct effect on the expression of ADP-glucose pyrophosphorylase (Müller-Rober et al., 1994) and other carbohydrate-responsive enzymes involved in sucrose and starch metabolism (Koch, 1996). Sugar accumulation also represses the expression of the sucrose transporter (Chiou and Bush, 1998; Li et al., 2003b). Sucrose is the main sugar transported through the phloem in *Citrus* (Zimmermann and Ziegler, 1975). We reported higher expression of the sucrose transporter and sucrose synthesis genes and lower expression of the starch synthesis genes in girdled shoots bearing one fruit, indicating that the synthesis and export of sucrose in the leaves is promoted when a strong sink is available to the source. In contrast, the expression of ADP-glucose pyrophosphorylase increased when no strong sinks were present, and the excess of assimilates had to be stored as starch in

the leaves. Sucrose synthase A has been associated with starch synthesis (Dejardin et al., 1997; Li et al. 2003a) due to its cleavage activity. We reported a decrease in its expression when starch accumulated in leaves after girdling, suggesting that this isoform is working in the direction of sucrose synthesis. However, this decrease was not observed in sucrose synthase 1, indicating a different regulation (Li et al., 2003c). The expression of both sucrose transporter 1 and sucrose phosphate synthase is strongly repressed after 8 h in vegetative girdled shoots relative to unifloral girdled shoots, indicating an inhibition of sucrose synthesis and phloem charge. This reduction was further maintained after 24 h. The repression of the sucrose transporter 1 gene was not triggered by soluble sugars as described by Li et al. (2003b). The expression of the sucrose transporter 2 was also inhibited after 8 h, but inhibition was not maintained after 24 h, suggesting a different regulatory mechanism. In addition, the expression levels of the sucrose transporter 2 isoform in leaves were similar to those of sucrose transporter 1, in contrast to the different physiological roles proposed for these transporters (Li et al., 2003b). From our study, the expression of the genes controlling sucrose and starch metabolism in the leaves changed before any changes in the accumulation of these carbohydrates, as described above for some of the photosynthetic parameters. Differences in starch turnover may play a role in sensing photoassimilate availability and regulation of photosynthetic and carbon metabolism gene expression, as suggested by Paul and Foyer (2001).

In conclusion, in the absence of sinks, a rapid modulation in the expression of genes implicated in adaptive changes in assimilate partitioning occurs. Sucrose synthesis and phloem charge is blocked in the leaves, and fixed carbon is channelled to starch production. Changes in starch and soluble sugar turnover, but not sugar content per se,

could provide the signal for the regulation of photosynthesis. In these conditions, non-stomatal limitations strongly inhibited the photosynthetic rate prior to any significant increase in carbohydrate levels.

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## LEGENDS FOR FIGURES

Figure 1. Effect of leaf-to-fruit ratio on the net $CO_2$ fixation rate in 'Salustiana' sweet orange trees. Values are means of 10 determinations in different trees. Measurements on girdled shoots bearing one fruit and 10 (●), 25 (▲) and 40 (■) leaves and unifloral shoots (□).

Figure 2. Effect of sink availability on the net $CO_2$ fixation rate in 'Salustiana' sweet orange trees. Values are mean of 10 determinations in different trees. Measures on girdled shoots with 10 leaves bearing one fruit (G10 UF, ▲) or vegetative (G10 VG, Δ); non-girdled shoots bearing one fruit (UF,●) or vegetative (VG, ○).

Figure 3. Effect of the presence of fruit in girdled shoots on the net $CO_2$ fixation rate in 'Salustiana' sweet orange trees during the first 24 h after girdling. Values are mean of 10 determinations in different trees. Measurements on girdled shoots with 10 leaves bearing one fruit (G10 UF, ▲) or vegetative (G10 VG, Δ). Girdling was performed between 0930 and 1000 h.

Figure 4. Relative gene expression in leaves of girdled shoots in 'Salustiana' sweet orange. Leaves of vegetative shoots sampled 2 h after girdling are used as a reference for expression levels. For each date and time, different letters indicate significant differences ($P < 0.05$). No letters indicates no differences. Each value is the mean of three independent determinations.

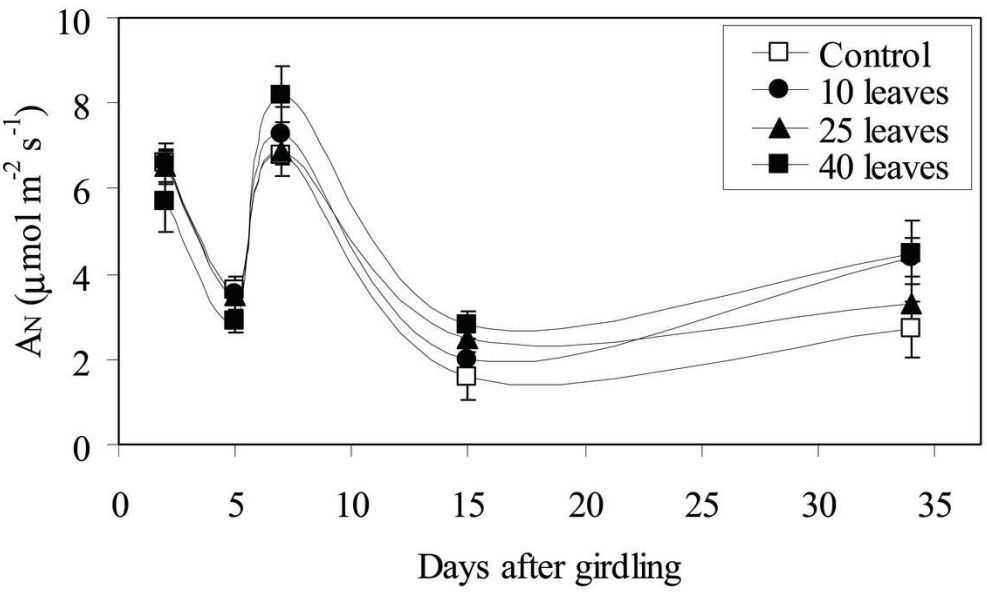


Figure 1. Effect of leaf-to-fruit ratio on the net CO2 fixation rate in 'Salustiana' sweet orange trees. Values are means of 10 determinations in different trees. Measurements on girdled shoots bearing one fruit and 10 (●), 25 (▲) and 40 (■) leaves and unifloral shoots (□).
48x30mm (600 x 600 DPI)

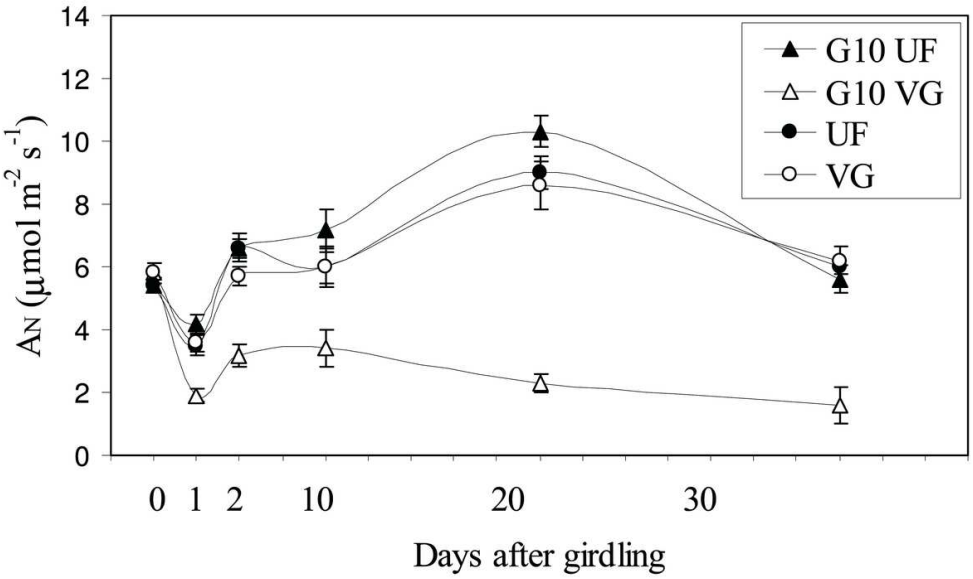


Figure 2. Effect of sink availability on the net CO2 fixation rate in 'Salustiana' sweet orange trees. Values are mean of 10 determinations in different trees. Measures on girdled shoots with 10 leaves bearing one fruit (G10 UF, ▲) or vegetative (G10 VG, Δ); non-girdled shoots bearing one fruit (UF,●) or vegetative (VG, ○).
51x32mm (600 x 600 DPI)

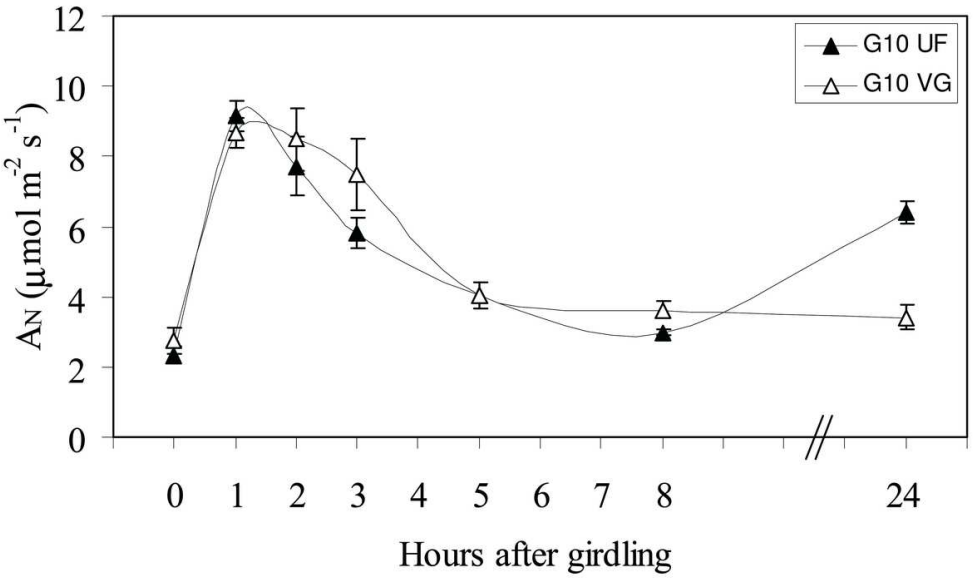


Figure 3. Effect of the presence of fruit in girdled shoots on the net CO2 fixation rate in 'Salustiana' sweet orange trees during the first 24 h after girdling. Values are mean of 10 determinations in different trees. Measurements on girdled shoots with 10 leaves bearing one fruit (G10 UF, ▲) or vegetative (G10 VG, Δ). Girdling was performed between 0930 and 1000 h.
50x32mm (600 x 600 DPI)

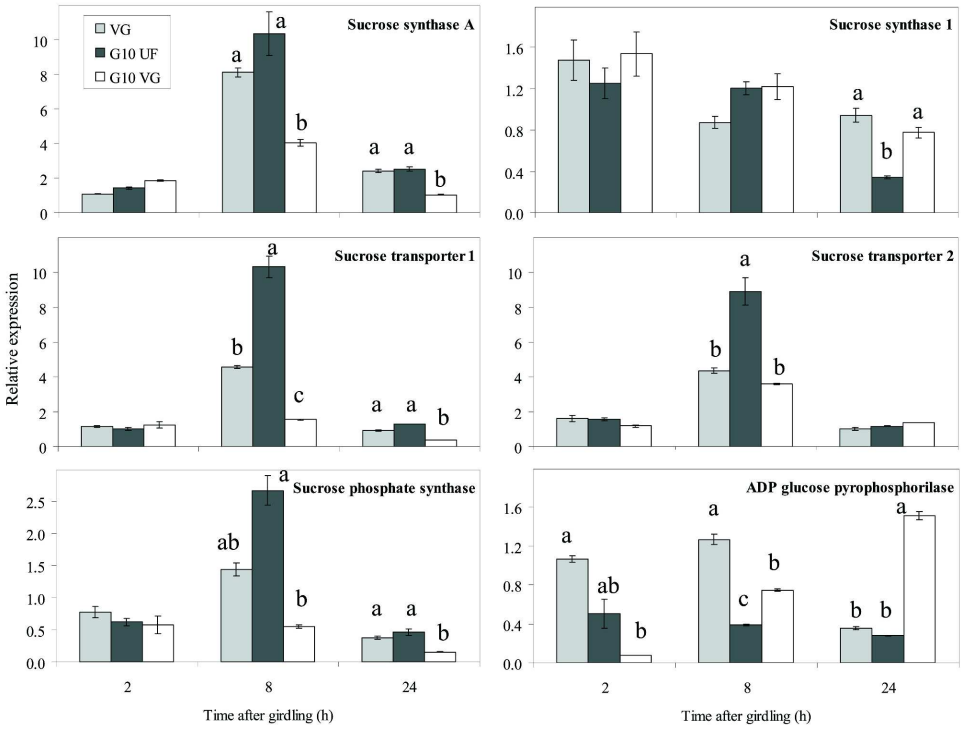


Figure 4. Relative gene expression in leaves of girdled shoots in 'Salustiana' sweet orange. Leaves of vegetative shoots sampled 2 h after girdling are used as a reference for expression levels. For each date and time, different letters indicate significant differences ($P < 0.05$). No letters indicates no differences. Each value is the mean of three independent determinations.
176x141mm (600 x 600 DPI)

Table 1. Set of primers used to amplify specific regions of genes implicated in sucrose and starch metabolism in *Citrus*.

| Gene | Accession | | Sequence |
|---|---|---|---|
| Glyceraldehyde-phospate dehydrogenase (GAPDH) | CX672747 | F | GGAAGGTCAAGATCGGAATCAA |
| | | R | CGTCCCTCTGCAAGATGACTCT |
| ADPG-glucose pyrophosphorylase | AF184597 | F | GTACCGATTAATGGCGAGTATGG |
| | | R | TGCTGCAGTTGACGGAGATG |
| Sucrose synthase A | AB021745 | F | TTGTGGACTTCCGACATTCG |
| | | R | TGACGCACCATGCTCGATAA |
| Sucrose synthase 1 | AB022092 | F | TGAGCCATTCAATGCCTCGT |
| | | R | TGGATGCATGCTCTCCTTGTC |
| Sucrose transporter 1 | AY098891 | F | TTGCGGAGGTGGCAACAT |
| | | R | CACTTAAGGCAGCCGCAACT |
| Sucrose transporter 2 | AY098894 | F | CCCAACGGAACCTCCAATTT |
| | | R | AAGGCCSSCCGAACTGAA |
| Sucrose-phosphate synthase | CB293478 | F | ATTCTAAGAGGTTCCGTAATGTATG |
| | | R | CTTCAAAGCTGCAGACAAATC |

Table 2. Influence of leaf-to-fruit ratio on carbohydrate content in 'Salustiana' sweet orange leaves. Data are the mean values of three independent extracts and three trees per extract.

| | Starch (% DW) | | | Soluble sugars (% DW) | | | Total sugars (% DW) | | |
|---|---|---|---|---|---|---|---|---|---|
| Days | 0 | 7 | 35 | 0 | 7 | 35 | 0 | 7 | 35 |
| Unifloral shoots | 9.7 | 5.4 ab | 4.5 a | 9.9 | 7.9 ab | 13.2 a | 19.6 | 13.2 | 17.7 a |
| Girdled | | | | | | | | | |
| 10 leaves | | 4.9 a | 6.7 ab | | 7.4 a | 13.5 a | | 12.2 | 20.3 a |
| 25 leaves | | 5.4 ab | 7.1 ab | | 9.0 b | 15.8 a | | 14.5 | 22.9 a |
| 40 leaves | | 6.2 b | 8.2 b | | 8.8 b | 20.9 b | | 14.9 | 29.0 b |

Shoots with a single fruit were girdled on June 20 above the number of leaves indicated. Measurements were performed after 7 and 35 days. Within each column different letters indicate significant differences ($P < 0.05$). No letters indicate no differences.

Table 3. The sink effect on leaf stomatal conductance ($g_s$), substomatal $CO_2$ concentration ($C_i$), maximum quantum efficiency of PSII photochemistry ($F_v/F_m$) and minimal ground fluorescence intensity ($F_o$) in 'Salustiana' sweet orange. In girdled shoots, ten leaves remained distal to the girdle. Shoots were girdled on June 28. Values are the mean of nine determinations in different shoots.

| | | $g_s$ mol $m^{-2}$ $s^{-1}$ | | | $C_i$ µmol $mol^{-1}$ | | | $F_v/F_m$ | | | $F_o$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Days after girdling | | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 |
| Treatment | Shoot | | | | | | | | | | | | |
| Ungirdled | Unifloral | 0.03 | 0.04 a | 0.04 a | 199 | 241 | 156 a | 0.78 | 0.79 a | 0.77 a | 852 | 822 b | 834 b |
| | Vegetative | 0.03 | 0.04 a | 0.05 a | 180 | 228 | 170 a | 0.78 | 0.79 a | 0.79 a | 876 | 838 b | 836 b |
| Girdled | Unifloral | | 0.06 a | 0.06 a | | 222 | 172 a | | 0.78 a | 0.79 a | | 788 b | 774 b |
| | Vegetative | | 0.02 b | 0.02 b | | 239 | 202 b | | 0.72 b | 0.70 b | | 1041 a | 1165 a |

Within each column, different letters indicate significant differences ($P < 0.05$). No letters indicate no differences.

Table 4. The sink effect on starch, soluble sugars and total carbohydrate content in 'Salustiana' sweet orange leaves. In girdled shoots, ten leaves remained distal to the girdle. Shoots were girdled on June 28. Data are the mean values of three independent extracts and three trees per extract.

| | | Starch (% DW) | | | Soluble sugars (% DW) | | | Total carbohydrates (%DW) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Days after girdling | | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | 2 |
| Treatment | Shoot | | | | | | | | | |
| Ungirdled | Unifloral | 10.5 | 9.6 | 9.9 a | 8.1 | 10.4 | 9.7 a | 18.6 | 20.0 | 19.6 a |
| | Vegetative | 8.6 | 8.4 | 8.8 a | 7.9 | 10.6 | 9.1 a | 16.5 | 19.0 | 17.8 a |
| Girdled | Unifloral | | 8.6 | 9.2 a | | 11.6 | 11.2 ab | | 20.2 | 20.4 a |
| | Vegetative | | 10.7 | 16.9 b | | 11.6 | 15.4 b | | 22.4 | 32.4 b |

Within each column, different letters indicate significant differences ($P < 0.05$). No letters indicate no differences.