

Seasonal effects on the relationship between photosynthesis and leaf carbohydrates in orange trees

Rafael V. Ribeiro^{A,E}, Eduardo C. Machado^A, Gustavo Habermann^B, Mauro G. Santos^C and Ricardo F. Oliveira^D

^ALaboratório de Fisiologia Vegetal 'Coaracy M. Franco', Centro de Pesquisa e Desenvolvimento em Ecofisiologia e Biofísica, Instituto Agronômico, IAC, PO Box 28, 13012-970, Campinas, SP, Brazil.

^BDepartamento de Botânica, Instituto de Biociências, Univ Estadual Paulista, UNESP, 13506-900, Rio Claro, SP, Brazil.

^CDepartamento de Botânica, Universidade Federal de Pernambuco, 50670-901, Recife, PE, Brazil.

^DDepartamento de Ciências Biológicas, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo, PO Box 9, 13418-900, Piracicaba, SP, Brazil.

^ECorresponding author. Email: rafael@iac.sp.gov.br

Abstract. To understand the effect of summer and winter on the relationships between leaf carbohydrate and photosynthesis in citrus trees growing in subtropical conditions, 'Valencia' orange trees were subjected to external manipulation of their carbohydrate concentration by exposing them to darkness and evaluating the maximal photosynthetic capacity. In addition, the relationships between carbohydrate and photosynthesis in the citrus leaves were studied under natural conditions. Exposing the leaves to dark conditions decreased the carbohydrate concentration and increased photosynthesis in both seasons, which is in accordance with the current model of carbohydrate regulation. Significant negative correlations were found between total non-structural carbohydrates and photosynthesis in both seasons. However, non-reducing sugars were the most important carbohydrate that apparently regulated photosynthesis on a typical summer day, whereas starch was important on a typical winter day. As a novelty, photosynthesis stimulation by carbohydrate consumption was approximately three times higher during the summer, i.e. the growing season. Under subtropical conditions, citrus leaves exhibited relatively high photosynthesis and high carbohydrate levels on the summer day, as well as a high nocturnal consumption of starch and soluble sugars. A positive association was determined between photosynthesis and photoassimilate consumption/exportation, even in leaves showing a high carbohydrate concentration. This paper provides evidence that photosynthesis in citrus leaves is regulated by an increase in sink demand rather than by the absolute carbohydrate concentration in leaves.

Additional keywords: *Citrus sinensis*, gas exchange, growth, seasonality, source–sink.

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Introduction

Under field conditions, citrus leaves show the highest photosynthetic rates during the spring when trees are flowering and fruiting (Ribeiro and Machado 2007). These phenological phases are characterised by a high demand of photoassimilate, with developing tissues causing a reduction in the starch concentration in leaves, stems and roots (Goldschmidt and Golomb 1982; Prado *et al.* 2007; Ramos 2009). Although high sink demand occurs during the spring, citrus leaves maintain high sugar concentration due to continuous photosynthetic activity throughout the year.

The non-structural carbohydrate availability in leaf tissues reaches more than 200 mg g⁻¹ during early spring when leaves show the highest CO₂ uptake (Ribeiro and Machado 2007). According to the current model of photosynthetic inhibition

by high leaf carbohydrate content (Iglesias *et al.* 2002) we would expect a downregulation of photosynthesis in citrus plants during the spring/summer season as a response to high sugar availability. However, this is not observed in citrus leaves under subtropical conditions (Ribeiro and Machado 2007). Recently, Nebauer *et al.* (2011) have reported that citrus photosynthesis is regulated by the turnover of starch and soluble carbohydrates rather than by their absolute concentration, adding more complexity to this interesting theme.

Artificial manipulation of the source–sink relationship, such as girdling (Iglesias *et al.* 2002), sucrose injection (Goldschmidt and Huber 1992), shoot removal (Syvertsen 1994; Iglesias *et al.* 2002) and de-fruiting (Syvertsen *et al.* 2003), supports the inhibition of photosynthesis by high concentration of carbohydrates in citrus leaves. However, plant

girdling may disturb the tree carbon status, affecting the carbon metabolism and water relations (De Schepper *et al.* 2010), which could influence the growth pattern as well as the relationship between photosynthesis and carbohydrates.

The role of sink demand in controlling the photosynthesis of citrus plants is not clear (Goldschmidt and Koch 1996), especially when plants are studied under natural conditions. Therefore, it would be innovative and less metabolically disturbing to study the source–sink relationships in citrus plants growing under natural conditions, without unnatural carbohydrate injections and without removing the fruit. In subtropical areas, it is well established that citrus trees show a high sink demand during the spring/summer season and an apparent reduction in growth during the winter season (Davies and Albrigo 1994; Ramos *et al.* 2010). Thus, a higher leaf carbohydrate demand would occur during the spring/summer season than in the winter season, when growth is restrained.

Using young ‘Valencia’ citrus trees growing under subtropical conditions, we measured leaf carbohydrate concentration, photosynthesis and total leaf area during the summer growing season and also during winter when there was no apparent growth. We tested the hypotheses that (i) artificially-induced reductions in leaf carbohydrate concentrations would stimulate the maximal photosynthetic capacity and that (ii) the relationship between carbohydrates and photosynthesis in citrus leaves is influenced by the growing season. In addition, we discuss the relationship between photoassimilates usage and photosynthesis in citrus leaves in subtropical conditions.

Materials and methods

Plant material and experimental set-up

One-year-old ‘Valencia’ sweet orange (*Citrus sinensis* (L.) Osb.) scions grafted onto ‘Cleopatra’ mandarin (*Citrus reticulata* Blanco) rootstocks were grown in plastic pots (36 L) containing a mixture of soil: sand: manure (2:1:1), which were fertilised according to van Raij *et al.* (1996). Measurements and samplings were taken in a group of four trees. The plants did not exhibit any symptom of nutritional deficiency in the summer or in the winter. Plants were grown in an experimental garden, in Piracicaba, SP, Brazil (22°42′S, 47°30′W, 576 m of altitude). This region has a Cwa-type climate, according to the Köppen classification, with rainy summers and dry winters and a mean air temperature higher than 22°C in the warmest month.

Aluminium envelopes were used to impose darkness for periods of 12 (overnight), 48 and 96 h over attached leaves in both winter and summer seasons. Afterwards, excised leaf discs (10 cm²) were used to evaluate photosynthetic rates as well as chlorophyll and carbohydrate concentrations. The dark period of 12 h was considered to be the reference because it represented the natural night-time period. Maximum photosynthetic capacity (measured under saturated light and air CO₂ concentration and also under optimum temperature) and chlorophyll *a* fluorescence were simultaneously measured in leaf discs, which were enclosed in an LD2/3 leaf chamber (Hansatech, King’s Lynn, UK). Inside the leaf chamber, each leaf disc was placed on a wet felt disc during the photosynthetic measurements. We used three replications (discs) from different trees to evaluate each of the

three darkness periods, which were imposed in July (winter) and February (summer). Samples were taken only on one day in February, a typical summer day and on one day in July, a typical winter day. The same trees were used for evaluation of the diurnal patterns of carbohydrates and actual photosynthesis of untreated leaves under natural conditions in both seasons. Four measurements for each evaluation time were taken in different trees to assess the diurnal patterns of the parameters mentioned above in 1 day.

Microclimate

The environmental conditions during the experimental period were monitored with an automatic weather station, equipped with sensors to measure the air temperature (minimum, mean and maximum values), relative humidity (probe model HMP45C, Campbell Scientific, Logan, UT, USA) and solar radiation (pyranometer model LI-200, Li-Cor, Lincoln, NE, USA).

Maximum photosynthetic capacity

Maximum photosynthetic capacity (A_P) was estimated by measuring the photosynthetic O₂ evolution on excised leaf discs with a leaf disc oxygen electrode (Hansatech). Measurements were taken at 35°C (Ribeiro *et al.* 2006) and the photosynthetic photon flux density (PPFD) of 1120 μmol m⁻² s⁻¹ was provided by an external light source (LS3, Hansatech). The A_P values were recorded using the Oxygraph measurement system (ver. 2.22, Hansatech). The saturated CO₂ concentration inside the chamber was generated by 0.2 cm³ of a carbonate/bicarbonate buffer solution (1 M, 1:19 v/v), according to Ribeiro *et al.* (2003).

*Chlorophyll *a* fluorescence*

A modulated fluorometer (FMS1, Hansatech) was used to evaluate the following photochemical variables: the maximum (F_v/F_m) and the actual ($\Delta F'/F_m'$) quantum yield of primary photochemistry; the apparent electron transport rate ($ETR_s' = \Delta F'/F_m' \times \text{PPFD} \times 0.5 \times 0.84$); and the non-photochemical [NPQ = $(F_m - F_m')/F_m$] and photochemical ($q_p = (F_m' - F_s')/(F_m' - F_o')$) quenchings (Roháček 2002). For the calculation of ETR_s' , 0.5 was used as the fraction of the excitation energy distributed to PSII and 0.84 was used as the fractional light absorbance (Björkman and Demmig 1987). Measurements of A_P and photochemistry were recorded after the full induction of photosynthesis, which occurred after 10 min of illumination.

Chlorophyll concentrations

The leaf chlorophyll concentrations were evaluated in the dark exposed leaves. The pigments were extracted by grinding leaf discs in 6 mL of acetone solution (80%, v/v) under dark conditions. The solution was centrifuged at 2000g for 5 min and the supernatant absorbance was measured with a spectrophotometer set to 646 and 663 nm. Chlorophylls *a* (chl *a*) and *b* (chl *b*) as well as the total chlorophyll (chl *a*+*b*) were determined according to the equations proposed by Lichtenthaler and Wellburn (1983).

Carbohydrates concentration

Leaf carbohydrate concentrations were measured after the evaluation of A_P in the leaf discs excised from the leaves that had been exposed to each of the three darkness periods (12, 48 and 96 h). For this, leaf tissues were rapidly frozen in liquid nitrogen, dried and then ground. Soluble sugars were extracted from samples of 75 mg of ground tissue after three cycles of extraction in an ethanol solution (80%, v/v) at 55°C. The reducing sugars (RS) were quantified by the Somogyi-Nelson method (Somogyi 1952) and the total soluble sugars (SS) were determined by the Anthrone method (Yemm and Willis 1954). After the extraction of ethanol-soluble sugars, the starch concentrations (St) were quantified in the insoluble fraction by the Anthrone method. The fraction of the total non-structural carbohydrates (TS) was expressed as the sum of SS and St (Syvertsen *et al.* 2003). The non-reducing sugars (NRS) concentration was calculated as $NRS = SS - RS$.

Using the same analytical methods described above, we also evaluated the diurnal dynamics of carbohydrates concentrations in leaves under natural conditions that were collected at sunrise (0600 or 0700 hours), 1000 hours, 1400 hours and at sunset (1800 or 1900 hours) in July (winter) and February (summer), from the same potted plants. An additional leaf sample was collected at sunrise of the following day to estimate the daily changes of leaf carbohydrates. The diurnal change was calculated as the difference between carbohydrate concentrations measured at sunset of day 1 and at sunrise of day 1, whereas the nocturnal change was the difference between carbohydrate concentrations measured at sunrise of day 2 and at sunset of day 1.

Actual photosynthesis

Additionally, the diurnal course of CO_2 assimilation (A_N) in sun-exposed and fully expanded leaves (~6 months old) was measured with an infrared gas analyser (LI-6400, Li-Cor). Measurements were taken in four leaves of four different potted trees and such data were recorded when the total coefficient of variation (CV) inside the leaf chamber was less than 0.5%, which happened after 3 min. The CV. was calculated by the Open software of the LI-6400 system and it considered the variations of air flow and concentrations of CO_2 and water vapour. The CO_2 concentration inside the leaf chamber was around $380 \mu\text{mol mol}^{-1}$. The air pumped into the IR gas analyser was passed through a buffering gallon (5 L) to reduce the time for measurement stabilisation. Measurements were taken under the natural fluctuation of air temperature and vapour pressure deficit (VPD). Measurements were taken on clear days and the PPFD used for each assessment was provided by an artificial light source (LI-6400-02 LED, Li-Cor), which was set to provide the same PPFD that was tracked by an external light sensor (LI-250, Li-Cor) installed on the leaf cuvette. Only data on the diurnal-integrated CO_2 assimilation (A_D) are shown, see the 'Results' section.

The leaf photoassimilate exportation/consumption (PEC) was calculated as $PEC = TS_{d1} + A_D - TS_{d2}$, where TS_{d1} and TS_{d2} are the leaf TS concentrations at sunrise of day 1 and day 2, respectively, and A_D is the diurnal-integrated CO_2 assimilation on day 1 ($\text{g CH}_2\text{O m}^{-2}$). The A_D value in $\text{g CO}_2 \text{m}^{-2}$ was converted to $\text{g CH}_2\text{O m}^{-2}$ using a factor of 0.68 as a

multiplier (Kalt-Torres *et al.* 1987). The total non-structural carbohydrates (TS) on a mass basis were converted to a leaf area basis using a specific leaf mass, which was evaluated in both seasons. The PEC was calculated using the diurnal CO_2 assimilation reported in a previous paper (Ribeiro *et al.* 2009a), which is shown in fig. 1.

The total leaf area (LA) of three plants was measured in both seasons using a digital planimeter (LI-3100, Li-Cor).

Data analysis

The data were subjected to the analysis of variance procedure either as a factorial design (season vs dark period) or as a random block design when comparing seasons. When significant differences were detected, mean values were compared by the Tukey's test ($P < 0.05$). The relationships between leaf sugar concentrations and photosynthesis were evaluated by using the Pearson's correlation coefficient (r) when statistical significance was detected ($P < 0.05$).

Results

Microclimate

As expected, air temperature and incoming solar radiation were significantly different between the winter and summer (Table 1). The cumulative rainfall during the summer and winter seasons was 487 and 43 mm respectively. This large difference is expected to affect the relative humidity, which was more elevated during the summer season. As citrus trees were potted and irrigated, the influence of water availability from rainfall was not considered in this study. In general, the summer was warmer than the winter season and the evaluation days were representative of such contrasting seasons. On the days measurements were performed, the minimum and mean air temperatures and solar radiation were higher ($+7.4^\circ\text{C}$, $+6.0^\circ\text{C}$ and $+8.8 \text{ MJ m}^{-2} \text{ day}^{-1}$ respectively) in the summer than in the winter.

Relationships between carbohydrates and maximum photosynthetic capacity in dark exposed leaves

Prolonged exposure to dark conditions led to increased A_P and the highest rates were observed after 96 h, regardless of the season (Fig. 1a). Relative to the 12 h of darkness (overnight), the A_P increased by ~1.6- and 1.3-times after 96 h of darkness on both the measured summer and winter days. On the typical

Table 1. Environmental conditions during the experimental period: entire seasons and evaluation dates

T, air temperature; RH, air relative humidity; Qg, solar radiation. Maximum, minimum and average values (\pm s.e.), considering all days of each season

Variables	Winter		Summer	
	Season	Day	Season	Day
T_{average} ($^\circ\text{C}$)	18.4 ± 0.2	17.8	22.7 ± 0.2	23.8
T_{maximum} ($^\circ\text{C}$)	26.5 ± 0.3	25.3	29.1 ± 0.3	30.9
T_{minimum} ($^\circ\text{C}$)	11.3 ± 0.3	11.1	18.1 ± 0.2	18.5
RH_{average} (%)	68.5 ± 1.2	72.3	79.9 ± 0.9	78.6
RH_{minimum} (%)	35.8 ± 1.5	40.3	50.9 ± 1.5	37.4
Qg ($\text{MJ m}^{-2} \text{ day}^{-1}$)	13.5 ± 0.3	13.2	18.6 ± 0.6	22.0

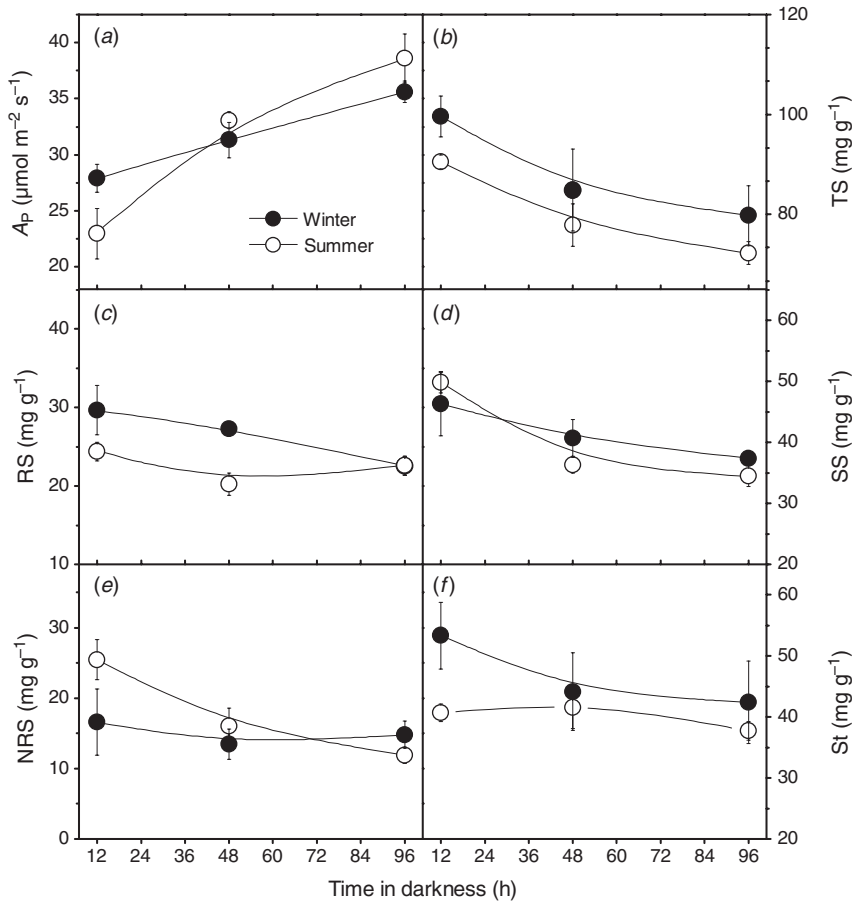


Fig. 1. (a) Photosynthetic O_2 evolution (A_p), (b) total non-structural carbohydrates (TS), (c) reducing sugars (RS), (d) soluble sugars (SS), (e) non-reducing sugars (NRS) and (f) starch (St) concentrations in response to darkness. Measurements were taken from leaf discs excised from sweet orange trees on typical winter (closed circles) and summer (open circles) days. Each symbol represents the mean value of three replications (\pm s.e.).

summer day, exposure to darkness reduced the concentration of TS, SS and NRS, whereas on the measured winter day significant decreases in TS and St were observed (Fig. 1).

The photochemical activity was higher on the summer day than on the winter day (Fig. 2). The highest values of $\Delta F'/F_m'$ and q_p were found on the summer day, regardless of the period under darkness. The $\Delta F'/F_m'$, q_p and ETR_s' tended to decrease with the increase of the period leaves remained in darkness only on the winter day. The largest differences in $\Delta F'/F_m'$, q_p and ETR_s' values between the seasons were observed after 96 h under dark conditions. However, the darkness and seasons did not affect NPQ or F_v/F_m . Similarly, neither the dark periods nor the seasons cause changes in chl *a*, chl *b* or chl *a + b* (Table 2).

Negative relationships were observed between leaf carbohydrates and maximum photosynthetic capacity in both seasons (Table 3), with the highest photosynthetic rates in leaves with reduced carbohydrate concentration. On the measured winter day, a significant negative correlation was determined between St concentration and A_p . Since starch is the main carbohydrate in leaf tissues, the negative relationship between TS and A_p was also significant. On the measured

summer day, significant correlations were found between NRS and A_p , SS and A_p and between TS and A_p . The highest slopes from the correlations were noticed in the summer season, i.e. 0.724 (summer) vs 0.218 (winter), for the relationship between TS and A_p . This pattern suggests high responsiveness of photosynthesis to changes in leaf carbohydrate concentration during the growing season (summer).

Carbohydrates and actual photosynthesis under natural conditions

The leaves exhibited higher concentrations of St, SS, NRS and TS on the summer day as compared with the winter day (Fig. 3a, b, d, e), whereas RS was similar in both seasons (Fig. 3c). In general, there was a large daily change in leaf carbohydrates, increasing from sunrise and reaching the highest values around midday in both seasons, regardless of the sugar type. The diurnal increase rate of St in leaves was higher on the winter day than on the summer day.

In the diurnal period, a higher accumulation of St was found on the winter day (Fig. 4a), whereas a higher increase in SS and

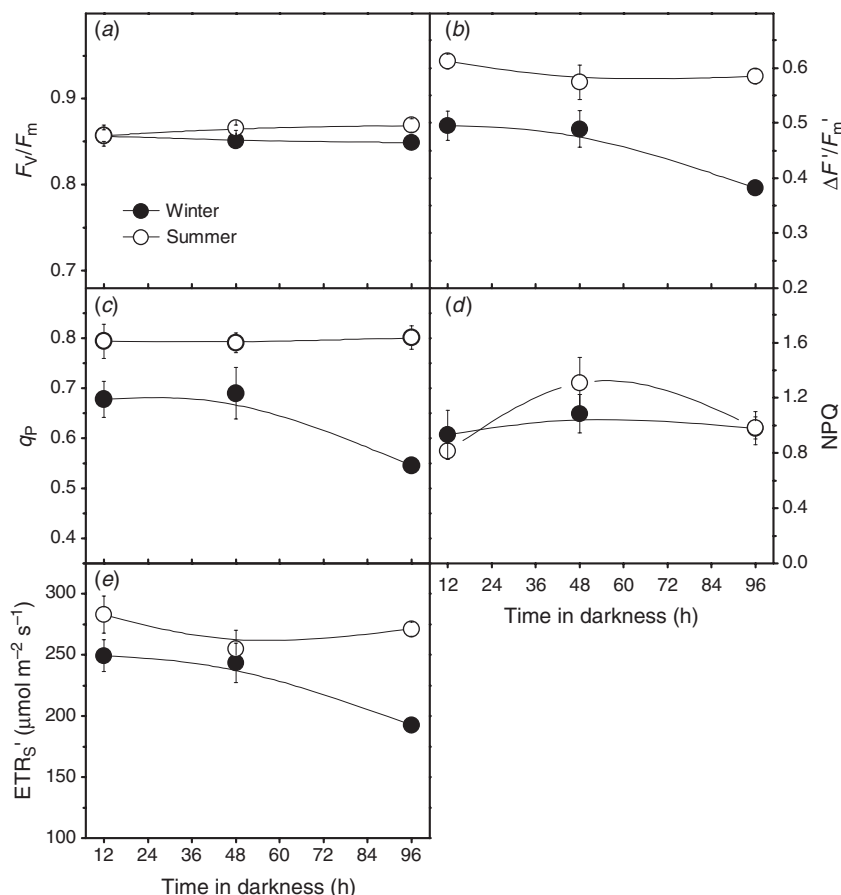


Fig. 2. (a) Maximum (F_v/F_m) and (b) actual ($\Delta F'/F_m'$) quantum yield of primary photochemistry, (c) photochemical (q_p) and (d) non-photochemical (NPQ) quenchings and (e) apparent electron transport rate (ETR_s') in response to darkness. Measurements were taken from leaf discs excised from sweet orange trees on typical winter (closed circles) and summer (open circles) days. Each symbol represents the mean value of three replications (\pm s.e.).

Table 2. Concentrations of chlorophylls (Chl) *a*, *b* and *a + b* in dark exposed leaves of sweet orange trees on typical winter and summer days

In each season, the same lower case letters indicate non-significant differences between darkness periods and the same upper case letters indicate non-significant differences between seasons for each darkness period (Tukey test, $P = 0.05$). $n = 4$ (\pm s.e.)

Season	Time in darkness (h)	Chl <i>a</i> ($\mu\text{g cm}^{-2}$)	Chl <i>b</i> ($\mu\text{g cm}^{-2}$)	Chl <i>a + b</i> ($\mu\text{g cm}^{-2}$)
Winter	12	15.63 \pm 0.66 ^{aA}	10.05 \pm 0.64 ^{aA}	25.68 \pm 1.30 ^{aA}
	48	15.51 \pm 0.89 ^{aA}	12.79 \pm 1.41 ^{aA}	29.72 \pm 2.11 ^{aA}
	96	17.32 \pm 0.40 ^{aA}	11.90 \pm 0.36 ^{aA}	29.22 \pm 0.77 ^{aA}
Summer	12	15.22 \pm 0.39 ^{aA}	9.96 \pm 0.38 ^{aA}	25.18 \pm 0.77 ^{aA}
	48	15.23 \pm 0.39 ^{aA}	10.11 \pm 0.33 ^{aA}	25.35 \pm 0.71 ^{aA}
	96	15.76 \pm 0.22 ^{aA}	10.59 \pm 0.22 ^{aA}	26.35 \pm 0.43 ^{aA}

RS was observed on the summer day (Fig. 4*b, c*). In the nocturnal period, the pattern observed for St was inverted, with leaves presenting higher St consumption on the summer day. Both diurnal and nocturnal changes of SS and RS were higher on the summer than on the winter day. Diurnal and nocturnal changes of NRS were the same for both seasons (Fig. 4*d*). The

largest difference between seasons was observed in the nocturnal consumption of St, which was approximately four times higher on the summer day than the winter day.

The high daily changes in the carbohydrate concentrations observed in the summer were in accordance with the leaf photoassimilate exportation/consumption. The amount of carbohydrates exported or consumed in leaves was approximately three times higher on the measured summer day than on the winter day (Fig. 5*a*). Accordingly, high A_D was observed on the summer day (Fig. 5*b*), which was \sim 2.9-times higher than on the winter day. Regarding the vegetative growth, plants showed 5.8-times more LA in the summer than in the winter (Fig. 5*c*). The specific leaf mass was not affected by season, varying from 77.2 ± 3.7 g DM m^{-2} in the winter to 75.7 ± 4.6 g DM m^{-2} in the summer.

Discussion

Maximum photosynthetic capacity as affected by leaf carbohydrates

There was a negative correlation between A_P and leaf carbohydrate concentrations on both the summer and

Table 3. Relationship between leaf carbohydrates and photosynthesis (A_p) in Valencia orange trees on typical winter and summer days

RS, reducing sugars; NRS, non-reducing sugars; SS, soluble sugars; St, starch; TS, total non-structural carbohydrates: $n=9$

Season	Relationship	Probability $>F$	Linear regression	r
Winter	RS vs A_p	$P > 0.05$	–	–
	NRS vs A_p	$P > 0.05$	–	–
	SS vs A_p	$P > 0.05$	–	–
	St vs A_p	$P \leq 0.05$	$A_p = (-0.249 \times St) + 43.22$	-0.69
	TS vs A_p	$P \leq 0.05$	$A_p = (-0.218 \times TS) + 50.81$	-0.74
Summer	RS vs A_p	$P > 0.05$	–	–
	NRS vs A_p	$P \leq 0.05$	$A_p = (-0.912 \times NRS) + 47.78$	-0.86
	SS vs A_p	$P \leq 0.05$	$A_p = (-0.901 \times SS) + 67.76$	-0.93
	St vs A_p	$P > 0.05$	–	–
	TS vs A_p	$P \leq 0.05$	$A_p = (-0.724 \times TS) + 89.58$	-0.90

winter days (Table 2). This relationship is well documented in tree species in which an external treatment artificially induces increases in leaf carbohydrate concentrations and reductions in photosynthesis (Goldschmidt and Huber 1992; Syvertsen 1994; Iglesias *et al.* 2002; Syvertsen *et al.* 2003; De Schepper *et al.* 2010). We observed the lowest A_p in leaves that exhibited the highest sugar concentrations (Table 2; Fig. 1). Low photosynthetic rates in leaves showing high carbohydrate concentrations may be ascribed to impaired CO_2 diffusion, decreased biochemical activity of photosynthesis and/or the downregulation of photosynthetic genes (Goldschmidt and Huber 1992; Koch 1996; Nakano *et al.* 2000; Paul and Foyer 2001; Paul and Pellny 2003).

Citrus shoot growth has a close relationship with the consumption of carbohydrates stored in mature leaves and roots (Bueno *et al.* 2011), so we may argue that the higher LA observed in the summer, in contrast with LA found in the winter

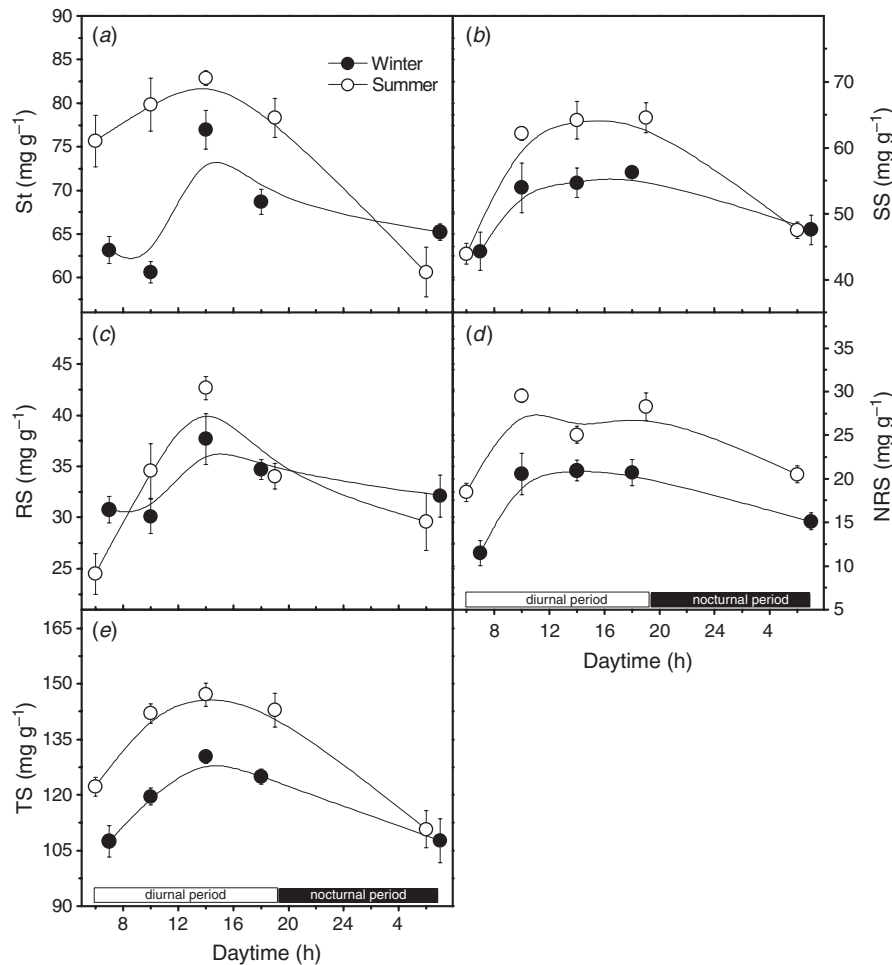


Fig. 3. Daily changes in (a) starch (St), (b) soluble sugars (SS), (c) reducing sugars (RS), (d) non-reducing sugars (NRS) and (e) total non-structural carbohydrates (TS) in leaves of sweet orange trees on typical winter (closed circles) and summer (open circles) days. Each symbol represents the mean value of four replications (\pm s.e.). White bar in the bottom position in (d) and (e) refers to the diurnal period and the black bar refers to the nocturnal period.

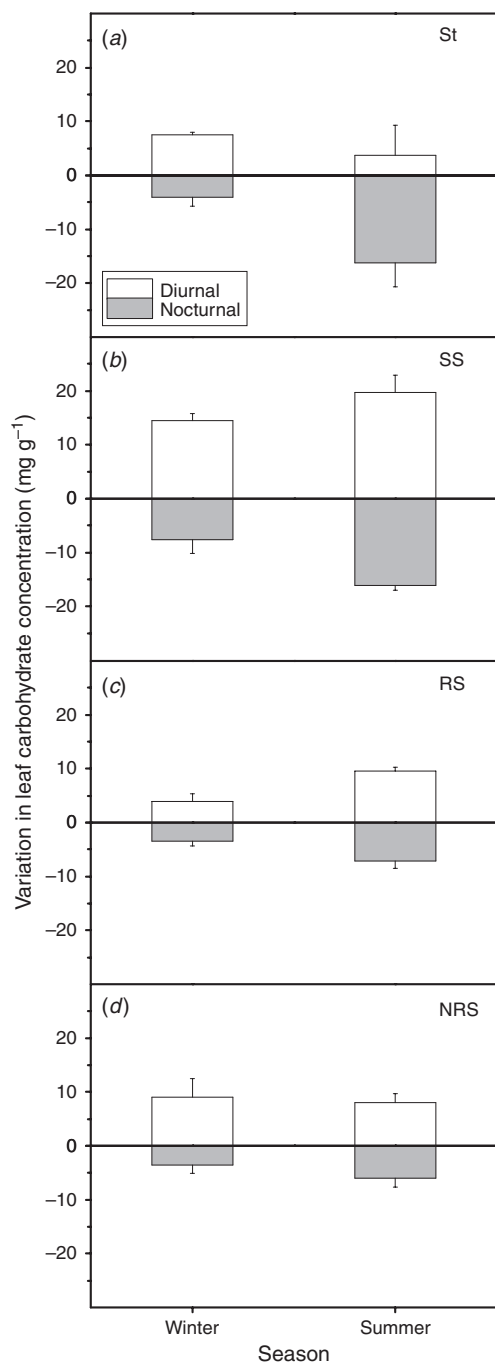


Fig. 4. Diurnal and nocturnal changes in the leaf concentrations of (a) starch (St), (b) soluble sugars (SS), (c) reducing sugars (RS) and (d) non-reducing sugars (NRS) of sweet orange trees on a typical winter and summer days. Each bar represents the mean value of four replications (\pm s.e.). Positive and negative values indicate the accumulation and consumption of carbohydrates respectively.

(Fig. 5c) is an indicative of plant growth and then an indirect measure of increased sink activity in the growing season. The strength of sinks regulates photosynthesis through the use of Calvin cycle end products, which represents a mechanism to ensure the appropriate balance between carbon uptake and

consumption (Paul and Pellny 2003; Smith and Stitt 2007). In this way, the maximum photosynthetic capacity of citrus leaves may be stimulated by increases in inorganic phosphorus (P_i) availability due to carbohydrate turnover (Bush 1999). Indeed, increases in A_P due to darkness cannot be ascribed to photochemical activity or chlorophyll concentrations because stimulation of A_P (Fig. 1a) occurred even with decreases in $\Delta F'/F_m'$, q_P and ETR_s' (Fig. 2) and no changes in leaf chlorophyll concentration (Table 2). Although the decreased photochemical activity during the winter may be justified by the low air temperature during the nocturnal period (Table 1), the low $\Delta F'/F_m'$, q_P and ETR_s' after 96 h of darkness are probable consequences of photochemistry-biochemistry uncoupling due to excessive darkness.

Therefore, our first hypothesis was confirmed; with citrus leaves presenting a large stimulation of maximum photosynthetic capacity when carbohydrate concentration was artificially reduced.

The relationship between carbohydrates and photosynthesis being affected by growing season

The dark conditions applied in the present study confirmed the relationship between carbohydrates and photosynthesis established in citrus species (Iglesias *et al.* 2002). However, we observed new relationships between distinct carbohydrate types and A_P values, which varied according to the season. Changes in St concentrations affected A_P on the measured winter day, whereas the changes in SS and NRS were more correlated with the changes of A_P on the measured summer day (Table 3).

As a remarkable increase in LA was observed from the winter to the summer (Fig. 5c) and as most of vegetative growth is well established to occur during the warmer months (Ramos *et al.* 2010), we argue that the sink activity was higher in February (summer) than in July (winter). During the summer (growing season), the NRS, sucrose among them, was the main carbohydrate type that was negatively related to photosynthesis (Table 3; Fig. 1e). Further, sucrose is the main sugar exported from leaves to other organs, such as roots, stems and young leaves of tree species during the growing season (Dickson 1991). Therefore, one would expect a significant influence of sucrose concentrations on photosynthesis, as determined in this study (Table 3). The high sucrose concentration in the phloem of mature leaves potentially inhibits the activity of proton-sucrose symporters, reducing the phloem loading (Bush 1999). As a consequence, leaves accumulate sucrose, the antiport transport of triose-phosphate and P_i through chloroplast membrane is reduced and then photosynthesis is impaired due to low P_i availability (Paul and Foyer 2001; Paul and Pellny 2003). Moreover, sucrose has been described as a potential signalling molecule regulating genes from carbohydrate metabolism and photosynthesis (Koch 1996; Li *et al.* 2003; Paul and Pellny 2003; Rolland *et al.* 2006; McCormick *et al.* 2006; Smith and Stitt 2007). High sucrose concentration in leaf tissues induces the expression of genes responsible for carbohydrate storage and usage, whereas it downregulates genes that are responsible for the photosynthetic activities (Ainsworth and Bush 2011).

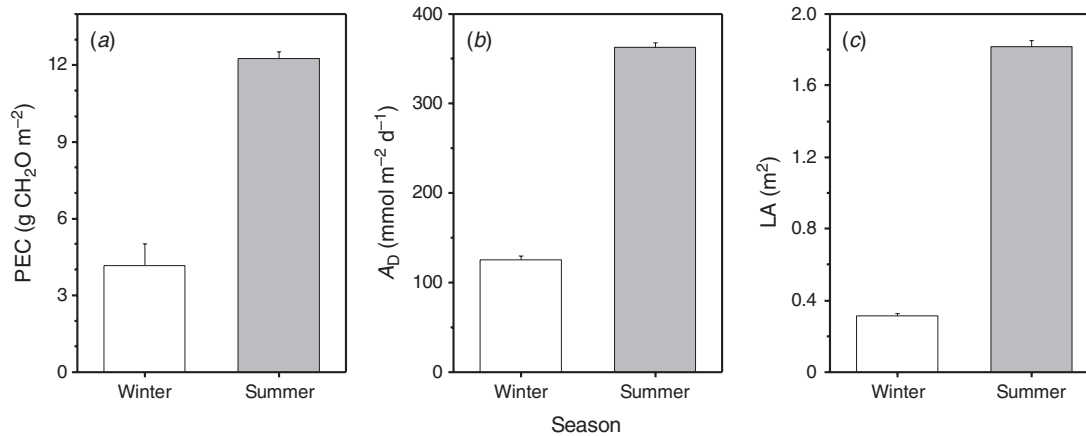


Fig. 5. (a) Leaf photoassimilate exportation/consumption (PEC), (b) leaf diurnal-integrated CO₂ assimilation (A_D) and (c) total leaf area (LA) of sweet orange trees in the winter and summer. Each bar represents the mean value of four (PEC) or five (A_D and LA) replications (\pm s.e.). PEC and A_D were sampled on typical winter and summer days.

Starch decreased by 20% on the winter day and non-reducing sugars decreased by 53% on the summer day and these events were apparently responsible for photosynthesis stimulation in citrus leaves after 96 h under dark conditions (Fig. 1e, f). On average, we found consumption rates of $0.16 \text{ mg g}^{-1} \text{ h}^{-1}$ for NRS on the summer day and $0.13 \text{ mg g}^{-1} \text{ h}^{-1}$ for St on the winter day. These reduction rates were higher than previously reported rates for citrus plants (Iglesias *et al.* 2002). Therefore, maximum photosynthetic capacities may show associations with specific carbohydrates depending on season.

The photosynthetic responses obtained herein revealed how specific carbohydrates may influence photosynthesis of citrus leaves in different seasons. Rather, the negative correlations between leaf carbohydrates and maximum photosynthetic capacity (Figs 3, 5b) do not determine what regulates photosynthesis in citrus leaves. Besides the probable regulation imposed by absolute contents of leaf carbohydrates, it is important to comment that the actual photosynthesis of citrus leaves is higher in the summer than in the winter due to increased ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity, augmented regeneration of RuBP dependent on electron transport and also due to elevated stomatal conductance (Habermann and Rodrigues 2009; Ribeiro *et al.* 2009a, 2009b). Such photosynthetic performance in summer conditions are clearly related to significant vegetative growth in subtropical conditions, as observed in leaf area of citrus trees (Fig. 5b, c).

A positive association was determined between the actual photosynthesis and the photoassimilate exportation/consumption, which occurred in parallel to a large increase in vegetative growth, given by the total leaf area in plants without external manipulation of the source-sink relationship (Fig. 5). Under subtropical conditions we know that citrus vegetative growth is intense during the spring/summer season (Ramos *et al.* 2010) and the demand of growing shoots cause significant impact on carbohydrate concentration of mature leaves, reducing the amount of carbohydrate reserves (Bueno *et al.* 2011).

According to Geiger *et al.* (2000) and Smith and Stitt (2007), there was no net accumulation of St during the diurnal period on the summer day. In this evaluation, however, we detected a large consumption of St during the night (Figs 3a, 4a). In addition, the nocturnal consumption of soluble sugars was higher on the warmer summer day than on the winter day (Figs 3b, 4b). These carbohydrate dynamics strongly agreed with the high demand for photoassimilates in the summer caused by intense vegetative growth during this season (Fig. 5).

The adjustment of carbon supply for plant growth is accomplished by the complex control of photoassimilate partitioning between sucrose and starch; thus, the plants have enough sucrose to support the immediate diurnal demand and sufficient starch to supply the nocturnal carbon demand (Smith and Stitt 2007). This pattern for the source-sink interaction was also detected in our data, with plants showing high sucrose concentrations in the diurnal period and high starch concentrations in the late evening on a typical summer day.

The occurrence of high actual photosynthesis and high photoassimilate usage in citrus trees suggests a probable upregulation of photosynthesis through increase in sink demand. This happens with leaf tissues showing high carbohydrate concentration (Fig. 3) coexisting with maximum photosynthetic capacity (Table 3; Fig. 1). This is a novel result and it is in agreement with a recent paper by Nebauer *et al.* (2011), in which citrus photosynthesis was demonstrated to be regulated by carbohydrate turnover and not by its absolute concentration. When there is no strong sink demand, genes related to sucrose synthesis and exportation were repressed in citrus leaves before any significant change in carbohydrate concentration (Nebauer *et al.* 2011), which happened in parallel with photosynthesis reduction.

A comparison between the coefficients from equations established by the linear correlations between TS and A_P (Table 3) indicated that the effects from changes in TS on A_P were approximately three times higher in the summer than in the winter (i.e. 0.724 vs 0.218). Therefore, our second hypothesis was also confirmed, with the relationship between

leaf carbohydrates and photosynthesis being affected by season. Such stimulatory effect was related to the vegetative growth of citrus trees, which was higher during the spring-summer season in comparison with the fall-winter.

Our data suggest that the pattern of photoassimilate consumption/production is an important element affecting the photosynthesis in broad leaf evergreen species growing in subtropical conditions. The carbon metabolism change between the summer and winter days could well represent what is importantly affecting the plant growth and the physiological performance of orange trees; not only when considering those selected days in both seasons, but over time.

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References

- Ainsworth EA, Bush DR (2011) Carbohydrate export from the leaf: a highly regulated process and target to enhance photosynthesis and productivity. *Plant Physiology* **155**, 64–69. doi:10.1104/pp.110.167684
- Björkman O, Demmig B (1987) Photon yield of O₂ evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* **170**, 489–504. doi:10.1007/BF00402983
- Bueno ACR, Prudente DA, Machado EC, Ribeiro RV (2011) Daily temperature amplitude affects the vegetative growth and carbon metabolism of orange trees in a rootstock-dependent manner. *Journal of Plant Growth Regulation* **30**. doi:10.1007/s00344-011-9240-x
- Bush DR (1999) Sugar transporters in plant biology. *Current Opinion in Plant Biology* **2**, 187–191. doi:10.1016/S1369-5266(99)80034-X
- Davies FS, Albrigo LG (1994) 'Citrus.' (CAB International: Wallingford, UK)
- De Schepper V, Steppe K, Van Labeke MC, Lemeur R (2010) Detailed analysis of double girdling effects on stem diameter variations and sap flow in young oak trees. *Environmental and Experimental Botany* **68**, 149–156. doi:10.1016/j.envexpbot.2009.11.012
- Dickson RE (1991) Assimilate distribution and storage. In 'Physiology of trees'. (Ed. AS Raghavendra) pp. 51–85. (John Wiley & Sons: New York)
- Geiger DR, Servaites JC, Fuchs MA (2000) Role of starch in carbon translocation and partitioning at the plant level. *Australian Journal of Plant Physiology* **27**, 571–582.
- Goldschmidt EE, Golomb A (1982) The carbohydrate balance of alternate-bearing citrus trees and the significance of reserves for flowering and fruiting. *Journal of the American Society for Horticultural Science* **107**, 206–208.
- Goldschmidt EE, Huber SC (1992) Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. *Plant Physiology* **99**, 1443–1448. doi:10.1104/pp.99.4.1443
- Goldschmidt EE, Koch KE (1996) Citrus. In 'Photoassimilate distribution in plants and crops: source-sink relationships'. (Eds E Zamski, AA Schaffer) pp. 797–823. (Marcel Dekker: New York)
- Habermann G, Rodrigues JD (2009) Leaf gas exchange and fruit yield in sweet orange trees as affected by citrus variegated chlorosis and environmental conditions. *Scientia Horticulturae* **122**, 69–76. doi:10.1016/j.scienta.2009.04.003
- Iglesias DJ, Lliso I, Tadeo FR, Talon M (2002) Regulation of photosynthesis through source-sink imbalance in citrus is mediated by carbohydrate content in leaves. *Physiologia Plantarum* **116**, 563–572. doi:10.1034/j.1399-3054.2002.1160416.x
- Kalt-Torres W, Keer PS, Usuda H, Huber SC (1987) Diurnal changes in maize leaf photosynthesis. 1. Carbon exchange rate, assimilate export rate, and enzyme activities. *Plant Physiology* **83**, 283–288. doi:10.1104/pp.83.2.283
- Koch KE (1996) Carbohydrate-modulated gene expression in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 509–540. doi:10.1146/annurev.arplant.47.1.509
- Li CY, Weiss D, Goldschmidt EE (2003) Effects of carbohydrate starvation on gene expression in citrus root. *Planta* **217**, 11–20.
- Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochemical Society Transactions* **11**, 591–592.
- McCormick AJ, Cramer MD, Watt DA (2006) Sink strength regulates photosynthesis in sugarcane. *New Phytologist* **171**, 759–770. doi:10.1111/j.1469-8137.2006.01785.x
- Nakano H, Muramatsu S, Makino A, Mae T (2000) Relationship between the suppression of photosynthesis and starch accumulation in the pod-removed bean. *Australian Journal of Plant Physiology* **27**, 167–173.
- Nebauer SG, Renau-Morata B, Guardiola JL, Molina R-V (2011) Photosynthesis down-regulation precedes carbohydrate accumulation under sink limitation in *Citrus*. *Tree Physiology* **31**, 169–177. doi:10.1093/treephys/tpq103
- Paul MJ, Foyer CH (2001) Sink regulation of photosynthesis. *Journal of Experimental Botany* **52**, 1383–1400. doi:10.1093/jexbot/52.360.1383
- Paul MJ, Pellny TK (2003) Carbon metabolite feedback regulation of leaf photosynthesis and development. *Journal of Experimental Botany* **54**, 539–547. doi:10.1093/jxb/erg052
- Prado AKS, Machado EC, Medina CL, Machado DFSP, Mazzafera P (2007) Flowering and fruit set in 'Valência' orange trees under different crop load status and with and without irrigation. *Bragantia* **66**, 173–182. [In Portuguese] doi:10.1590/S0006-87052007000200001
- Ramos RA (2009) Thermal regime and source-sink relationship in young sweet orange plants: carbohydrate dynamics, photosynthesis and growth. Masters dissertation, Agronomic Institute. [In Portuguese]
- Ramos RA, Ribeiro RV, Machado EC, Machado RS (2010) Seasonal variation in vegetative growth of Hamlin sweet orange grafted on Swingle citrumelo plants, in Limeira, São Paulo State. *Acta Scientiarum Agronomy* **32**, 539–545. [In Portuguese]
- Ribeiro RV, Machado EC (2007) Some aspects of citrus ecophysiology in subtropical climates: re-visiting photosynthesis under natural conditions. *Brazilian Journal of Plant Physiology* **19**, 393–411. doi:10.1590/S1677-04202007000400009
- Ribeiro RV, Machado EC, Oliveira RF (2003) Early photosynthetic responses of sweet orange plants infected with *Xylella fastidiosa*. *Physiological and Molecular Plant Pathology* **62**, 167–173. doi:10.1016/S0885-5765(03)00038-9
- Ribeiro RV, Machado EC, Oliveira RF (2006) Temperature response of photosynthesis and its interaction with light intensity in sweet orange leaf discs under non-photorespiratory condition. *Ciência e Agrotecnologia* **30**, 670–678. doi:10.1590/S1413-70542006000400012
- Ribeiro RV, Machado EC, Santos MG, Oliveira RF (2009a) Photosynthesis and water relations of well-watered orange plants as affected by winter and summer conditions. *Photosynthetica* **47**, 215–222. doi:10.1007/s11099-009-0035-2
- Ribeiro RV, Machado EC, Santos MG, Oliveira RF (2009b) Seasonal and diurnal changes in photosynthetic limitation of young sweet orange trees. *Environmental and Experimental Botany* **66**, 203–211. doi:10.1016/j.envexpbot.2009.03.011

- Roháček K (2002) Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationships. *Photosynthetica* **40**, 13–29. doi:10.1023/A:1020125719386
- Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annual Review of Plant Biology* **57**, 675–709. doi:10.1146/annurev.arplant.57.032905.105441
- Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. *Plant, Cell & Environment* **30**, 1126–1149. doi:10.1111/j.1365-3040.2007.01708.x
- Somogyi M (1952) Notes on sugar determination. *Journal of Biological Chemistry* **195**, 19–23.
- Syvertsen JP (1994) Partial shoot removal increases net CO₂ assimilation and alters water relations of *Citrus* seedlings. *Tree Physiology* **14**, 497–508.
- Syvertsen JP, Goñi C, Otero A (2003) Fruit load and canopy shading affect leaf characteristics and net gas exchange of 'Spring' navel orange trees. *Tree Physiology* **23**, 899–906. doi:10.1093/treephys/23.13.899
- van Raij B, Cantarella H, Quaggio JA, Furlani AMC (1996) Recomendações de adubação e calagem para o Estado de São Paulo. Instituto Agronômico e Fundação IAC, Campinas.
- Yemm EW, Willis AJ (1954) The estimation of carbohydrates in plant extracts by anthrone. *Biochemical Journal* **57**, 508–514.