Research paper

Biomass partitioning and photosynthesis in the quest for nitrogen- use efficiency for citrus tree species

Veronica L. Dovis [1,4], Norma M. Erismann[2], Eduardo C. Machado[2], Jose A. Quaggio[3],
Rodrigo M. Boaretto[1] and Dirceu Mattos Júnior[1,4]

[1]Centro de Citricultura Sylvio Moreira, Instituto Agronômico (IAC), Rod Anhanguera km 158, 13490-000, Cordeirópolis, Brasil; [2]Laboratório de Fisiologia Vegetal Coaracy M. Franco, Centro de Pesquisa e Desenvolvimento em Ecofisiologia e Biofísica, Instituto Agronômico (IAC), Av Doutor Theodureto de Almeida Camargo 1500, 13075-630, Campinas, Brasil; [3]Centro de Solos e Recursos Ambientais, Instituto Agronômico (IAC), Av Barão de Itapura 1481, 13020-902, Campinas, Brasil; [4]Corresponding authors V.L.Dovis (veronica@gmail.com); D.Mattos (ddm@ccsm.br)

Optimizing the use of nitrogen (N) for food production is a major challenge in agricultural systems. The transformation of N into crop production results from intricate pathways, depending on plants, as well as the environment and fertilization regimes, which affect the N-use efficiency (NUE) of plants. In this context, lemon trees [*Citrus limon* (L.) Burm. f.] attain maximum harvest index at lower leaf N concentrations compared with sweet orange trees [*Citrus sinensis* (L.) Osbeck], and the processes governing these plant responses are not well known. The aim of this study was to understand how the higher NUE in lemons trees is constructed based on growth and biomass partitioning evaluation, as well as photochemical and biochemical characteristics of photosynthesis. To attain this goal, we evaluated growth, photosynthesis and biochemical characteristics in lemon and sweet orange trees under two different N levels over 14 months. We hypothesized that higher NUE in lemon trees is affected by plant capacity to grow with economy on nutrient resources. Furthermore, lemon trees could be more efficient in CO_2 assimilation in non-limiting environmental conditions. We found that higher NUE in lemon trees was explained in part by the ability of trees to invest greater biomass in leaves instead of roots, even though this species exhibited lower relative recovery efficiency of N from the substrate than the sweet orange. We also found that lemon trees had a higher relative growth rate than sweet oranges, despite the fact that net CO_2 assimilation and dark respiration were similar between the two species. As a consequence, we suggested that lemons could exhibit a lower biomass construction cost than oranges. Because lemon presented lower N concentration than sweet orange trees, the former exhibited better photosynthetic N-use efficiency (PNUE: 55–120 mmol CO_2 g N^{-1} day^{-1}) compared with the sweet orange (PNUE: 31–68 mmol CO_2 g N^{-1} day^{-1}). Lemon trees also exhibited a higher relative rate of electron transport per unit of chlorophyll (ETR/chlor: 350–850) compared with orange trees (ETR/chlor: 300–550) at both low and at high N supply. These characteristics were likely associated with transport facilitation of CO_2 to the catalytic sites of plants. In fact, improved growth of lemon trees results from an array of events explained mostly by increase in leaf area and associated low construction cost despite N supply.

Keywords: CO_2 assimilation, lemon tree, orange tree, relative growth rate, root growth, specific leaf area.

Introduction

World food production was estimated to have increased 40% after the onset of the green revolution in the 1960s, resulting mostly from the use of synthetic nitrogen (N) fertilizers (Keeney and Hatfield 2008). Consequently, reactive N in the biosphere increased by 50%, becoming an environmental concern (Keeney and Hatfield 2008, Fowler et al. 2013) and stimulating the search for increased N-use efficiency (NUE, kg biomass kg^{-1} of N) in crops (Zhang et al. 2015).

The citrus industry occupies a prominent role in the world economy, with trees grown over approximately 8.0 million hectares in more than 140 countries, and oranges, mandarins,

limes lemons and grapefruits represent 22% of the total of the eight main fruits cultivated (FAO). The production of citrus fruits is largely regulated by N supply, promoted by fertilization of orchards, with treatments typically 180–240 kg ha^{-1} year^{-1} of N (Cantarella et al. 2003, Alva et al. 2006, 2008, Morgan et al. 2006, Martínez-Alcántara et al. 2012, Quaggio et al. 2014).

Nitrogen-use efficiency for citrus fruit production is estimated to be above 380 kg fresh fruits kg^{-1} of N applied in the USA and Brazil (Alva et al. 2006, Quaggio et al. 2014), although it is below 200 kg of fresh fruits kg^{-1} of N in Asian countries (Qin et al. 2016). Significant gains in NUE involves tackling increasing fertilizer use (Mattos et al. 2003, Quaggio et al. 2014), improved nutrient acquisition by roots (Scholberg et al. 2002, Miller and Cramer 2004) and/or enhanced biomass production (Hirel et al. 2011, Santa-María et al. 2015).

The transformation of N into an agricultural product results from complex processes, depending on plants, the environment and fertilizer regimes, with different efficiency factors and definitions for each part of the process (Fageria and Baligar 2005, Sorgonà et al. 2006, Benincasa et al. 2011, Xu et al. 2012, Qin et al. 2016). Thus, as a function of the available N (NO$_3$$^-$ and NH$_4$$^+$) for root uptake, two main components have to be considered for the estimation of NUE: (i) N recovery efficiency (NR$_{ec}$E) and (ii) N efficiency ratio (NER). These components represent the proportion of N acquired per unit N applied via fertilization, and the biomass produced per unit N taken up by plants, respectively (Sorgonà et al. 2006, Benincasa et al. 2011, Xu et al. 2012). Moreover, NR$_{ec}$E is affected by the plant and soil, as well as fertilizer characteristics, application method and environmental factors. The NER depends on plant internal processes, and how these are affected by the environment.

Up to 85% of the applied N can be lost in the soil–plant–atmosphere system in citrus orchards, increasing risks for the environment and production costs for growers (Alva et al. 2006, Menino et al. 2007, Boaretto et al. 2013). Comparing citrus species, it is interesting to note that maximum fruit yield of sweet orange trees (*Citrus sinensis*) is attained with leaf N concentration of 28 g kg^{-1} of N (dry mass basis; Quaggio et al. 1998, 2014), whereas the same yield for lemon trees is attained at a concentration of 18 g kg^{-1} of N (dry mass basis; Quaggio et al. 2002).

Lemon trees grown under the same N fertilization level are more vigorous and exhibit a greater NER compared with orange trees (Boaretto et al. 2010, Dovis et al. 2016), which plant vigor is defined in terms of rapid growth. Lemon trees exhibit higher photosynthetic nitrogen-use efficiency (PNUE, assimilation of CO$_2$ g^{-1} of leaf N; Romero-Aranda et al. 1997, Dovis et al. 2016), which leads to questions as to the underlying cause of this effect. Notably, these citrus species differ in leaf anatomy (Romero-Aranda et al. 1997) and specific leaf area

(SLA; Boaretto et al. 2010, Dovis et al. 2016), characteristics which could affect CO$_2$ conductance in the mesophyll and consequently plant photosynthesis (Tomás et al. 2013, Buckley and Warren 2014).

Unraveling processes that determine differences in NUE in these species would firstly represent an opportunity to increase productivity and quality of agricultural products, with fine-tuned fertilization programs, associated with economic and social growth, and environmental protection (Weih et al. 2011). Secondly, it would represent an opportunity to assist breeding programs seeking genotypes that use resources more efficiently in modern agriculture (van Bueren and Struik 2017), especially in perennial plants that have been less studied. We hypothesized that greater NUE in lemon trees is a result of the improved capacity of N-use for biomass construction and hence for higher CO$_2$ assimilations by N unit, when in non-limiting environmental conditions. Therefore, this study evaluated the biomass production, N distribution and photosynthetic efficiency in two species of citrus with contrasting horticultural performance, along the same environmental conditions of different growing seasons and N availability for plant uptake.

Materials and methods

Plant material and experimental set-up

The experiment was carried out in a screen house covered with transparent plastic film in Cordeirópolis, São Paulo State, Brazil (22°27′40″S–47°24′4″O; 639 m of altitude). Nine-month-old Lisbon lemon [*Citrus limon* (L.) Burm. f.] and Valencia sweet orange trees [*C. sinensis* (L.) Osbeck] transplanted into 42 l plastic pots filled with a mixture of sand and pine bark substrate, were used. Both species were grafted onto Swingle citrumelo [*Citrus paradisi* Macfad. x *Poncirus trifoliata* (L.) Raf.]. After transplanting, all plants were pruned at 30 cm from graft and a set of four plants for every species was destructively harvested. Plants were fertilized biweekly with a complete nutrient solution, with annual doses per plant of: 16.8 g calcium, 5.5 g phosphorus, 6.6 g potassium, 2.0 g magnesium 2.4 g sulfur, and either 27 g of N (sweet orange: S.Or-N27, or lemon: Le-N27 plants) or 7 g N (sweet orange: S.Or-N7, or lemon: Le-N7). Nitrogen was supplied as a combination of Ca (NO$_3$)$_2$·4H$_2$O and NH$_4$NO$_3$ to achieve the 1.5 NO$_3$$^-$:NH$_4$$^+$ ratio. Regular foliar application of micronutrients was according to Quaggio et al. (2003). Plants were drip irrigated four times a day to maintain the substrate at a maximum water storage capacity.

Environmental conditions inside the screen house were monitored by a Vantage Pro2 automatic weather station (Davies Instruments, Hayward, CA, USA). Maximum, minimum and mean temperatures, total radiation (Qg, MJ m^{-2} day^{-1}) and air humidity were registered every 30 min. During the experimental period, maximum solar radiation was also recorded and

converted into photosynthetic photon flux density (PPFD; Assis and Mendez 1989, Anjorin et al. 2014) by:

$$PPFD = 2.23 \times Qg.$$

At transplanting, on 10 November 2014, lemon and sweet orange plants were destructively evaluated for biomass production, to establish the experimental starting point. After that, additional plant evaluations were conducted: (i) 8 months after transplantation, on a winter day (on 8 July 2015; cold nights); (ii) 11 months after transplanting, on a spring day (on 20 October 2015; hot and dry days); and (iii) 14 months after transplanting, on a summer day (on 12 January 2016; hot and bright days). The time between transplanting and the first evaluation in July was identified as Period 1 (P1 = 240 days), the time between the July and October evaluations was identified as Period 2 (P2 = 103 days) and the time between October and January was identified as Period 3 (P3 = 83 days).

Photosynthesis: gas exchange and photochemistry

Gas exchange and chlorophyll a fluorescence was measured using an LI-6400 infrared gas analyzer (IRGA; LI-COR, Inc., Lincoln, NE, USA), with an integrated LCF6400-40 modulated fluorimeter (LI-COR Inc.). Measurements were taken from a completely exposed mature leaf for each replicate, between the third and fifth position from the end of the youngest branches. Measurements were performed at 2 h intervals, from 07:30 to 17:30h, with 380 μmol CO_2 mol^{-1} at the leaf chamber and temperature not controlled. The PPFD was measured at the beginning of every evaluation interval and was fixed for all measurements in that same interval. Air (T_{Air}) and leaf (T_{Leaf}) temperatures, vapor pressure difference between leaf and air (VPD_{L-A}), relative humidity in the chamber (% RH), net CO_2 assimilation (P_n, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}), transpiration (E, mmol m^{-2} s^{-1}) and intercellular CO_2 concentration (C_i, μmol mol^{-1}) were measured. The apparent carboxylation efficiency (P_n/C_i) and water-use efficiency (WUE; P_n/E) were calculated. Diurnal CO_2 assimilation (P_d) was determined from integration of the diurnal curve of P_n, being expressed in mmol CO_2 m^{-2} day^{-1}. The PNUE was calculated as P_d (mmol CO_2 m^{-2} day^{-1})/N (g m^{-2} leaf). Dark respiration (R_d) was also evaluated at 05:30 and 13:00h. All measurements were taken with a coefficient of variation (CV) lower than 5% and when temporal stability was achieved.

Measurements of chlorophyll a fluorescence were obtained in dark-adapted (overnight at 05:30 h) and light-adapted leaf tissues, by the method of saturation pulse (λ = 630 nm, $Q \sim 6000$ μmol m^{-2} s^{-1}, 0.8 s). From the fluorescence signals, maximum quantum efficiency of photosystem II (PSII) photochemistry (F_v/F_m, where $F_v = F_m - F_0$) was calculated, using the maximum (F_m) and minimum (F_0) fluorescence values measured from dark-adapted leaves at ambient CO_2 partial pressure. The parameters for PSII efficiencies and photochemical quenchings were calculated according to Roháček et al. (2008) and apparent electron transport rate (ETR) was calculated according to Schreiber et al. (1998). We estimated the number of electrons transported through PSII per molecule of CO_2 fixed by the ratio ETR/P_n. After photosynthesis measurements, leaves were sampled for nutrient and carbohydrate analyses, and plants were harvested, separated into component organs with dry weights determined.

Biomass partitioning and parameters calculations

After gas exchange measurements were performed, samples of leaves, branches and roots were collected between 12:30 and 13:30h for carbohydrate and nitrogen analysis. After that, all leaves were detached and leaf area (LA) was measured using a LI-3100C Area Meter (LI-COR Inc.). Weight of total dry matter (TDM) was determined after drying leaves (LDM), branches (BDM) and roots (RDM) in forced-air circulation at 55 °C. Parameters for plant growth analysis were calculated as described by Hunt et al. (2002): relative growth rate (RGR) was the biomass produced per unit dry mass per day (mg g^{-1} day^{-1}); unit leaf rate (ULR) was the biomass produced per unit LA per day (g m^{-2} day^{-1}); SLA was the ratio of LA to LDM (m^2 kg^{-1}) and leaf weight fraction (LWF) was LDM as a fraction of TDM (g g^{-1}):

$$RGR = \left(\log_e TDM_2 - \log_e TDM_1 \right) \div (t_2 - t_1)$$

$$ULR \approx \left[(TDM_2 - TDM_1) \times \left(\log_e LA_2 - \log_e LA_1 \right) \right] \div \left[(LA_2 - LA_1) \times (t_2 - t_1) \right],$$

$$SLA = LA \div LDM$$

$$LWF = LDM \div TDM,$$

where t is the time between consecutive harvests. Nitrogen-use efficiency definitions and parameters were in accordance with Sorgonà et al. (2006). The NER, which indicated how much biomass was produced per unit of nitrogen absorbed, was calculated as TDM divided by total nitrogen accumulation by the plant (g TDM g^{-1} N). Nitrogen utilization efficiency (NU$_t$E) was defined as the amount of biomass per unit of N present in the biomass, and was calculated as TDM divided by nitrogen concentration (kg^2 TDM g^{-1} N). Finally, nitrogen uptake efficiency (NU$_p$E), represented the relative ability to acquire N with minimal investment of fixed carbon in roots, and was calculated as total nitrogen accumulation divided by RDM (g N kg^{-1} RDM). Calculations were made for the periods P1, P2 and P3. All variables were calculated using the difference between the final and the initial biomasses and N accumulation for each period.

Table 1. Environmental conditions during the experimental periods of plant growth. T_{mean} (mean $\pm$ SD medium temperature), T_{max} (mean $\pm$ SD maximum temperature), T_{min} (mean $\pm$ SD minimum temperature) and average of global solar radiation during of three experimental periods. Data recorded with automatic weather station between 10 November 2014 and 12 January 2016.

	Period 1 (P1) Summer–autumn	Period 2 (P2) Winter–spring	Period 3 (P3) Spring–summer
Experimental period	10 November to 8 July	9 July to 20 October	21 October to 12 January
Days within period	240	103	83
T_{mean} (°C)	23.9 $\pm$ 2.4	22.2 $\pm$ 2.6	25.4 $\pm$ 1.3
T_{max} (°C)	31.4 $\pm$ 3.1	32.2 $\pm$ 2.6	33.1 $\pm$ 2.5
T_{min} (°C)	18.5 $\pm$ 3.6	16.1 $\pm$ 2.2	20.4 $\pm$ 1.1
Qg (MJ m^{-2} day^{-1})	19.4 $\pm$ 6.9	19.5 $\pm$ 6.2	20.8 $\pm$ 6.8
Frequency of occurrence	% of days within periods		
T_{air} < 13 °C	3.6	8.8	0
25 °C < T_{air} < 35 °C	73.6	63.2	66.2
T_{air} > 35 °C	16.4	22.8	31.9
Qg < 15 MJ m^{-2} day^{-1}	26.6	23.3	20.5
Qg > 25 MJ m^{-2} day^{-1}	21.8	20.4	32.5

Measurement of chlorophyll, nitrogen concentrations, total non-structural carbohydrate and total amino acids

Chlorophyll quantification was performed on fresh leaf tissue at 09:00 after dimethyl sulfoxide extraction, with incubation for 4 h at 64 °C (Wellburn 1994). Then plants were harvested, and leaves, branches and roots were dried and ground to pass through a 200-mesh sieve. Total N concentrations of each plant part were measured according to Malavolta et al. (1997), and soluble carbohydrates were extracted by the MCW method (methanol:chloroform:water) and their concentration was determined by the phenol-sulfuric acid method (Dubois et al. 1956, Bieleski and Turner 1966). Starch was determined enzymatically according to Amaral et al. (2007). Quantification of total amino acids was performed in MCW extracts by the ninhydrin method (Yemm and Cocking 1955).

Statistical analysis

The experiment was arranged in a completely factorial design, with 15 replicates for each treatment. Five uniform plants of each treatment were randomly selected for measurement and destructively harvested at each of the three evaluation periods. Data were subjected to analysis of variance (ANOVA) in a factorial design, with two sources of variation, citrus species and N- level. Mean values were compared using Tukey's test when significance was detected (P < 0.05). Different periods were individually analyzed for each variable (n = 5). When no significant differences were detected between evaluated periods, statistical analysis was performed pooling the values for the three periods for the same treatment (n = 15).

Results

Environmental conditions during experiment conduction

The conditions in the screen house varied during the three periods of the experiment (Table 1). The first period (P1,
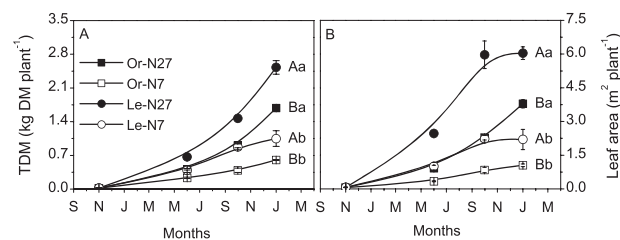

Figure 1. Growth of citrus trees submitted to different N supply levels during 14 months, from November 2014 to January 2016. (A) TDM accumulation per plant, and (B) LA accumulation per plant (m^2 plant^{-1}). Capital letters indicate statistically significant differences between species, and lowercase letters indicate statistically significant differences between N levels for the same species (Tukey's test; P < 0.05). Vertical bars indicate standard errors of the mean (n = 5). Legend: Or, sweet orange cv Valencia; Le, lemon cv Lisbon; N27, 27 g of N plant^{-1}; N7, 7 g of N plant^{-1}.

summer–autumn) was characterized by a few days with T_{min} < 13 °C and also a few days with T_{max} > 35 °C. The second period (P2, winter–spring) was characterized by an increasing number of days with temperatures <13 °C or >35 °C, compared with P1. In the third period (P3, spring–summer), T_{max} > 35 °C was more common (frequency = 32% within the period), while there were no days with temperatures <13 °C (Table 1). In P3, days with high radiation intensity were more frequent (32.5% of days with Qg > 25 MJ m^{-2} day^{-1}), which was more than 10% of the frequency observed in the other periods (Table 1).

Plant growth and biomass partitioning

Plants supplied with the highest dose of N exhibited an exponential pattern of TDM accumulation, with a greater increase for lemon trees (Le-N27) in comparison with orange trees (Or-N27), during the 14-month period of this study. Trees supplied with the lowest dose of N exhibited low and almost linear growth
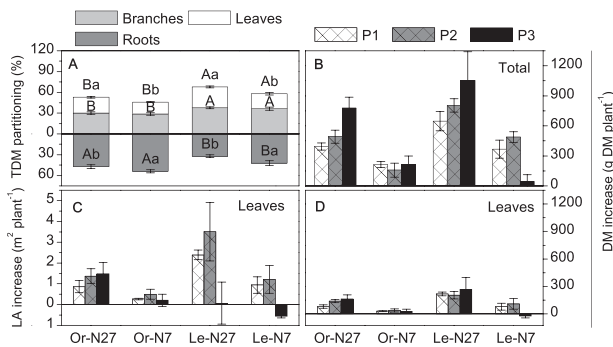
Figure 2. Growth of citrus trees exposed to different N supply levels during 14 months, from November 2014 to January 2016. (A) TDM partitioning into plant organs at final harvest, (B) TDM increase in each period (DM increase), (C) LA increase (LA increase) and (D) leaf dry matter increase in each period of growth. Capital letters indicate statistically significant differences between species, and lowercase letters indicate statistically significant differences between N levels for the same species (Tukey's test; $P < 0.05$). Vertical bars indicate standard errors of the mean ($n = 5$). Legend: Or, sweet orange cv Valencia; Le, lemon cv Lisbon; N27, 27 g of N plant^{-1}; N7, 7 g of N plant^{-1}; P1, November to July; P2, July to October; P3, October to January.

over time (Figure 1A). These patterns of TDM accumulation were also observed for LA (Figure 1B).

At the end of the experiment, Le-N27 had accumulated 48.6% more TDM and 49.8% more LA than Or-N27. With the lowest nutrient supply level, Le-N7 trees were also more efficient in their production of dry mass, accumulating 71.3% more TDM and 92.4% more LA than Or-N7 trees. Lemon trees exhibited a greater fraction of the TDM (overall mean for both N levels) invested in leaf growth (~25%) when compared with orange trees (20%; Figure 2A). Furthermore, when plants were submitted to a limited supply of N, the biomass partitioning into leaves decreased 6.4% ($P < 0.05$), while root biomass partitioning increased 8.3% ($P < 0.05$) in both species, without changes in TDM invested in branches (Figure 2A).

Growth analysis also revealed that trees under the N27 fertilization level exhibited progressive DM increases of leaves, branches and roots from P1 to P3; whereas, those under the N7 level exhibited less pronounced DM increases (Figure 2B). The biomass increase in lemon trees was particularly affected in P3 (spring–summer), with Le-N7 exhibiting only a minor increase in TDM due to RDM and BDM increase (data not shown), associated to observed early leaf senescence, as demonstrated by the reduction in LA (Figure 2C and D).

Moreover, Le-N27 did not show a significant LA increase in P3 (Figure 2C), despite increases in LDM and (Figure 2D). Mean SLA over all of the studied periods was greater ($P < 0.05$) for lemon trees (10.7–14.0 m^2 kg^{-1} DM) than orange trees (9.8–11.0 m^2 kg^{-1} DM). Furthermore, SLA in lemon trees was variable between periods while the same was constant in sweet orange ($P < 0.05$) (data not shown).

The RGR and ULR varied for both citrus species and N levels, with mean values decreasing from P1 to P3, resulting not

only from an increased plant size, but also by a less favorable environmental growth conditions in P2 and P3 (Figure 3). Differences between species were observed in RGR for the evaluated periods, in which, during P1 (summer–autumn) lemon trees exhibited 15% greater RGR than sweet orange trees (Figure 3A). Minor differences in the RGR of trees were still observed during P2 (winter–spring). However, greater variation was observed for lemon trees during P3, especially for those grown with limited N supply. In these conditions, the difference in RGR was large; with Le-N7 plants exhibiting a 57% lower RGR than Or-N7 plants (Figure 3A).

The ULR did not vary between species and N levels in P1 and P2 (Figure 3B). Decrease in URL was only observed in lemon trees in P3 (Figure 3B), indicating that there was a negative effect of environmental conditions for lemon growth during this period (Figure 1), when more warmer days and higher intensity of radiation were registered, compared with P1 and P2.

Nitrogen concentration, accumulation and use efficiency

Differences in concentration of total N in plant tissues were observed, with lemon trees exhibiting lower levels in both the aboveground parts and the roots, compared with orange trees under the same N levels (Table 2). Notably, N contents were the same for both species within each N level (N27 = 21.7 g N plant^{-1}, and N7 = 6.3 g N plant^{-1}), taking into account greater biomass accumulation by lemons trees compared with orange trees. However, in terms of total N partitioning, lemon trees accumulated less N in roots (<47%) and more N in leaves (>53%) compared with orange trees, independent of the N supply level and corresponding with biomass partitioning (Figure 4A). Lemon trees developed greater LA per unit of N allocated in leaves (m^2 g^{-1} of N) under both N supply regimes, demonstrating superior capacity for leaf construction (Figure 4B).

Estimates of N-use by trees highlighted differences between plant responses grown under the two N levels, N27 and N7. For the N27 level, sweet orange and lemon trees exhibited different values for NER, NU$_t$E and NU$_p$E during all evaluated periods. However, under the N7 level, trees differed only between species, but not between time periods (Figure 5). Because of this, NER, NU$_t$E and NU$_p$E were calculated as an average for P1, P2 and P3. Overall efficiency ratio calculated as TDM per unit of N (NER) was 1.3- to 2.8-fold higher for lemon trees than orange trees (Figure 5A and D). However, when NER is broken down into NU$_p$E (uptake efficiency) and NU$_t$E (transformation efficiency), it is highlighted that NU$_t$E in lemon trees was responsible for higher NER since NU$_t$E was 2.0 to 2.8-fold than orange trees (Figure 5B and E). On the other hand, NU$_p$E in orange tress was 1.2- to 2.4 higher than lemon trees (Figure 5C and F). These results indicated that NU$_t$E was responsible, at least in part, for the better NUE in lemon trees.
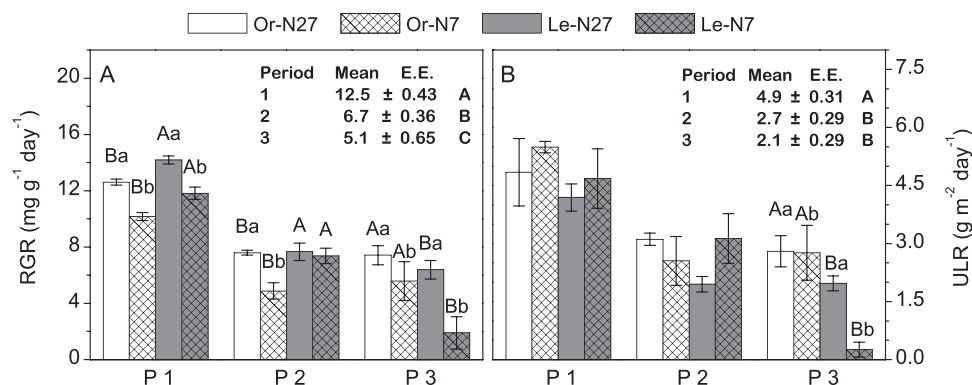
Figure 3. Growth analysis of citrus trees exposed to different N supply levels for 14 months, from November 2014 to January 2016. (A) RGR, and (B) ULR. Capital letters indicate statistically significant differences between species, lowercase letters indicate statistically significant differences between N levels for the same species and no letters mean no statistical differences (Tukey's test; $P < 0.05$). Vertical bars indicate standard errors of the mean ($n = 5$). Legend: Or, sweet orange cv Valencia; Le, lemon cv Lisbon; N27, 27 g of N plant^{-1}; N7, 7 g of N plant^{-1}; P1, summer–autumn; P2, winter–spring; P3, spring–summer.

Table 2. Nitrogen concentration in citrus trees exposed to different N supply levels and evaluated 7 (July), 11 (October) and 14 (January) months after transplanting. Means of data from the three evaluation dates are presented.

Species	N level	Leaves[1]		Branches		Roots	
		g kg^{-1} DM					
Sweet orange	N27	29.1 ± 1.62	Aa	8.7 ± 0.55	Aa	12.4 ± 0.47	Aa
	N7	19.5 ± 0.77	Ab	5.9 ± 0.29	Ab	9.7 ± 0.79	Ab
Lemon	N27	20.1 ± 0.99	Ba	5.7 ± 0.25	Ba	10.3 ± 0.53	Ba
	N7	12.0 ± 0.45	Bb	3.7 ± 0.33	Bb	8.0 ± 0.34	Bb

[1]Capital letters in columns indicate statistically significant differences between species for the same N level, and lowercase letters in columns indicate statistically significant differences between N levels for the same species (Tukey's test; $P < 0.05$). Mean ± standard error ($n = 15$). N27, 27 g of N plant^{-1}, N7, 7 g of N plant^{-1}.
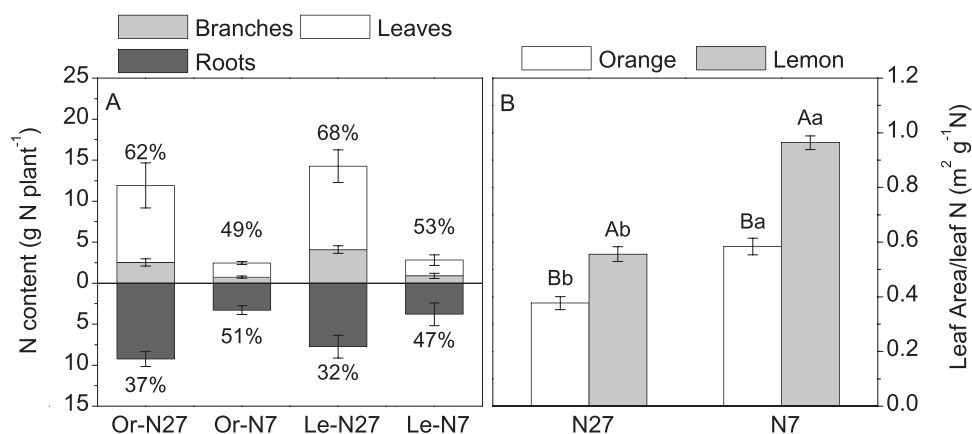

Figure 4. Nitrogen relations in citrus trees exposed to different N supply levels during 14 months after transplanting. (A) Nitrogen partitioning, and (B) LA developed per unit of nutrient allocated in leaves. Above ground percentages represent leaves + branches. Capital letters indicate statistically significant differences between species, and lowercase letters indicate statistically significant differences between N levels for the same species (Tukey's test; $P < 0.05$). Vertical bars indicate standard errors of the mean ($n = 5$). Legend: Or, sweet orange cv Valencia; Le, lemon cv Lisbon; N27, 27 g of N plant^{-1}; N7, 7 g of N plant^{-1}.

Diurnal and seasonal changes in gas exchange and chlorophyll fluorescence

Environmental conditions during days of physiological evaluations benefited P_d (see Figure 7) in July more than in October

or January (Table 2), when T_{max} of the air was approximately 28 °C, T_{Leaf} was 28–30 °C, RH in the air was 51% and VPD$_{L-A}$ was 1.8–2.1 kPa, favoring g_s and increasing P_n. Moreover, Qg and maximum solar radiation in January were higher than levels
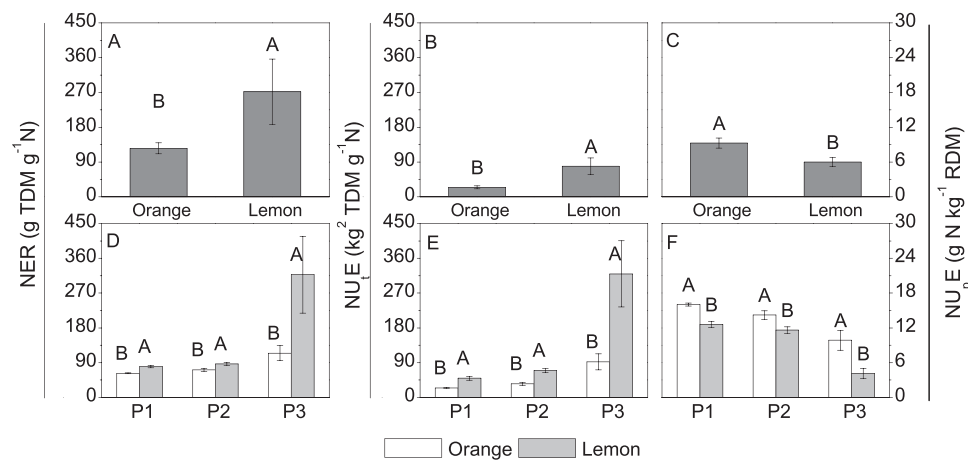
Figure 5. (A), D. NER (g TDM g^{-1} N); (B), E. NU$_t$E (kg^2 TDM g^{-1} N); and (C), F. NU$_p$E (g N kg^{-1} RDM) in sweet orange cv Valencia (Orange) and lemon trees cv Lisbon (Lemon), exposed to N levels, N7 and N27. (A, B and C). Evaluated for N7 level treatment, values of variables evaluated at P1, P2 and P3 were used to determine the mean (n = 15). (D, E and F). Evaluated for N27 level treatment, values of variables evaluated at P1, P2 and P3 were used to determine the mean (n = 5). Legend: P1, summer–autumn; P2, winter–spring; P3, spring–summer. Capital letters indicate statistically significant differences between species (Tukey's test; $P < 0.05$).

Table 3. Environmental conditions during days of photosynthetic and chlorophyll a fluorescence evaluation.

Variables	Environmental conditions during the physiological evaluations					
	1 July	16 October		7 January		
T_{max} (°C)	27.8	41.2		35.7		
T_{min} (°C)	13.6	22.9		19.1		
T_{med} (°C)	19.2	30.6		26.7		
Min RH (%)	51	21		43		
Qg (MJ m^{-2} day^{-1})	12.9	26.0		30.0		
	Morning	Midday	Morning	Midday	Morning	Midday
VPD (kPa)	1.8 ± 0.4	2.1 ± 0.6	2.1 ± 0.4	6.2 ± 0.4	2.3 ± 0.3	4.9 ± 0.9
T_{Leaf} (°C)	27.9 ± 0.5	29.6 ± 0.6	29.8 ± 1.9	43.5 ± 0.5	31.2 ± 1.2	39.9 ± 2.7
PPFD (μmol m^{-2} s^{-1})	1000	1400	600	1000	1000	1500
Max Rad (μmol m^{-2} s^{-1})	397 ± 47	495 ± 183	433 ± 265	1050 ± 346	584 ± 270	1617 ± 98

T_{max}, maximum air temperature; T_{min}, minimum air temperature; T_{med}, medium air temperature; Min RH, minimum relative humidity; Qg, global solar radiation; recorded at the weather station. VPD, leaf-to-air vapor pressure difference; T_{Leaf}, leaf temperature; PPFD, fixed photosynthetic photon flux density, measured in a LI-COR 6400 chamber in the morning (09:00–10:00h) and at midday (12:00–13:00h); Max Rad, maximum solar radiation (Max rad) registered at the weather station. Data in the lower half of the table are presented as means ± SD.

observed in the other periods. Furthermore, mean leaf temperature in January was 4.2 °C higher than ambient temperature (Table 3), with that in lemon up to 2.5 °C higher than orange trees (see Figure S1 available as Supplementary Data at *Tree Physiology* Online).

Nevertheless, no differences in gas exchange variables (P_n and P_n/C_i) between species were observed in July and in October, despite differences of N supply level (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). Diurnal CO_2 assimilation (P_d) was 2–48% higher in July than in October or January (Figure 6), especially due to a more intense reduction in P_n from midday onwards (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). Differences in P_d between species were only observed in January (Figure 6) when lemon trees had lower P_d than orange

trees. Lemon trees exhibited a higher PNUE than orange trees, both in July and in October. Yet environmental conditions already described for January limited CO_2 assimilation and RGR and ULR of lemon trees, compared with sweet orange trees (Table 3; Figures 3 and 6).

The highest levels of ETR of PSII were always attained with the N27 compared with the N7 supply, as well in orange trees compared with lemon trees, in October and January (Figure 7). The ETR in orange trees under the N27 level increased 21% with the global solar radiation from July to January. Meanwhile, the ETR in lemon trees increased only 5% during the same period, indicating that the reduction in chlorophyll concentration negatively affected plant capacity to transfer electrons from the chlorophyll reaction center of PSII through the electron transport chain (Figure 7). Furthermore, leaf chlorophyll concentration
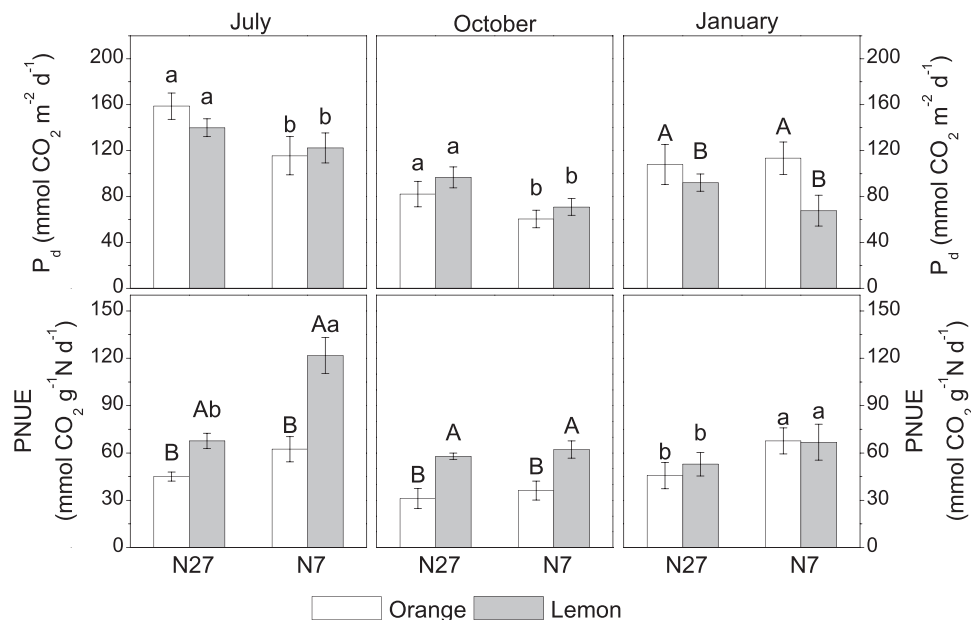
Figure 6. Integrated diurnal CO_2 assimilation (P_d) and PNUE in sweet orange trees cv Valencia (Orange) and lemon trees cv Lisbon (Lemon), exposed to N levels, N7 and N27, and evaluated 7 (July), 11 (October) and 14 (January) months after transplanting. Capital letters indicate statistically significant differences between species, and lowercase letters indicate statistically significant differences between N levels for the same species. Mean ± standard error ($n = 5$; Tukey's test; $P < 0.05$).

directly correlated with the N level, which was expressed by the greener color of leaves of orange trees compared with lemon trees. Despite the lower values of ETR in lemon trees, the ETR to chlorophyll ratio was up to 17–100% higher for this species (Figure 7), ranging from 300 to 598 µmol g^{-1} s^{-1} in orange trees and 352 to 835 µmol g^{-1} s^{-1} in lemon trees.

The system of light capture and energy transfer of trees was responsive to differences between the two N supply levels and species. Higher values of F_v/F_m and qP were observed with N27 compared with N7, during the three periods (Figure 7). Lower values of F_v/F_m were observed in July for the N7 treatment, when orange trees attained 0.71 whereas lemon trees attained only 0.62. Lemon trees consistently exhibited values of PSII maximum efficiency (F'_v/F'_m) from 6.5 to 13.3% lower than oranges in October and January. The reduction in F_v/F_m did not affect qP in October, even though in January, the decrease was more pronounced in lemons (Figure 7).

Carbohydrate and free amino acid concentrations

Concentration of soluble sugars in plant leaves did not differ between species or N levels, except for lemon trees in January, when grown with N27 or N7 (Figure 8). However, more marked differences related to citrus species and N supply level were observed in branches and roots, especially in July, with higher levels in lemon trees compared with orange trees of up to 20% and 46%, respectively. In October, metabolites concentrations (soluble sugars and amino acids) were lower than in July and January, related to a reduction in P_d observed in that period. In addition, starch concentration was higher in leaves and branches

of lemon trees compared with orange trees (Figure 9). Such differences correlated with RGR ($P < 0.05$), and were highest in P1, compared with P2 and P3, for all treatment combinations (see Figure S2 available as Supplementary Data at *Tree Physiology* Online). On the other hand, due to conditions that probably limited growth of lemon trees in P3, higher concentrations of starch occurred in leaves and branches in January and were to some extent also associated with the N7 level (Figure 9).

The level of free amino acids in leaves reflected the nutritional status of trees, with total N being lower in lemons compared with orange trees (Table 2 and Figure 8). Interestingly, such differences became more evident when comparing citrus species and N supply levels in January, which were also especially associated with early leaf senescence in lemon trees under the N7 level (Figure 2). The concentration of free amino acids in plants also increased in January, suggesting that leaf senescence resulted from N remobilization from mature leaves to sink tissues when NU$_p$E decreased (Figure 5C and F).

Discussion

Biomass and N partitioning in trees

The highest NUE in lemon trees resulted from the increase in biomass and N partition to leaf production, as well the plasticity in SLA determined under varying environmental conditions and nutrient supply. The SLA plasticity in this species could be associated with efficiency in biomass construction cost. Another characteristic responsible for lower construction cost was likely
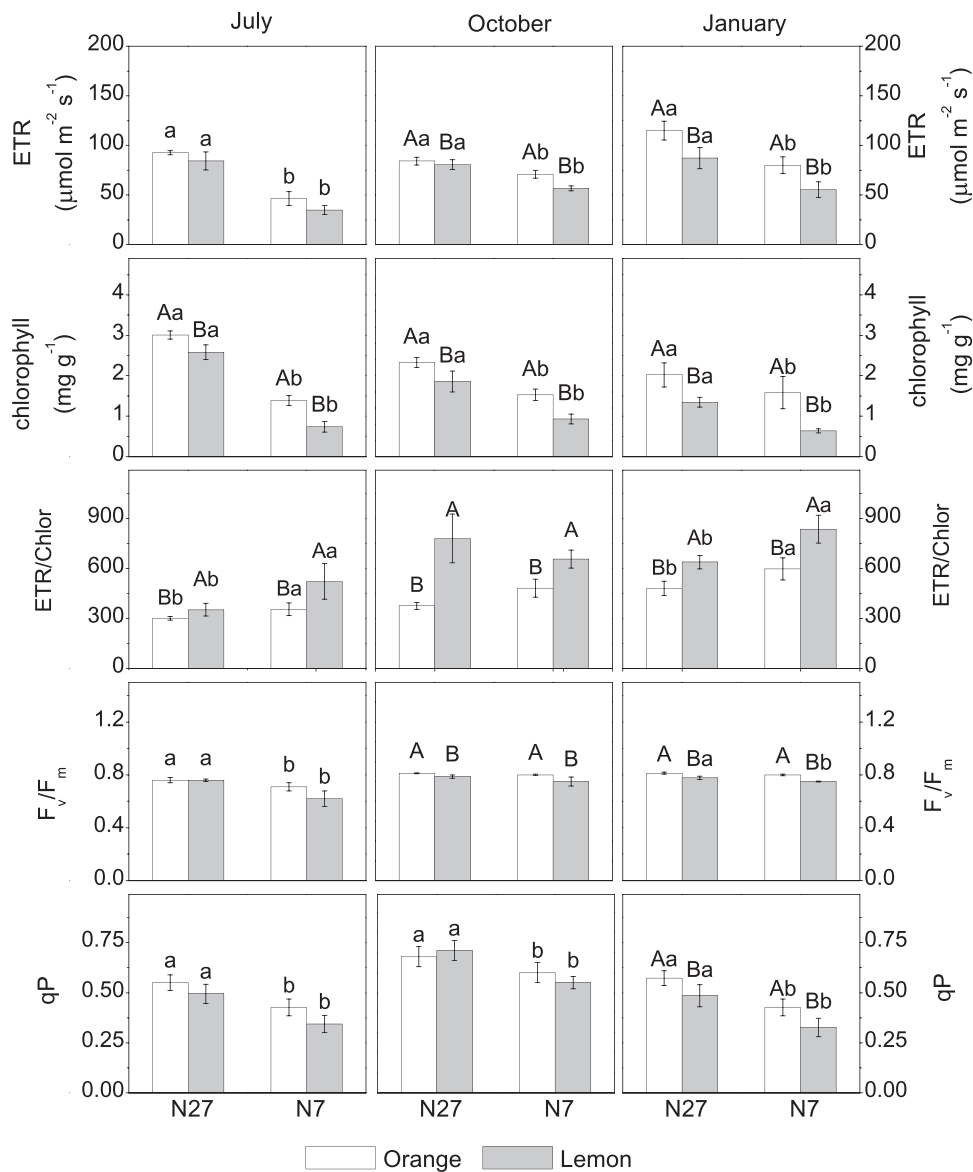
Figure 7. The ETR (μmol electrons m^{-2} s^{-1}), total chlorophyll concentration (mg g^{-1}), ratio between ETR and total chlorophyll concentration (ETR/chlor, μmol electrons g^{-1} chlorophyll), maximum quantum efficiency of PSII photochemistry (F_v/F_m) and photochemical quenching (qP) in leaves of sweet orange trees cv Valencia (Orange) and lemon trees cv Lisboa (Lemon), exposed to N levels, N7 and N27, and evaluated 7 (July), 11 (October) and 14 (January) months after transplanting, in the morning. Capital letters indicate statistically significant differences between species, and lowercase letters indicate statistically significant differences between N levels for the same species. Mean ± standard error ($n = 5$; Tukey's test; $P < 0.05$).

the highest ratio of LA/N also found in lemon trees. These could be most relevant traits for enhanced growth (RGR) and resource use (NU$_t$E) of lemon trees.

In fact, lemon trees exhibited more vigorous growth than sweet orange trees (Figure 1) and this was associated with a differential strategy of N-use, based on the preference for allocation of biomass (Figure 2) and N into leaves instead of roots (Figure 4). Such definition correlating growth and nutrient-use efficiency is a critical issue for seeking agricultural sustainability (Lynch 2013). Moreover, lemon trees seem to be less sensitive to N starvation than sweet orange trees. Since this former species exhibited a reduction in leaf N concentration of

approximately 40% in respect to the optimum level. However, the final reduction in total biomass was only 59%. In sweet orange trees, the leaf N concentration was reduced by 33%, whereas the decrease in total biomass was 63%.

Furthermore, lemon trees achieved better growth compared with orange trees during late autumn and early spring, when photosynthesis was similar between both species (Figures 3 and 6). CO$_2$ assimilation and growth rate in *Citrus* is favored by temperatures between 25 and 35 °C (Machado et al. 2005, Pimentel et al. 2007, Ribeiro et al. 2009, Dovis et al. 2014). By contrast, we observed differences between species in photosynthesis as well as RGR and ULR only in the summer
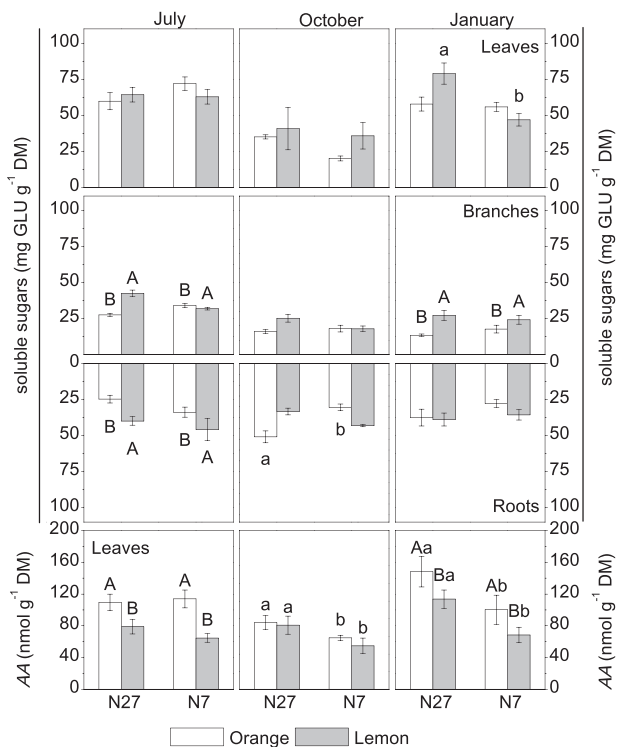
Figure 8. Soluble sugar concentrations (mg GLU g^{-1} DM) in leaves, branches and roots, and free amino acid concentrations (*AA* nmol g^{-1} DM) in leaves of sweet orange trees cv Valencia (Orange) and lemon trees cv Lisbon (Lemon), exposed to N levels, N7 and N27, and evaluated 7 (July), 11 (October) and 14 (January) months after transplanting. Capital letters indicate statistically significant differences between species, and lowercase letters indicate statistically significant differences between N levels for the same species. Mean ± standard error (n = 5; Tukey's test; P < 0.05).
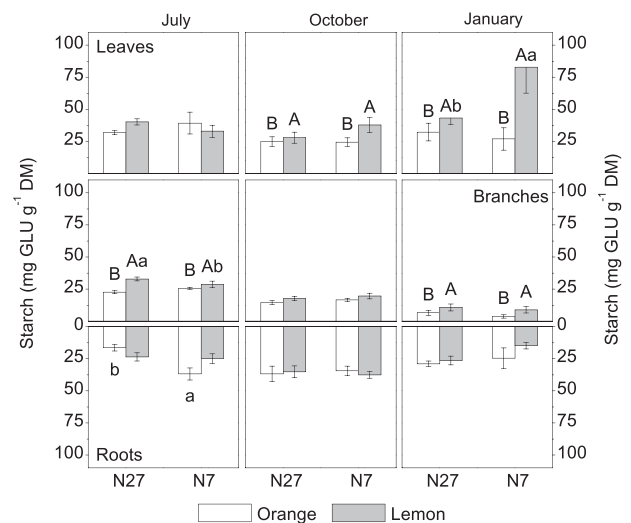

Figure 9. Starch concentration (mg GLU g^{-1} DM) in leaves, branches and roots of sweet orange trees cv Valencia (Orange) and lemon trees cv Lisbon (Lemon), exposed to N levels, N7 and N27, and evaluated 7 (July), 11 (October) and 14 (January) months after transplanting. Capital letters indicate statistically significant differences between species, and lowercase letters indicate statistically significant differences between N levels for the same species. Mean ± standard error (n = 5; Tukey's test; P < 0.05).

season what resulted from a Qg of 30.0 MJ m^{-2} day^{-1} and a maximum PPFD of 1600 µmol m^{-2} s^{-1} at midday, and secondly by high temperatures. Such conditions, at least in part, were limiting for growth of lemon trees.

Since, lemon trees exhibited higher seasonal variation in SLA than orange trees, which indicated a more efficient plant regulation of leaf construction costs (C_c; grams of glucose necessary to synthesize 1 g leaf tissue). This was supported by the fact SLA negatively correlated with C_c and positively correlated with nitrogen-use, as reported by Feng et al. (2008).

Lower C_c and similar photosynthesis implied in a similar capacity for capturing light, CO_2 and N per unit LA, and a shorter payback for biomass plant production in lemon trees, thus providing more available energy for new leaf construction (Villar and Merino 2001). Moreover, increased SLA in lemon trees compared with sweet orange trees is reported to be proportional to better nitrogen-use and increased fruit production (Boaretto et al. 2010).

The highest ratio between LA and N investment in leaves of lemon trees (Figure 4B; m^2 g^{-1} of N) likely explained improved nutrient use (NER and NU$_t$E) (Figure 5) of these compared with orange trees, since the photochemical process of light energy capture that drives carbon assimilation occurs mainly on the leaf surface (Walker et al. 2014). This characteristic demonstrated an important mechanistic response of plants to N starvation (Bondada and Syvertsen 2003, Warren 2004). A combination of greater biomass allocation for leaf production and lower investment of N per unit area resulted in faster development of leaves in lemon trees.

The calculated RGR for citrus trees was similar to that reported for perennial coffee plants growing in different environmental conditions (Cavatte et al. 2012), which is lower than described for fast-growing species (Stitt and Zeeman 2012). Considering the relationship between RGR and ULR, it might be argued that greater growth of lemon trees, at least in part, resulted from improved leaf growth or lower C_c, but not from improved photosynthesis. Furthermore, lemon trees were efficient in transforming N assimilated into biomass, even though they had lower recovery values of N per root unit (Figure 5). Thus, the lower investment of lemon trees in root biomass and lower uptake efficiency (NU$_p$E) was compensated by a higher nutrient use (NU$_t$E) compared with orange trees.

Modulation of horticultural responses of canopy varieties by different rootstocks is described in the literature, being associated with modifications in the canopy organization (Mesquita et al. 2016, Tworkoski and Fazio 2016), vigor (Quaggio et al. 2003), responses to nutrient supply (Mattos et al. 2017, Hippler et al. 2016), tolerance to biotic and abiotic stresses (Souza et al. 2017, Hippler et al. 2018), tolerance to water

and cold stress (Santana-Vieira et al. 2016), and fruit yield and quality (Schinor et al. 2013, Bowman et al. 2016). Even so, this study first described the different growth patterns of the Swingle citrumelo rootstock resulting from whether the scion was lemon or the sweet orange variety.

To understand NUE of *Citrus*, this current study firstly demonstrated the response of lemon trees in relation to root and shoot growth, and N partitioning. From this point, further studies on signaling mechanisms related to cytokinins and sucrose transport might allow a more comprehensive explanation of the modulation of tree growth (van der Werf and Nagel 1996, Roitsch and Ehneß 2000, Sakakibara et al. 2006, McAllister et al. 2012).

Photosynthetic nitrogen-use efficiency

Lemon trees exhibited lower N and chlorophyll concentrations in leaves, even though effects observed in different photosynthesis components were not translated into a reduction of CO_2 assimilation. Thus, higher PNUE was observed in this species. Part of this higher PNUE (Figure 6) was due to the improved efficiency of transport and use of light (Table 1, Figures 2 and 3), except during the limiting environmental growth condition found in the hot summer, when high radiation apparently affected P_n and P_d by diffusive and metabolic aspects, and impaired the horticultural advantage observed in the late autumn and early spring.

Despite the lower leaf chlorophyll concentration in lemon trees, those always sustained higher levels of ETR/chlorophyll than sweet orange trees (Figure 7). *Citrus* species exhibit light saturation at 700–800 µmol m^{-2} s^{-1} (Ribeiro et al. 2009). Nevertheless, lemon trees were more sensitive to the combination of high temperature and excess radiation than orange trees, again, indicating the sensitivity to high temperatures (Guo et al. 2006). During the hot summer, lemon trees had lower F_v/F_m and F_v'/F_m', indicating a reduction in the availability of oxidized Q_A, even though not producing chronic photoinhibition (Critchley 1998). Photoinhibition was observed only for plants supplied with low N and in the late autumn, with values lower than 0.72 in both species (Crithley 1998). Noteworthy, increasing N and magnesium levels could hinder such negative effects in lemon trees (Boaretto et al. 2020).

In the hot summer, we observed reduction in the proportion of energy used for photochemical work (qP), according to Baker (2008). These effects, added to the leaf temperature increase and consequent stomatal closure observed in the summer, led to a reduction in rate of growth by unit of leaf (ULR).

Taking into account that 64–68 mol of protein-N constitute the light-harvesting complex II (LHCII) and photosystems are associated with 1 mol of chlorophyll (Evans 1989), as well as the known molecular weights of chlorophyll *a* and *b* (Porra et al. 1989), we could estimate the amount of N in trees related to light-harvesting and electron transport processes. The

amount of N allocated for these components in lemon trees was 1.96 mg of N per g of leaf fresh weight (FW; 16.7% of total N), and for orange trees 2.48 mg of N per g of leaf FW (16.4% of total N). Despite differences in the amount of N allocated to light-harvesting and electron transport, the proportion of total leaf N was similar for both species. These disagree with previous studies that showed positive correlation between SLA and PNUE with reduction in the investment of N in cell walls and proportional increase of N allocation for photosynthesis (Feng et al. 2008). In the current study, there was a proportional reduction of the light-harvesting components, with an increase in their efficiency of use.

P_n and P_d were more affected in lemon than sweet orange trees in the hot and sunny summer as a consequence of stomatal closure and reduction in P_n/C_i with an increase in leaf temperature. Therefore, the reduction in P_n was a result of effects on diffusive, metabolic and photochemical mechanisms in trees. Since the most significant environmental difference in that period was the high radiation intensity, and considering that maximum air temperature was almost 5 °C lower and humidity was 2-fold higher than in the early spring, we can assume that high radiation and photoinhibition associated with lower chlorophyll levels were limiting to photosynthesis in lemon trees.

Metabolizable carbohydrates

The highest concentrations of soluble sugars and starch were observed in branches of lemon trees in July (Figures 8 and 9) compared with oranges, despite no differences in CO_2 assimilation being observed (Figure 6), which suggested that more energy was available for growth of lemons. Coincidentally, the highest growth rate was recorded in P1 (summer–autumn), which positively correlated with starch and soluble sugars in branches ($P < 0.05$) (see Figure S2 available as Supplementary Data at *Tree Physiology* Online).

The concentration of free amino acids correlated with the concentration of N in leaves and with NU_pE, which was lower in lemon trees compared with orange trees. In addition, the increase of free amino acids in the hot summer, together with the increase in soluble sugars in leaves and reduction in growth, could be associated with an increase in photorespiration (Igarashi et al. 2006, Masclaux-Daubresse et al. 2010). Since there was a reduction in the efficiency of N uptake by roots in this period, the increase of free amino acids could be accounted for by remobilization, and not assimilation of new absorbed N.

Conclusion

The higher NUE in lemon trees compared with sweet orange trees was shown to be a multifactorial trait, regulated by the ability of the plant to invest greater proportion of biomass in leaves and with improved nitrogen utilization (NU_tE), even

though nutrient uptake per unit of root (NU_pE) of lemon trees was proportionally lower compared with orange trees. The higher PNUE probably resulted from an increase in the efficiency of chlorophyll use for transportation of energy (ETR/chlor). Furthermore, lemon trees were less sensitive to varying N supply than sweet orange trees, when grown in otherwise favorable environmental conditions. This last conclusion was revealed by maintenance of LA of lemon trees in a limited N environment, in comparison with the decrease for sweet orange trees. However, higher temperatures and severe nitrogen deficiency affected photosynthesis of lemon trees, and led to reduced growth when trees reached a greater size at the end of the experiment.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* online.

Acknowledgments

Conflict of interest

None declared.

Authors' contributions

D.M.J., R.M.B. and V.L.D. formulated the hypothesis, and designed and conducted the study; E.C.M., N.M.E. and V.L.D. implemented methodology for physiological and biochemical analyses; V.L.D. and R.M.B. conducted the experiment and collected data; D.M.J., E.C.M., N.M.E. and V.L.D. conducted data analysis/interpretation, and manuscript writing/revision; R.M.B. and J.A.Q. assisted in manuscript revision.

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