LUCAS DE ÁVILA SILVA

PHYSIOLOGICAL AND METABOLIC SHIFTS ASSOCIATED WITH YIELD-RELATED PARAMETERS IN PEPPER (*Capsicum chinense*) SWAYED BY NITROGEN SUPPLY

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Plant Physiology Graduate Program for the obtention of the degree of *Doctor Scientiae.*

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To my mother Marilda, my father Carlos Antônio (*in memoriam*), my wife Patricia, and my sister Nathália, for the love, encouragement, and support.

I dedicate.

"*I have no idols. I admire work, dedication, and competence*."

Ayrton Senna da Silva

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CONTENTS

ABSTRACT

SILVA, Lucas de Ávila, D.Sc., Universidade Federal de Viçosa, February, 2019. **Physiological and metabolic shifts associated with yield-related parameters in pepper (*Capsicum chinense*) swayed by nitrogen supply**. Adviser: Adriano Nunes Nesi. Co-adviser: Agustín Zsögön.

Among the essential elements for plants, nitrogen (N) is highlighted due to the large amounts uptake and the close relationship between carbon (C) and N metabolism. Despite its importance, little is known about the role of the N supply on physiological and metabolic traits as well as its relation with the yield-related parameters in Capsicum. For this, in this work, the natural variation of fruit set, fruit size, and leaf morphology between two commercial cultivars of *C. chinense*, Biquinho and Habanero, was explored. First, plants of both cultivars were submitted to increasing doses of N. In this study (Chapter 1), both cultivars increased the biomass allocation to leaves in conditions of higher N supply. Plants growing under N-deficiency produced a lower number of flowers and heavier fruits. Contrarily, plants under high N condition tended to decrease their CO_2 assimilation rates, harvest index and fruit weight. The continuous formation of new reproductive sinks was as an important factor to tolerate the N excess. The results suggest that N supply acts on the sucrose supply to different organs and can influence yield-related traits between Capsicum cultivars. Subsequently, the same cultivars were submitted to moderate N deficiency or N sufficiency combined with unshaded or shaded conditions to better understand the role of source strength on fruit set at the metabolic level (Chapter 2). The metabolic balance of different metabolites at source leaves and flowers during the flowering period was assessed. Higher fruit set was mainly related to the export of both sucrose and amino acids from source leaves to flowers. Additionally, starch turnover in source leaves but not in flowers played a central role on the sucrose supply to sink organs at night. As biometric parameters used to evaluate the relationship between the leaf area and mass such as the specific leaf area (SLA) are often related to growth, the behavior of SLA in relation to the N supply was assessed in the Chapter 3. Overall, we observed that both genotypes showed a decrease in SLA according to the increase of N supply. Our results suggest the common SLA

behavior occur due to the role of N metabolism-related metabolites variation, in special amino acids, protein, and starch, influencing the leaf density. However, in the range of moderate N deficiency to N sufficiency, the genotypes showed a distinct response of SLA in relation to the N supply due to the genotypic alterations on palisade and spongy parenchyma. Taken as a whole, the results indicated important metabolic and physiological traits associated with growth- or yield-related parameters of Capsicum plants. However, further studies are necessary to identify genes related to the traits highlighted in the studies presented, using mapping techniques together with populations of landrace accessions, as reviewed in Chapter 4. Following the identification of the genes, important traits can be tested and used in breeding programs of Capsicum and other plant species.

RESUMO

SILVA, Lucas de Ávila, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Mudanças fisiológicas e metabólicas associadas a parâmetros relacionados à produtividade em pimenta (*Capsicum chinense*) influenciadas pelo suprimento de nitrogênio**. Orientador: Adriano Nunes Nesi. Coorientador: Agustín Zsögön.

Entre os elementos essenciais para as plantas, o nitrogênio (N) tem destaque devido à grande quantidade absorvida e à estreita relação entre o metabolismo do carbono (C) e N. Apesar da importância, pouco se conhece sobre o papel do suprimento de N sobre as características fisiológicas e metabólicas bem como sua relação com os parâmetros relacionados à produtividade de Capsicum. Para isso, neste trabalho, explorou-se a variação natural da frutificação efetiva, tamanho de frutos e morfologia foliar entre duas cultivares comerciais de *C. chinense*, Biquinho e Habanero. Primeiramente, plantas dos dois cultivares foram submetidas a doses crescentes de N. Nesse estudo (Capítulo 1), ambos cultivares aumentaram a alocação de biomassa para folhas em condições de maior oferta de N. Plantas crescendo sob deficiência de N produziram menor número de flores, porém frutos mais pesados. Por outro lado, plantas sob maiores doses de N tenderam a diminuir sua taxa de assimilação de CO_2, índice de colheita e peso dos frutos. A contínua formação de novos drenos reprodutivos mostrou-se como um importante fator na tolerância ao excesso de N. Os resultados sugerem que o suprimento de N atua na partição da sacarose para diferentes órgãos e pode influenciar as características relacionadas ao rendimento entre cultivares de Capsicum. Posteriormente, os dois cultivares foram submetidos a moderada deficiência de N ou suficiência de N combinado com condições não sombreadas ou sombreadas para melhor compreender o papel da força da fonte na frutificação efetiva à nível metabólico (Capítulo 2). Avaliou-se o balanço metabólico de diferentes metabólitos nas folhas fontes e flores durante o período de floração. A maior frutificação efetiva foi relacionada principalmente à exportação de sacarose e aminoácidos das folhas fonte para as flores. Além disso, o *turnover* de amido nas folhas fonte, mas não nas flores, teve um papel central no suprimento de sacarose para órgãos dreno à noite. Como parâmetros biométricos utilizados para avaliar a relação entre a área foliar e a massa, como área foliar específica (AFE), são

frequentemente relacionados ao crescimento, avaliou-se a relação entre AFE e suprimento de N no Capítulo 3. No geral, observou-se que os dois cultivares utilizados apresentaram uma diminuição na AFE de acordo com o aumento da oferta de N. Os resultados sugerem que o comportamento comum da AFE ocorre devido à variação de metabólitos relacionados ao metabolismo do N, em especial o nível de aminoácidos, proteína e amido, influenciando a densidade foliar. No entanto, na faixa de deficiência moderada de N até a suficiência de N, os cultivares apresentaram uma resposta distinta da AFE em relação ao suprimento de N em função das alterações genotípicas no parênquima esponjoso e paliçádico. De modo geral, os resultados indicaram importantes traços metabólicos e fisiológicos associados com parâmetros relacionados à produtividade ou crescimento de plantas de Capsicum. Entretanto, novas pesquisas são necessárias para identificar os genes relacionados com os traços destacados nos estudos apresentados, fazendo uso de técnicas de mapeamento juntamente com populações naturais ou crioulas, como revisado no Capítulo 4. A partir da identificação dos genes, traços importantes poderão ser testados e utilizados em programas de melhoramento de Capsicum e outras espécies de plantas.

GENERAL INTRODUCTION

Capsicum species are considered as important economic and nutritional group of plants worldwide (Pickersgill 1969; Eshbaugh 1975, Rufino & Penteado 2006). *Capsicum* ssp. have been used for fresh consumption, spices, dyes, and medicinal purposes. The global production of fresh fruits was estimated at 34.5 million tons cultivated in 1.9 million hectares in 2016 (*www.fao.org*). Bolivia is considered as the center of origin of Capsicum genus (Eshbaugh 1993). The species from this genus spread through the pre-Holocene Americas mainly via dispersal by birds or river flows (Nimmakayala *et al.* 2016). About 32 species are reported in the genus, among them, only *C. annuum*, *C. assamicum*, *C. baccatum*, *C. frutescence*, *C. chinense,* and *C. pubescens* are domesticated and cultivated (Smith & Heiser 1957; Ramchiary *et al.* 2014). Domesticated forms probably appeared in early agricultural sites in the coastal of Peru and Mexico (Pickersgill 1969, 1997; Davenport 1970; Jarret & Berke 2008; Kraft *et al.* 2014). Currently, *C. annuum* and *C. chinense* have been widely utilized on a global scale and they are considered the most cultivated peppers (Eshbaugh 1975, 1993; DeWitt & Bosland 1996).

Brazil is one of the most important biodiversity hotspots of domesticated and wild species of Capsicum (Barboza & Bianchetti 2005). In particular, the Amazon region seems to be the major area of biodiversity for *C. chinense* (Pickersgill 1971; McLeod *et al.* 1983; Reifschneider 2000). *C. chinense* has a great genetic diversity resulting in a vast variety of size, color, shape, and pungency levels in fruits (Jarret & Berke 2008; Finger *et al.* 2010; Baba *et al.* 2016; Moreira *et al.* 2018). Furthermore, this species has a considerable natural variation for metabolic and physiological traits (Rosado-Souza *et al.* 2015).

Natural variation is an important tool to assess new insights into the regulation of physiological and biochemical networks as well as their correlations with phenotypic traits (Cross *et al.* 2006; Keurentjes & Sulpice 2009; Driever *et al.* 2014; Rosado-Souza *et al.* 2015; Nunes-Nesi *et al.* 2016). For example, arabidopsis growth traits have been related to starch content at the end of the light period (Sulpice *et al.* 2009) and to ATP consumption associated with protein turnover (Ishihara *et al.* 2017). Indeed, there is a well-known relationship between yield and physiological traits (Long *et al.* 2006; Fischer 2007; Zhang *et al.* 2013; Furbank *et al.*

2015) as well as a close link between growth and metabolism (Schauer *et al.* 2006, 2008; Lisec *et al.* 2008; Fernie & Schauer 2009; Riedelsheimer *et al.* 2012; Chen *et al.* 2016; Fusari *et al.* 2017; Kumar *et al.* 2017).

Exploring a panel of 49 *C. chinense* accessions, some physiological and metabolic traits correlated with yield- and growth-related parameters (Rosado-Souza *et al.* 2015). N is one of the nutrients that most influence plant growth and yield (Ohyama 2010). Although N has a key role in pepper fruit yield (Stroehlein & Oebker 1979; Johnson & Decoteau 1996; Medina-Lara *et al.* 2008), there are no studies exploring the N effect on physiological, metabolic and yield-related parameters. On the other hand, the shading effect on physiological, metabolic and some yield-related parameters is better characterized (Turner & Wien 1994; Aloni *et al.* 1996). Here, I worked with different N supply and occasionally shading conditon to better understand the physiological and metabolic shifts associated with yield-related parameters in this species.

Previous studies have evaluated photosynthesis and partition of C metabolism-related metabolites in sink organs of contrasting *C. annuum* genotypes in terms of flower abscission rate to explain the fruit set behavior under shading (Turner & Wien 1994; Aloni *et al.* 1996). These studies indicated that partitioning of photoassimilates to flower is an important trait related to fruit set. Based on these studies, I evaluated gas exchange, chlorophyll *a* fluorescence parameters, C and N metabolism-related metabolites in sink and source organs to explain the variation in yield-related parameters such as number of flowers, fruit set, and individual fruit weight according to the N supply. For this, I used two *C. chinense* cultivars Biquinho and Habanero, contrasting in terms of fruit set, fruit size and leaf morphology.

In the Chapter 1, I evaluated the differences between source and sink tissues of Biquinho and Habanero plants under increaseasing N doses to gain insights into the effect of N supply on photosynthesis, carbon allocation, and yield-related traits of *C. chinense*. As fruit set is a yield-related parameter with huge variation among Capsicum cultivars, in the Chapter 2 I focused on the metabolic shifts related to fruit set. After, due to the natural variation in leaf morphology, I further investigated the effect of the N supply on the specific leaf area (SLA) of both cultivars via determination of primary metabolites and anatomical analysis. Last of all, the integration of metabolomic approaches and mapping methods were reviewed aiming the identification of metabolic quantitative trait loci (mQTL). I believe that these set of studies presented here will contribute to understanding the source-sink relation of Capsicum (Sonnewald & Fernie 2018) as well as revealing interestingly physiological and metabolic traits for breeding programs (Fernandez *et al.* 2016).

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CHAPTER 1

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THE TRADEOFF BETWEEN PHOTOSYNTHESIS, CARBON ALLOCATION, AND YIELD-RELATED TRAITS IN CHILI PEPPER IN RESPONSE TO NITROGEN

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HIGHLIGHTS:

- A Capsicum cultivar with higher fruit set and lower fruit size is less affected by excess N;
- Vegetative and reproductive organs play important roles in responses to N supply;
- Reproductive sink strength is an important factor for plant tolerance to NH_4^+ toxicity.

ABSTRACT

Different from other species, yield-related traits of *Capsicum chinense* are highly dependent on coordination between vegetative and reproductive growth, since the formation of new reproductive tissues occurs in new sympodial bifurcations. In this study, we used two *C. chinense* cultivars (Biquinho and Habanero), contrasting for fruit size and fruit set, to investigate the responses of nitrogen (N) deficiency and excess on growth, photosynthesis, carbon (C) and N metabolisms as well as yield-related traits. Both cultivars increased the biomass allocation to leaves in conditions of higher N supply and exhibited a parabolic behavior for fruit biomass allocation. Plants growing under N-deficiency produced a lower number of flowers and heavier fruits. Contrarily, plants under high N condition tended to decrease their CO_2 assimilation rate, harvest index and fruit weight. Biquinho, the cultivar with lower fruit size and higher fruit set, was initially less affected by excess of N due to its continuous formation of new reproductive sinks in relation to Habanero (which has lower fruit set and higher fruit size). The results suggest that N supply acts on the sucrose supply to different organs and can differentially influence yield-related traits between Capsicum cultivars with contrasting source-sink relations.

Key words: fruit set, yield, sucrose, flowering, metabolism, ammonium

1. INTRODUCTION

Efficient use of nitrogen (N) is a key challenge in global agriculture [1]. World agriculture has succeeded in increasing three-fold the production of plant proteins in the past half century, however synthetic N fertilizer input increased nine times during the same period [2,3]. Thus, N use efficiency is globally decreasing, while it is expected that global N fertilizer consumption will increase around 2.7 fold by 2050 to sustain food demand [3–6]. In the current scenario, approximately 2% of world energy use is dedicated to the industrial production of N through the Haber-Bosch process [7]. A remarkable imbalance exists on the distribution of N-containing fertilizers, which are in excess in some regions, leading to environmental pollution, while in other regions the supply is not sufficient to support crop requirements and thus close the yield gap [1,7,8].

At the whole plant level, N and C metabolisms are highly interconnected and mutually regulated [9,10]. N is a key constituent of major biomolecules, such as nucleotides, chlorophylls, amino acids and proteins. In leaves, more than 60% of total N is allocated to proteins of the photosynthetic apparatus [11] and up to 75% of N is found in the chloroplasts of mesophyll cells [12–14]. N nutrition can also influence fruit and seed quality, mainly by altering the quantity and proportion of proteins and amino acids, but it can also alter the content of vitamins, soluble sugar, and soluble solids in horticultural crops [15]. Plants grown under severe N deficiency often contain lower levels of nitrate and N containing compounds such as amino acids and protein [16,17]. N deprivation leads to alterations in C metabolism, such as decreased levels of malate and other organic acids and increased levels of starch [16,17] and thus promotes drastic metabolic changes that severely affects crop yield [18,19]. Moreover, N-deficiency impact crop development with an acceleration of leaf maturation and senescence [20].

Unlike N-deficiency, the effects of N excess are poorly understood [21]. Excess of N is mainly characterized by ammonium (NH_4^+) toxicity symptoms such as leaf chlorosis, ion imbalance, hormone deregulation, disorder in pH regulation, changes in metabolite levels, reduced photosynthetic rate and biomass production [21–26]. Plants can use different strategies to counteract NH_4^+ toxicity symptoms, *e.g.* by enhancing its metabolic assimilation, increasing its efflux outside the cells or its sequestration within the vacuole [22,27].

Generally, plant yield is reduced under N deficiency, and it increases linearly with the supply of N until it reaches a plateau defined by the genetic potential [28]. In pepper (*Capsicum* spp.), fruit yield is strongly influenced by N supply [29–31]. N-deficiency leads to a decrease in biomass production and lower number of flowers, fruits and consequently lower fruit yield [29,30,32]. On the other hand, N-excess decreases the number of flowers and fruits, and thus fruit yield [30,31]. However, biomass production in plants under N-excess is variable and can decrease, stabilize or increase [29,31,32]. Genotype-dependent fruit size variation is also an important parameter influencing fruit set and yield. Fruit set of cultivars with smaller fruits is higher compared to cultivars with bigger fruits; in addition, there is a strong correlation between the relative fluctuations in fruit set and fruit yield [33]. In cultivars with large fruits, new flowers set fruit depending on the competition between fast-growing fruits while cultivars with small fruits have high fruit set although fast-growing fruits are always present [33–35].

Considering that in pepper plants the formation of new reproductive tissues is highly dependent on the vegetative growth, because flowers and fruits are produced in new sympodial bifurcations [36], and that fruits allocate as much as 29% of the total N [30], we examined the association between N supply and yield related traits. We hypothesized that responses to N supply displayed by Capsicum are a function of the fruit number and size displayed by the plant. Hence, to address this question we used two *C. chinense* cultivars contrasting for fruit set and fruit size to assess the relation between the N supply and yield-related parameters. We investigated physiological traits such as photosynthetic parameters, C and N allocation and assessed their impact on yield-related parameters. In addition, metabolic analysis was performed in flowers, sink and source leaves to explore the N responses displayed by Capsicum plants.

2. MATERIAL AND METHODS

2.1.Plant growth conditions

Seeds of Biquinho and Habanero, obtained from TopSeed®, were sown and grown for 42 days on a commercial substrate (Topstrato® HT). The seedlings were then transplanted to pots containing 5 dm^3 of a Yellow-Red Oxisol soil. Before transplantation, the soil used for cultivation was sieved, homogenized and its acidity neutralized by applying $CaCO_3$ and $MgCO_3$ (molar ratio of 3.5:1). The soil was fertilized with nutrients according to Novais, Neves & Barros (1991), with the exception of N. The total amount of nutrients was 300, 150, 40, 0.81, 1.33, 1.55, 3.66, 0.15 and 4.00 mg dm^{-3} for P, K, S, B, Cu, Fe, Mn, Mo, and Zn, respectively. Due to the low N content of the soil, 5 mg dm^{-3} of this nutrient was applied as NH_4NO_3 to ensure the initial development of the plants after transplantation. To avoid leaching of N from the soil, plastic bags were used to cover the base of the pots.

Following the development of the first sympodial unit, which corresponds to the reproductive differentiation, we applied 0, 20, 50, 125 and 312.5 mg dm^{-3} of N, using NH_4NO_3 as N source. NH_4NO_3 was supplied every twenty days based on Pinto et al. (2006), so five times till the final harvest (Fig. S1). The experiment was performed in a greenhouse with a temperature of 25 ± 5 °C and an average irradiance of 500 µmol photons m^{-2} s^{-1}. The photoperiod was 12h daylight / 12h night and water was supplied daily.

2.2. Plant biometric growth parameters and yield traits

To estimate the growth parameters plant height was measured weekly. In addition, all flowers were labelled within two or three days after anthesis during the flowering period in order to follow the rate of production of flowers and fruit set. At 91 days after flowering (DAF) fruits, leaves, branches, and roots were harvested and dry mass was determined as previously described [39]. At the same time, the number of fruits and the fresh weight of fruits per plant were determined. To compare the difference between older leaves (formed before reproductive differentiation) and younger ones (formed after reproductive differentiation), leaves from above and below the first sympodial unit were harvested separately and the leaf area was measured using a planimeter (Li 3100C Li-Cor, Inc., Lincoln, NE, USA). Harvest index (HI) was determined as the fraction of the dry weight of fruits in relation to the total plant dry biomass [40].

2.3. Measurements of photosynthetic parameters

Since the second fertilization, gas exchange and chlorophyll *a* fluorescence analyses were performed five days after each fertilization (Fig. S1). All parameters were measured on fully expanded leaves of the second sympodial unit from the apex (Fig. S2). Gas exchange analyses were performed using a portable infrared gas analyzer (Li 6400XT, Li-Cor, Inc., Lincoln, NE, USA) equipped with an integrated fluorescence chamber under standard conditions (flow rate: 300 µmol s^{-1}, light intensity: 1000 µmol photons m^{-2} s^{-1}, block temperature: 25 °C, reference [CO_2]: 400 ppm and relative humidity: 60%) All measurements were performed on attached leaves from 8:00 to 12:00 a.m. in the greenhouse. Dark respiration (R_d) was determined after at least two hours into the dark period, on the same leaf previously used to determine the net carbon assimilation (A).

Chlorophyll-*a* fluorescence parameters were determined in leaves during the light period, as estimated minimum fluorescence of light-adapted leaves ($F_0' = F_0/(F_v/F_m + F_0/F_{m'})$ [41], maximum fluorescence of light-adapted leaves ($F_{m'}$), apparent electron transport rate (ETR = Φ_{PSII} x RFA x 0.5 x 0.84) [42], effective quantum yield of PSII (Φ_{PSII}) [42], photochemical extinction coefficient (qL = ($F_{m'}$-F) x $F_{0'}$/($F_{m'}$-$F_{0'}$) x F) [43], quantum dissipation yield ((Φ_{NPQ} = (F/$F_{m'}$) - (F/F_m)) and quantum efficiency of PSII uncontrolled energy dissipation (Φ_{NO} = F/F_m). Φ_{NPQ}, Φ_{PSII}, and Φ_{NO} were calculated considering $\Phi_{NPQ}+ \Phi_{PSII}+\Phi_{NO}=1$ [42–44]. In addition, minimum (F_0) and maximum (F_m) fluorescence in leaves adapted to the dark were determined at night, and potential quantum yield of PSII (F_v/F_m) was calculated [45].

2.4.Total N and C levels and metabolite analyses

At the end of the experiment (91 DAF), the total N and C levels were determined in dried leaf samples [46]. To compare the difference between older leaves (formed before reproductive differentiation) and younger ones (formed after reproductive differentiation), the total C and N levels were determined in leaves above and below the first sympodial unit (upper and lower leaves), at the exception of the plants under N deprivation where there was not sufficient amount of material for the analyses. The total N amount was calculated by multiplying the N content by the foliar dry weight as adapted from Peoples et al. (1989).

For metabolite analyses source and sink leaf samples were harvested 24 hours before the second and the fourth N fertilization, always in the middle of the light period. The first fully expanded leaves of the third sympodial unit from the apex, were considered as source leaves. In contrast, young leaves, considered only those not yet fully expanded, were treated as sink leaves [48]. In addition, samples from flowers were collected 2 days after the anthesis, at noon time and before the fourth fertilization (Fig. S2). All samples were snap frozen in liquid nitrogen and stored at -80 °C.

Metabolite extraction was performed by grinding the tissue in liquid nitrogen followed by the determination of the sample fresh weight and ethanol extraction [49]. In the ethanol soluble extracts we determined sucrose, fructose, glucose [50], malate [51], NH_4^+ [22], total amino acids [49], nitrate [52], chlorophyll *a* and *b* [53]. Starch and protein levels were determined in the ethanol insoluble fraction, as previously described [49].

At 88 DAF, three and six discs of 1.03 cm^2 were collected from source leaves below and above the first sympodial unit, respectively for the determination of electrolyte leakage [54].

2.5.Statistical analyses

The experiment was performed in a completely randomized block design with five replicates, considering the factorial accession (F1) versus N dose (F2). The normality of the data was verified by the Shapiro-Wilk test after the variables were analyzed by two-way ANOVA. Separation of significant mean differences was achieved by Tukey's test at $P \leq 0.05$ using the software GENES [55]. The graphs, regression analyses and Pearson's correlations were performed using Sigma Stat software v.2.0 (SPSS Inc., Chicago, IL, USA). F and *t*-test were performed to validate the regression models and coefficients, respectively.

3. RESULTS

3.1.Leaf area and biomass allocation

Total leaf area (LA) increased steadily with increasing N doses for Habanero but peaked at 50 mg dm^{-3} for Biquinho and decreased at 312.5 mg dm^{-3} (Fig. 1A). A higher proportion of leaf area was produced after flowering in Biquinho than in Habanero (Fig 1B). Plants under no N supply had similar behavior in biomass allocation (Fig 2A). The cultivars were contrasting in fruit and root biomass allocation (Fig 2A). In general, Biquinho had higher biomass allocation in fruits and lower biomass allocation in roots than Habanero. Biquinho also had higher relative biomass allocation in fruits while Habanero had higher relative biomass allocation in leaves and roots (Fig. 2B). However, both cultivars increase their relative biomass allocation in leaves with increasing N doses (Fig. 2B). On the other hand, both cultivars decrease their relative biomass allocation in root with increasing N doses (Fig. 2B). Both cultivars showed highest vegetative development until 10 weeks after planting (Fig. S3 and S4).

3.2.C and N levels in leaves

The C level in lower leaves (formed before the reproductive differentiation) did not differ between treatments (Fig. 3A and B). In upper leaves (formed after the reproductive differentiation), there were no differences between cultivars, but significant changes were observed among the N doses. In Biquinho, C level increased in plants under 0 to 50 mg of N dm^{-3} and remained invariable for higher N doses. In Habanero, a different pattern was observed, C level increased in plants under 20 to 125 mg of N dm^{-3} and stabilized. N level in leaves of the lower and upper parts of the plants was different between the cultivars only at 125 mg of N dm^{-3} (Fig. 3C and D). As expected, N levels increased and the C:N ratio decreased with the increase in N supply (Fig. 3C, D, E and F). Interestingly, leaves from the upper part of the plants exhibited higher N level than lower leaves in both cultivars.

The percentage of N present in the upper part leaves was significantly higher in Biquinho than in Habanero plants for all N doses (Fig. 4A). The largest differences were observed for plants not supplied with N after transplantation. In Habanero leaves, the percentage of N was highest for the 50 mg dm^{-3} N dose. In Biquinho plants, the percentage of N present in leaves formed after the beginning of the flowering period did not vary between N doses, at the exception of N deprivation

To evaluate possible oxidative damage caused by the limitation or excess of N, we further evaluated electrolyte leakage in leaves (Fig. 4B). Globally, N doses had no impact on electrolyte leakage and no differences were observed between both cultivars, with the exception of leaves from Biquinho plants grown under the highest N dose, which showed higher electrolyte leakage. Under N deprivation, the two cultivars differed in leaf abscission, with drop for Biquinho leaves developed before the flowering while Habanero plants kept all leaves over their life cycle. Leaves also fell in Biquinho plants under the highest N supply after the fifth N fertilization (Fig. S5, S6, S7, and S8).

3.3.Fruit related traits

Under N deprivation neither cultivar produced significant amounts of flowers and fruit set was thus null. For other N doses, the number of flowers and fruits was significantly lower in Habanero than in Biquinho plants (Fig. 5A and B; Fig. S9 and S10). Flower number, fruit number and fruit set responded to N fertilization in Biquinho but not in Habanero plants (Fig 5A-C). In contrast, Biquinho fruit fresh weight was lower in comparison with the fruits of Habanero plants for all N doses (Fig. 5D). Both cultivars exhibited lower fruit weight with increased N doses. Total yield per plant was higher in Biquinho than in Habanero and strongly influenced by N level (Fig 5E). Yield decreased considerably in plants from both cultivars when subjected to the highest N dose. Harvest index showed a similar response as fruit yield for both cultivars (Fig 5F).

Lastly, regression analyses were performed for all yield-related parameters in response to the applied N doses (Table S1). The number of flowers, number of fruits, fruit set, fresh weight of fruits and harvest index in both cultivars were best explained by quadratic models (Fig. S11). Nevertheless, in both cultivars, N supply and individual fruit weight were negatively related in a linear manner, indicating that the increase in N dose reduces the individual fruit weight (Fig. S12). Altogether, these analyses revealed that the optimal theoretical N doses for the yield of fresh fruits were 74.75 and 51.50 mg of N dm^{-3} for Biquinho and Habanero, respectively.

3.4.Gas exchange and chlorophyll a fluorescence parameters

Given the large differences in yield-related parameters of the cultivars when grown under different N doses, we next analyzed the effect of different N doses on the photosynthetic rates of plants from both cultivars. At 65 DAF, Biquinho plants exhibited higher net CO_2 assimilation rates (*A*) than Habanero, regardless of N dose (Fig. 6A). Both cultivars showed the same behavior for *A* in response to changes in N doses. As expected, the lowest *A* values were observed for plants grown under N deprivation. But low values were also observed under N excess condition (312.5 mg of N dm^{-3}). The highest *A* values were observed in plants under 20 and 50 mg of N dm^{-3}. In general, Biquinho plants also displayed higher photosynthetic rates than Habanero during all cultivation period (Table S2 and Fig. S13), suggesting a higher photosynthetic capacity. It was also observed that *A* gradually decreased over time in Biquinho plants under N deprivation and N excess conditions, while in Habanero plants lower *A* values were already observed 25 DAF under the same conditions

(Table S2 and Fig. S13). Similar behaviors were observed for stomatal conductance (g_s) (Fig. 6B, S13 and Table S2), suggesting that the observed changes in A were determined by g_s (Fig. 9).

We then evaluated chlorophyll fluorescence parameters. The decrease in A and g_s observed in Biquinho leaves of plants grown under N deficiency and N excess was matched by similar decreases in some of the fluorescence parameters (Table S2), *e.g.* the quantum efficiency of open PSII reaction centers (*Fv'/Fm'*) at 65 DAF (Fig. 6C). In both cultivars the lowest *Fv'/Fm'* was observed in leaves from plants grown under N deprivation, followed by the values observed for the highest N dose, indicating that under these two extreme conditions plants could be experiencing oxidative stress. Biquinho plants displayed similar *Fv'/Fm'* values under 20, 50 and 125 mg of N dm^{-3}, while in Habanero the highest value was observed under 50 mg of N dm^{-3}. The maximum quantum yield of PSII (*Fv/Fm*) did not vary in Biquinho plants regardless of N dose but decreased in Habanero plants under no N supply, 125 and 312.5 mg of N dm^{-3} (Fig. 6D).

Dark respiration (R_d) and electron transport rate (*ETR*) showed little variation in response to N doses for both cultivars (Table S2 and Fig. S13). Biquinho plants under higher N supply (125 and 312.5 mg of N dm^{-3}) showed lower R_d values than Habanero plants over time. *ETR* was highest in Biquinho plants grown under 20, 50 and 125 mg of N dm^{-3} and only decreased at 85 DAF for the plants grown under 20 mg of N dm^{-3}. In Habanero, the *ETR* was highest at 25 DAF for plants grown under 125 mg of N dm^{-3} but decreased during the crop. For all other time points, Habanero plants grown under 50 mg of N dm^{-3} displayed the highest *ETR*.

3.5.Nitrogen metabolism related metabolites in leaves and flowers

To gain more insight into the N allocation in leaves and flowers, we next decided to extend this study to the major compounds involved in N metabolism. In both cultivars, with the increase in N doses, NH_4^+ content increased in the source and sink leaves as well as in flowers (Fig. 7A and B). NH_4^+ content in these three organs was higher in Habanero plants than in Biquinho plants at 125 and 312.5 mg of N dm^{-3} doses. In Habanero, the differences were more pronounced in source leaves. NO_3^- levels were low in both cultivars for doses up to 50 mg of N dm^{-3} (Fig. 7C and D). At the dose of 125 mg of N dm^{-3}, higher levels of NO_3^- were found in source and sink leaves of Habanero plants; at the dose of 312.5 mg of N dm^{-3} there was a higher accumulation of NO_3^- in the source leaves of Biquinho compared to Habanero. In flower tissues, there was no significant difference in the levels of NO_3^- between the cultivars, with exception of Habanero plants under the highest N dose that showed higher accumulation.

Marked differences were observed for the amino acid levels between both accessions. In plants grown under N-deprivation or under low N supply (20 mg of N dm^{-3}), higher content of free amino acids was observed in Biquinho than Habanero leaves (Fig. 7E and F). Plants under 20, 50 and 125 mg of N dm^{-3} showed higher amino acid content in Biquinho flowers. In plants under 125 and 312.5 mg of N dm^{-3} higher amino acid contents were observed in Habanero source leaves, but no differences were observed for sink leaves.

Before the second fertilization, the protein content in Biquinho was the same for all N doses in source and sink leaves, but steadily increased in Habanero with increasing N doses (Fig. S14). Protein contents in Biquinho and Habanero before the fourth fertilization only varied in the source leaves of plants under the highest N dose and sink leaves of plants under 125 and 312.5 mg of N dm^{-3} (Fig. 7G and H). There was no significant change in protein content in flowers (Fig. 7G and H). Concerning levels of chlorophylls *a* and *b* determined in source (Fig. 7I, 7J, S15) and sink (Fig. S16) leaves, before the second fertilization (19 DAF), Habanero showed lower values of chlorophyll *a* in source leaves of plants under low N supply. Habanero also had lower values of chlorophyll *b* for all N doses (Fig. S15). Before the fourth fertilization (59 DAF), chlorophyll *a* and *b* contents in source leaves were the same in both accessions for doses up to 50 mg of N dm^{-3}, and lower in Habanero for 125 and 312.5 mg of N dm^{-3} doses (Fig. 7I and J).

3.6. Carbon metabolism related metabolites in leaves and flowers

Variations in glucose and fructose levels were observed between both cultivars, mainly in leaves. Biquinho showed higher levels of glucose than Habanero whilst Habanero showed more fructose (Fig. 8A, B, C and D). Sucrose levels differed in all organs between the two cultivars (Fig. 8E and F). Before the second fertilization, there was already a difference for all N doses, which was more pronounced in sink leaves of Biquinho plants, which exhibited higher sucrose content than Habanero (Fig. S17). Before the fourth fertilization, higher levels of sucrose were observed in Biquinho than in Habanero for all N doses in sink leaves as well as in flowers of plants growing under up to 20 mg of N dm^{-3}. Sucrose levels in source leaves of plants from both cultivars were much more stable than in sink leaves, decreasing slightly for the 312.5 mg of N dm^{-3} dose compared to other N doses for Biquinho, whilst the lowest levels were observed for the N deprived treatment in Habanero. As the N dose increased for both cultivars, sucrose levels also increased in sink leaves, while in flowers the levels remained unaltered. A regression could be adjusted to describe the sucrose content in sink leaves ($P<0.01$) while in flowers sucrose content was constant in both cultivars under different N supply (Fig. S18).

Starch levels in source and sink leaves at 19 DAF decreased with the increase in N dose, in both cultivars (Fig. S17). In source leaf tissues of Habanero plants growing under 0, 20 and 50 mg dm^{-3} of N, starch levels were higher than in Biquinho. A similar pattern was observed at later time points, before the fourth fertilization (Fig. 8G and H). For both time points, Biquinho sink leaves had the same or more starch than source leaves, in contrast to Habanero plants where sink leaves contained less starch than source leaves. The starch content in flowers was less affected by the applied N doses. The starch content in flowers of Biquinho plants was higher than in flowers of Habanero plants under 50, 125 and 312.5 mg of N dm^{-3}. A regression could be adjusted to describe the starch content in sink leaves for both cultivars under different N supply ($P<0.01$) (Fig. S19). In flowers, a linear regression could be adjusted for Biquinho to describe the starch content ($P<0.05$) while it was constant to Habanero.

Since malate is known as carbon source related to growth [56,57], the levels of these organic acid were evaluated in all three organs. Clear differences in malate levels were observed between cultivars (Fig. 8I and J). Malate levels were remarkably higher in source leaves and flowers of Biquinho plants. In flowers from both accessions, the levels of malate were increased in plants grown under 20 mg of N dm^{-3} than under higher N doses. Otherwise, malate levels were similar in sink leaves of both accessions for all N doses. They were also similar in source leaves of Habanero plants, but not in source leaves of Biquinho where they were reduced in plants under 125 and 312.5 mg of N dm^{-3}. A power regression could be adjusted to describe the malate content in sink leaves ($P<0.01$) while the malate content in flowers was constant for both cultivars under different N supply (Fig. S20).

3.7.Pearson correlation analysis of some evaluated traits

Lastly, we highlighted some evaluated traits (relative dry weight of leaves, N content in upper leaves, the yield of fresh fruits, A and g_s) to confirm some of their relationships using all combinations between cultivars and N doses. The relative dry weight of leaves correlated positively with the increment of the N content in upper leaves (Fig. 9A). The A correlated positively with g_s and yield of fresh fruits (Fig. 9B and C). In addition, g_s correlated with the malate content in source leaves (Fig. 9D).

4. DISCUSSION

4.1.Biomass responses to N dose in C. chinense cultivars

In this study we demonstrated that Biquinho and Habanero cultivars exhibit differences in plant architecture and allocation of C and N between young and old leaves. Habanero plants grown under N-excess condition were smaller with compact architecture (Fig. 2 and S6) whereas in Biquinho plants, the source leaves fell off after the last fertilization, most likely as a consequence of toxic NH_4^+ effects observed in these plants (Fig. 1, 2, S5, S7 and S8). On the other hand, high N supply led to accumulation of NH_4^+ and NO_3^- in Habanero plants. NH_4^+ and NO_3^- generally accumulated in source leaves of plants under the highest N dose, suggesting these ions were sequestrated within the vacuole and enhanced their metabolic assimilation in free amino acids to avoid the possible toxicity symptoms [9,22,27]. It has been suggested that accumulation of free amino acids can be associated with carbohydrate limitation due to the higher demand of C skeletons, reducing equivalents and ATP for NH_4^+ assimilation [9,22,24]. In agreement, in this study, we observed accumulation of amino acids in leaves of plants grown under the highest N dose, in particular in the source leaves of both cultivars, suggesting a limited supply of carbohydrates to sink tissues. Interestingly, the accumulation of amino acids in source leaves was prominent in Habanero, but much less in Biquinho plants. Moreover, in Biquinho plants, the level of NH_4^+ in leaves and flowers remained lower than in Habanero plants, suggesting that Biquinho could cope with high N doses, at least during the first part of the crop. In addition, sucrose levels were much higher in the flowers of Biquinho than those of Habanero, again suggesting that high N doses were more tolerated by Biquinho than Habanero, at least during the first weeks of the crop. Indeed, photosynthesis was maintained for longer a period in Biquinho over the life cycle (Fig. S13) and thus, ensuring a higher production of C blocks to supply the reproductive organs with both carbohydrates and amino acids. Several studies suggest that a larger number of reproductive sink tissues have higher N and C demand [58–60]. Indeed, pepper fruits are an important N sink since they can allocate up to 29% of the total N [30]. However, this alleviation of N excess did not last for the whole plant growth period and the yield in Biquinho was also strongly affected by N excess (Fig. 3). Interestingly, this reduction in yield was not explained by fruit drop, but by a sharp decrease in fruit weight, which suggests an impairment in C and N supply to the fruits towards the end of the plant life cycle.

It was also verified that N-deficiency affected markedly leaf biomass and fruit production of both cultivars. Other studies have indicated that N-deficiency results in accumulation of carbohydrates in leaves and higher carbon allocated to roots, leading to an increase of the root-to-shoot biomass ratio [61–63]. Higher carbon allocation to roots probably results from continual growth of this organ to access N in the soil solution [19], which is linked to the content and signaling role of sugars and NO_3^- in both shoots and roots [64–66]. Plants under low N condition display decreased growth rate, likely due to an insufficient amino acid pool required to sustain protein synthesis necessary for the formation of new tissues, and as a result show increased starch content in leaves [19,67]. In addition to the growth reduction, similar to the perennial species [68], Capsicum might prioritize the C storage over growth to ensure the survival for a long-term, as suggested by the starch reduction over time in plants under N-deprivation (Fig. 6 and S17). Hence, the cultivars displayed differences in their biomass allocation; however, both increased their relative biomass allocation in leaves with increasing the N doses (Fig. 2 and 9). N deficiency was marked by biomass decrease and starch accumulation in leaves to both cultivars. On the other hand, the high N supply led an accumulation of NH_4^+ in tissues and increased the vegetative growth for both cultivars.

4.2.Biquinho and Habanero have contrasting photosynthetic performance

There is a large natural variation in *C. chinense* for photosynthetic and chlorophyll *a* fluorescence parameters [39], which is also observed in other Solanaceae species [69]. In this study, we observed that Biquinho plants have higher A and g_s than Habanero (Fig. 6, S13, and Table S2), for all N doses. Since the cultivars showed similar R_d (Table S2, Figure S13), these results suggest higher source strength in Biquinho than Habanero [48]. This higher A might be related to the high g_s, as both traits followed the same pattern for all N doses (Fig. 6, 8 and 9). These results suggest that the higher *A* observed in Biquinho plants might be partially related to increased stomatal aperture. Interestingly, in this study there was a positive correlation between malate content in source leaves and g_s (Fig. 9), indicating this metabolite may be an important influencer of the source strength. This is in close agreement with previous studies suggesting that malate plays a key regulatory role in stomatal function [51,70–72]. In Arabidopsis, the malate transport from apoplast and its accumulation in guard cells have been associated with stomatal opening due to an increased in osmotic pressure [70,72–75]. Thus, despite the fact that this association was not observed in other accessions of *C. chinense* [39], the obtained results led us to hypothesize that malate is a key metabolite for stomatal regulation also in *C. chinense*.

In agreement with our results (Fig. 9) photosynthetic rates and sink strength, represented by the fruits, are closely associated in several crops [76–81]. However, Biquinho and Habanero cultivars, despite showing comparable vegetative biomass production, exhibited contrasting fruit yield, which was markedly higher in Biquinho plants, independently of the N dose, with the exception of N deprivation where both cultivars could not produce almost any fruits (Fig. 5). Thus high photosynthetic rates, and especially their longer maintenance during the plant life cycle, might be partly explained by the fruit biomass produced by this cultivar [81,82].

We expected a reduction of photosynthesis in N-deficient plants (20 mg of N dm^{-3}) as a direct consequence of the feedback limitation due to the accumulation of soluble carbohydrates. Instead, under N-deficiency, plants maintained *A* levels and probably avoided inhibition by accumulating starch. Interestingly, Biquinho plants under low N supply were able to maintain high *A* despite an almost 50% reduction in protein amounts in their source leaves as compared to higher N doses. This led us to hypothesize that in Capsicum, enzymes related with photosynthetic apparatus are present in excess and a decrease in the amount of these proteins can be compensated via postranslational regulation mechanisms, which have been previously described for other species (for a review see [83]). Noteworthy, we observed that plants from both cultivars grown under higher N supply had decreased CO_2 assimilation rates (Fig. 6 and Table S2). The excessive N supply also affects CO_2 assimilation through effects on photosynthetic components (Fig. 6 and 7). We observed damage indirectly *via* fluorescence emission parameters (Fig. 6, S13 and Table S2), probably due to the negative effects on photosystems of NH_4^+ accumulation [21,84,85]. The lower values of fluorescence emission parameters also suggest an incapacity of the redox metabolism to minimize the oxidative stress caused by NH_4^+ accumulation over long-term periods (Table S2) [86].

4.3. C and N allocation strategy in C. chinense cultivars Biquinho and Habanero

It is known that higher N levels are observed in young over old plant tissues. Even though both cultivars exhibited the same behavior of C and N allocation in leaves (Fig. 3), Biquinho and Habanero showed different strategies in leaf production, with different amount of leaves being formed before and after the first sympodial unit (Fig. 1). In contrast to the situation observed in Habanero plants, Biquinho plants have higher leaf area and allocate more C and N to leaves formed after the reproductive differentiation (Fig. 4). It suggests that Biquinho keeps a higher photosynthetic area near to the reproductive sinks. In soybean, a study using leaf pruning showed that there was a reduction in the seed yield when the source-to-sink distance increased [87]. Further studies should be performed with these contrasting cultivars to clarify how and to which extension the source-sink distance and composition can influence Capsicum yield.

4.4. The role of N in yield associated traits

N is one of the nutrients that most influence plant growth and yield [88]. As expected, for both cultivars, all yield components evaluated displayed a parabolic response (Fig. S11) according to the deficiency, adequate and toxicity of the N supply [29–31]. Interestingly, we observed for both cultivars that individual fruit weight decreases linearly according to the increase of the N supply (Fig. S12). Similar effects have been documented in *Prunus domestica* L. [89], where the reduction in fruit size was explained by an increased vegetative growth. New leaves have significative sink strength [48] while the competition between fruit and vegetative growth can reduce the fruit weight [90,91], probably due to a source limitation effect [92].

In chili peppers excessive N treatments stimulate vegetative growth and reduce the number of flowers [31]. The results showed here confirm this pattern and suggest that the balance of the sucrose content between sink leaves and flowers acts as a regulator (Fig. S18). Sucrose is the major transport carbohydrate in most of the higher plants and the capacity for sinks tissues to develop and grow is stimulated by the supply of assimilates [93–97]. Vegetative growth increases in response to higher N supply [31,65,98]; on the other hand, the flowering process is dependent on sucrose supply. In *Arabidopsis*, sucrose availability in the aerial part of the plant allowed flowering in complete darkness [99]. In tomato, optimal amounts of sucrose, cytokinins and N levels were necessary to promote flowering [100]. In fact, sucrose metabolism, acquisition and allocation within and between organs, are genetically linked to the metabolism of inorganic and organic N [17,65,93,101,102].

According to our results, N supply acts on the sucrose supply to sink leaves. Sink leaves would receive more sucrose than flowers in Biquinho and Habanero plants for doses higher than 73.50 and 52.50 mg of N dm^{-3}, respectively (Fig. S18), then stimulating more the vegetative than the reproductive development. Interestingly, highest yields of fresh fruits would be obtained in Biquinho and Habanero plants for almost the same N doses (74.75 and 51.50 mg of N dm^{-3}, respectively; Table S1). Thus, these data suggest that the highest fresh yield of fruits would be obtained in plants balancing equally their sucrose supply between sink leaves and flowers. Indeed, the formation of new reproductive tissues is dependent of the vegetative growth since pepper plants produce flowers and fruits in new sympodial bifurcations [36]. Consequently, the yield is dependent on an adequate coordination between vegetative and reproductive growth [103] and this regulation is likely related to sucrose levels present in these organs.

Strong floral sinks are vital for the establishment of new fruits [58]. Under shade conditions, it has been shown that carbohydrate amounts in flowers, in particular sucrose, are positively associated with fruit set in Capsicum [48,104]. Thus the amounts of sucrose taken up by the flower and sucrose synthase activity were related with the increase of carbohydrate concentration in flowers, suggesting that sucrose importation by flowers as a key factor to fruit set of pepper [48,104]. In our study, both cultivars under N-deficiency accumulated starch in sink leaves and malate in flowers (Fig. S19 and S20). It suggests malate as an important C-source to the anapleurotic flux replenishing intermediates of the TCA cycle in flowers [56]. As a sink organ, the accumulation of malate might indicate lower TCA cycle turnover and energy limitation to flowers. Lower fruit set in plants under the highest N supply occurred because of the imbalance between vegetative and reproductive growth, and because of a decrease in photosynthetic rates. In agreement, a study on six Capsicum cultivars showed that when vegetative sink strength was decreased or source strength was increased, fruit-set and fruit weight increased [90]. Internal competition for assimilates between different organs has been reported as critical for the control of flower and young fruit abortion in pepper plants [35,48,90,105].

5. CONCLUSIONS

Here we used Biquinho and Habanero cultivars, contrasting in terms of fruit set and fruit size, to understand the behavior of *C. chinense* yield-related parameters under different N supply. The cultivars displayed moderate differences in their vegetative biomass according to the N supply. While N-deficiency is marked by biomass decrease and starch accumulation in leaves, N-excess increased NH_4^+ levels and the relative biomass allocation in leaves, mainly in cv. Habanero, which has lower fruit set and higher fruit size. Thus, we propose that the new reproductive sink strength is an important factor for plant tolerance to toxic NH_4^+ levels. Moreover, we observed that N-deficiency in Capsicum plants reduces the fruit yield through the lower development of new reproductive and vegetative tissues. Plants under N-deficiency have a lower number of flowers but heavier fruits. In contrast, plants under higher N supply have lower CO_2 assimilation and favor the vegetative instead reproductive growth probably due to an imbalance in sucrose supply. Under N excess, the lower CO_2 fixation and the internal competition for assimilates between the vegetative and reproductive organs can be responsible for the decrease in the weight of individual fruits and the decrease in the number of flowers, fruit set and yield.

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FIGURES

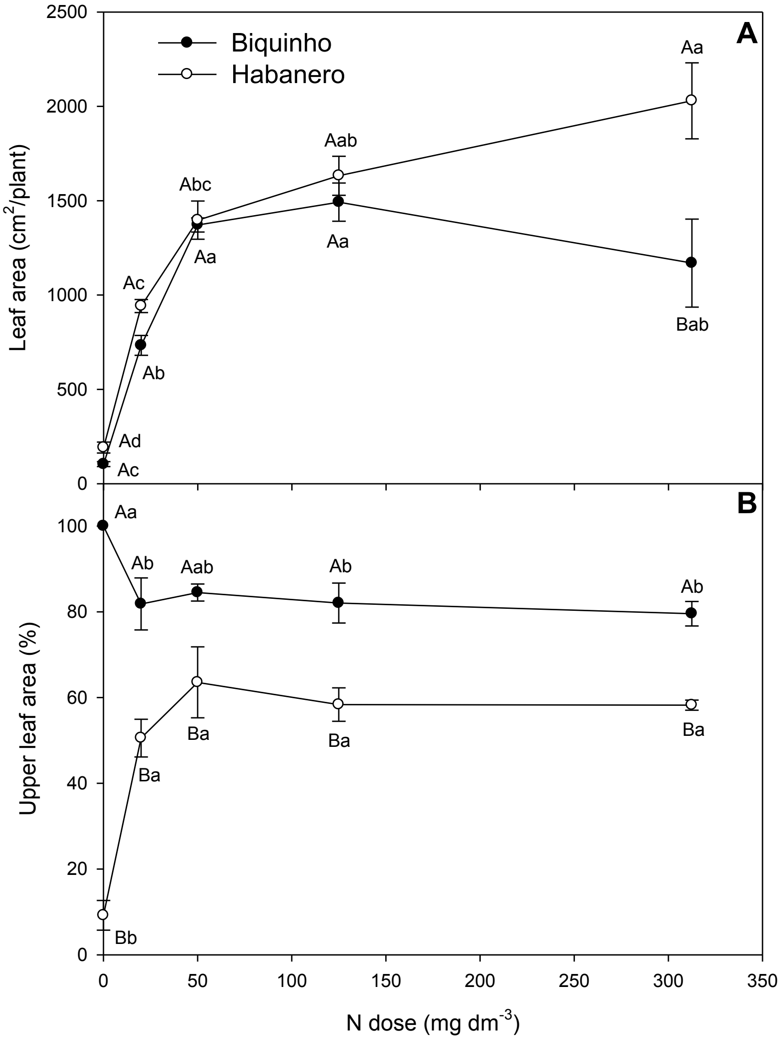

Figure 1. Leaf area (A) and the relative amount of leaves above the first sympodial unit (leaves formed after flowering) (B) of two *C. chinense* cultivars, Biquinho and Habanero, submitted to different nitrogen regimes. The evaluations were performed 91 days after flowering. Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each N dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

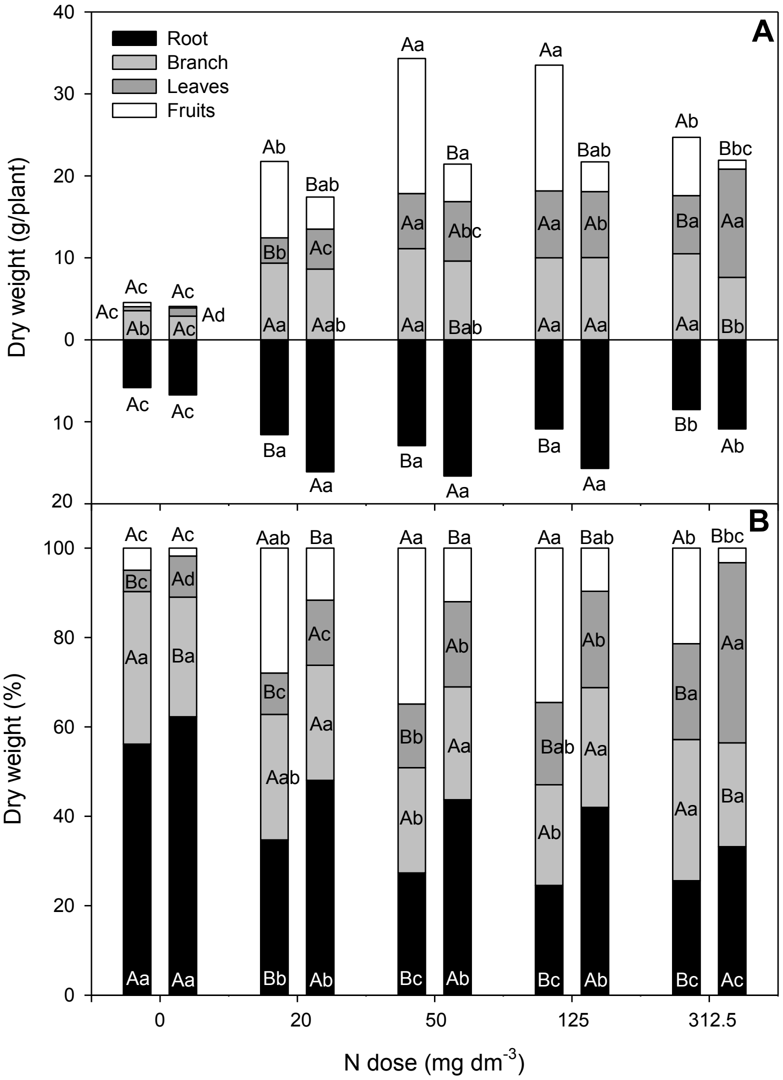

Figure 2. Dry weight (A) and relative dry weight (B) of root, branch, leaves and fruits of two *C. chinense* cultivars, Biquinho (left) and Habanero (right), submitted to different nitrogen regimes. The evaluations were performed 91 days after flowering. Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each N dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

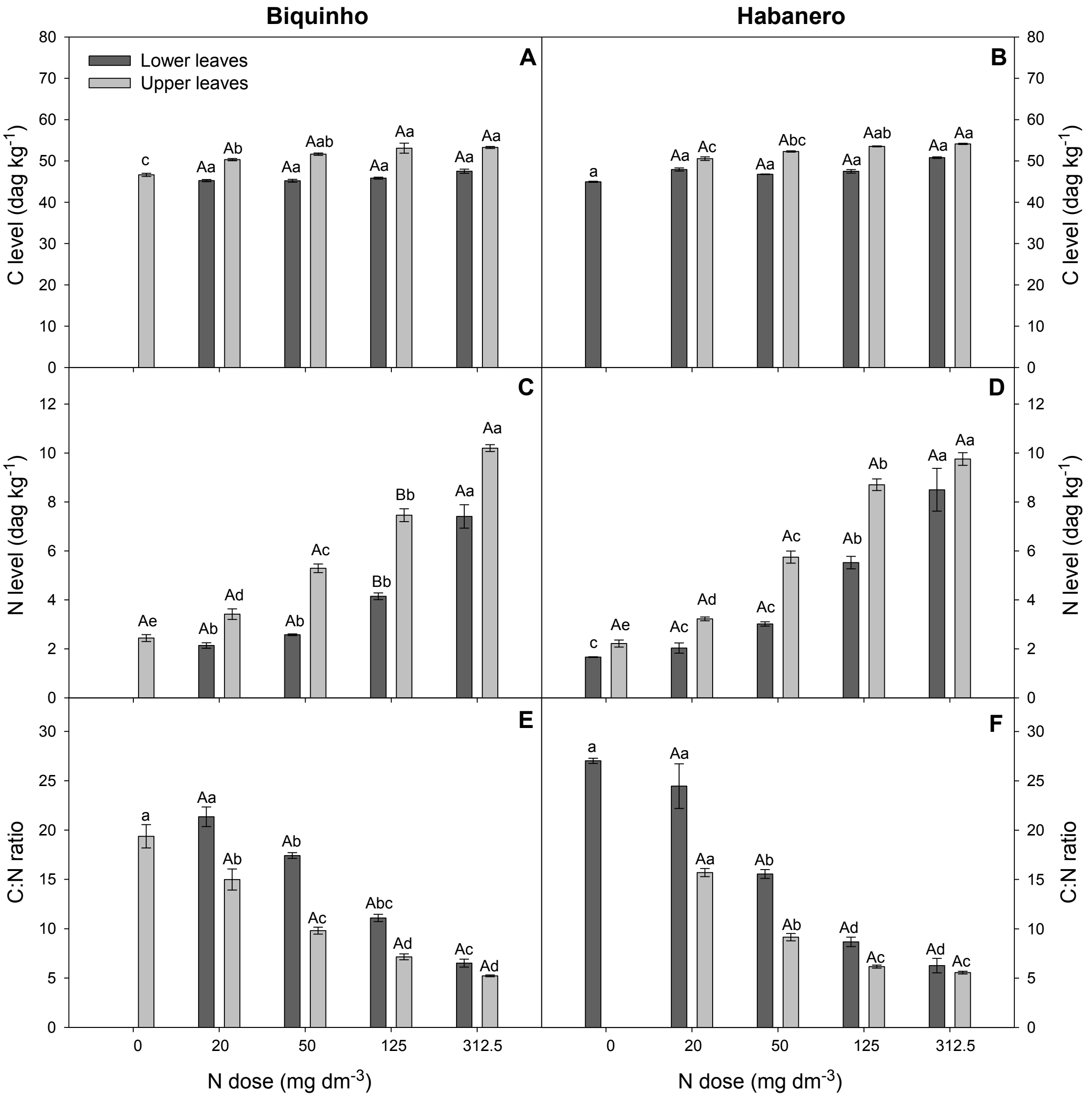

Figure 3. Carbon and nitrogen level in leaves of two *C. chinense* cultivars, Biquinho and Habanero, submitted to different nitrogen regimes. The evaluations were performed 91 days after flowering. A and B are carbon level in leaves of Biquinho and Habanero, respectively. C and D are nitrogen level in leaves of Biquinho and Habanero, respectively. E and F are C:N ratio in leaves of Biquinho and Habanero, respectively. Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each N dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

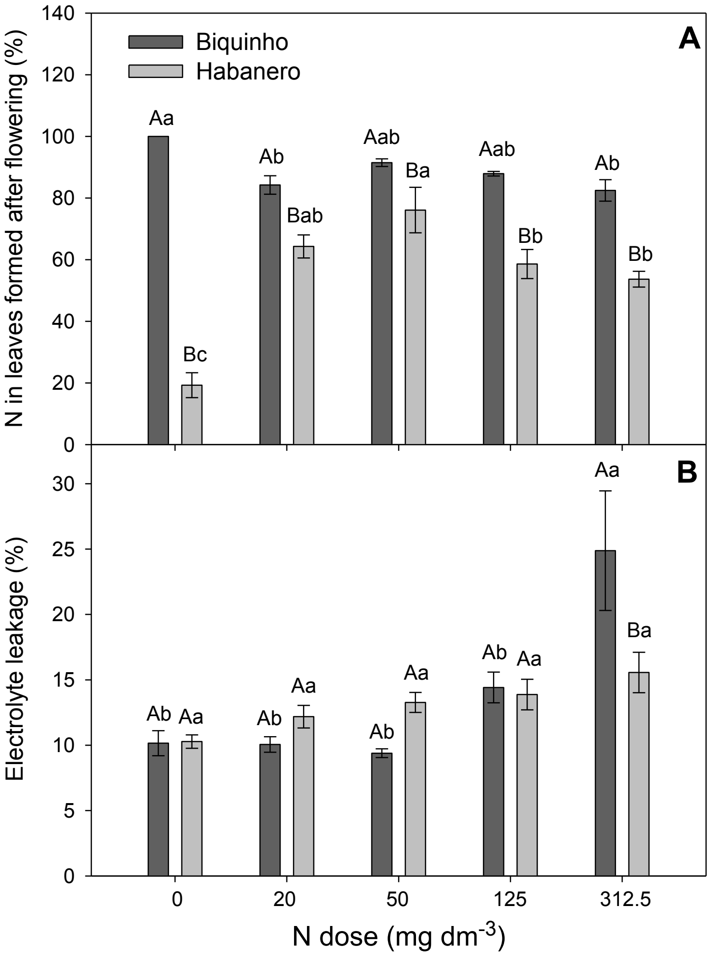

Figure 4. Relative amount of nitrogen immobilized above the first sympodial unit (A) and electrolyte leakage of leaves (B) in leaves of two *C. chinense* cultivars, Biquinho and Habanero, submitted to different nitrogen regimes. The evaluations were performed 91 and 88 days after flowering, respectively. Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each N dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

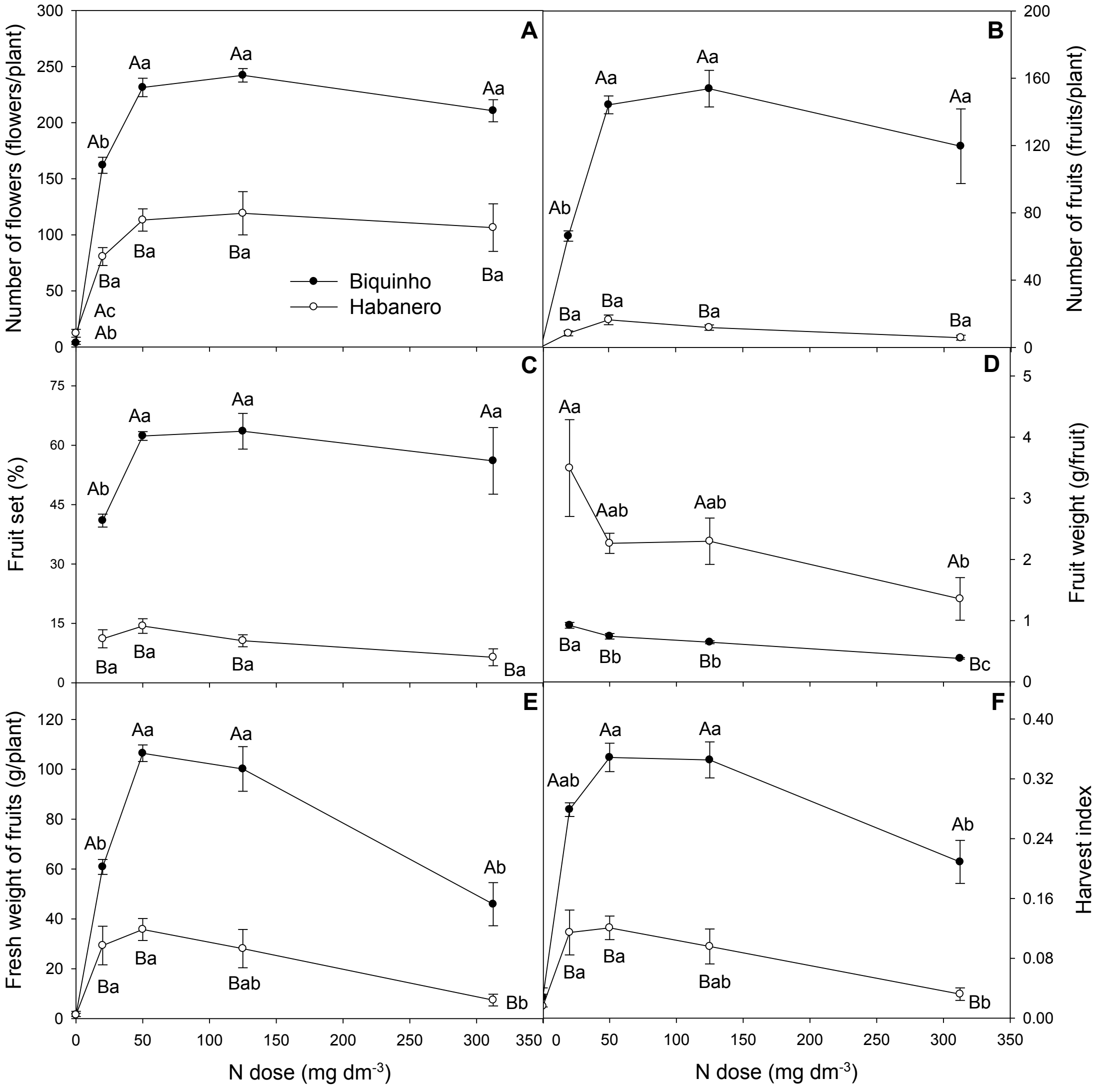

Figure 5. Yield parameters of two *C. chinense* cultivars, Biquinho and Habanero, submitted to different nitrogen regimes. The evaluations were performed 91 days after flowering. A, number of flowers. B, number of fruits. C, fruit set. D, individual fruit weight. E, fresh weight of fruits. F, harvest index. Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each N dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

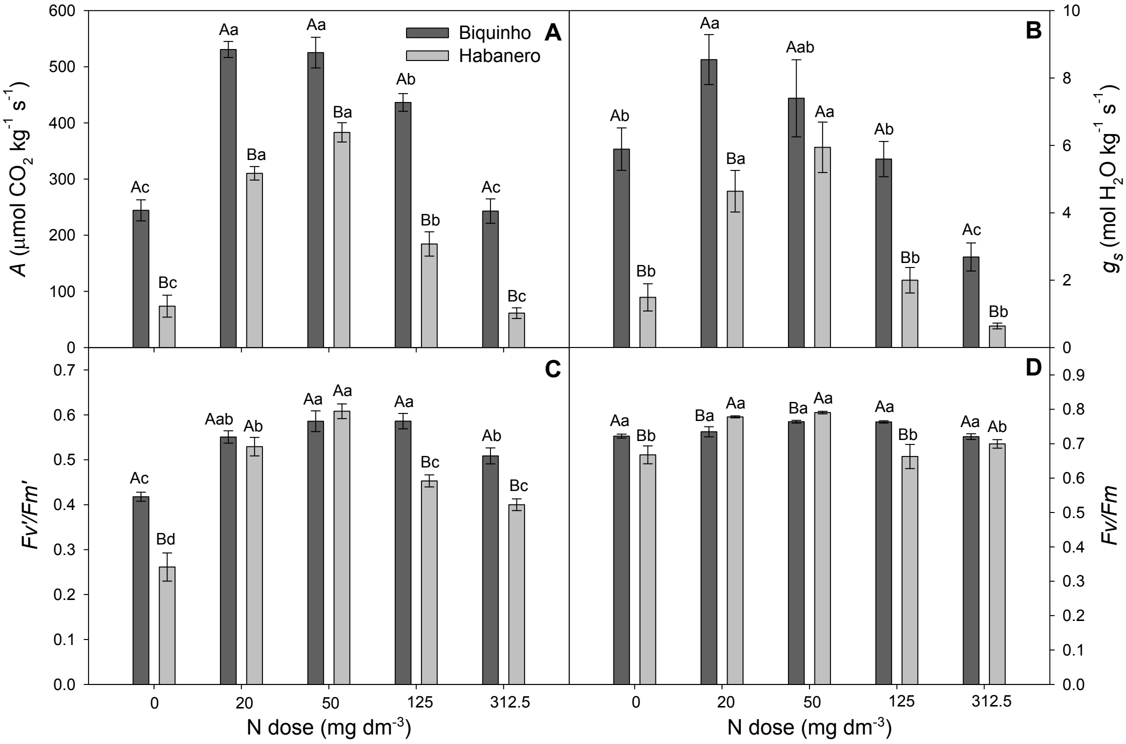

Figure 6. Gas exchange and chlorophyll *a* related parameters of two *C. chinense* cultivars, Biquinho and Habanero, submitted to different nitrogen regimes. The evaluations were performed 65 days after flowering. A, net carbon assimilation rate. B, gas exchange. C, quantum efficiency of *open* PSII reaction centers. D, maximum quantum yield of PSII. Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each N dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

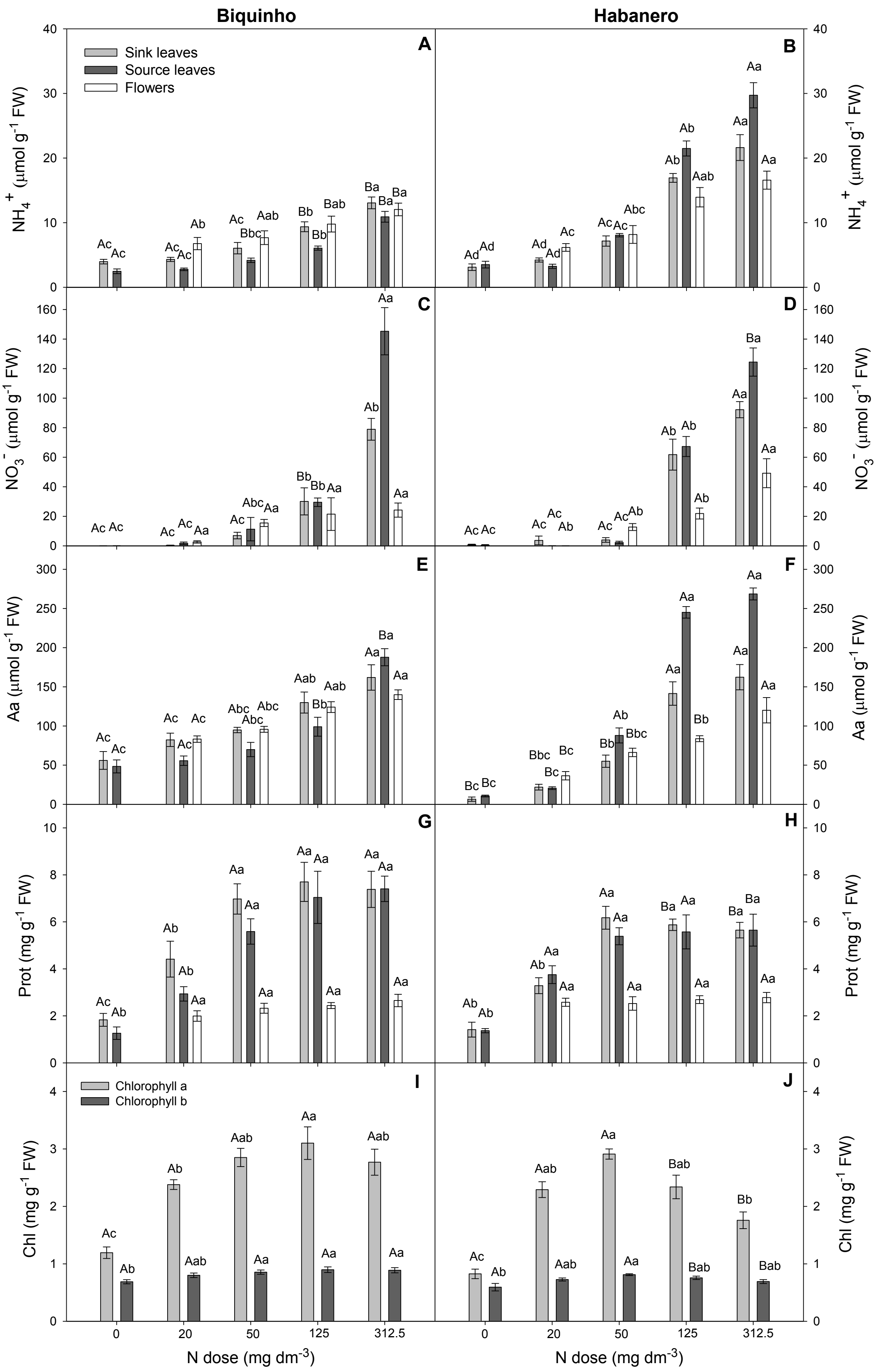
Biquinho
Habanero
Sink leaves
Source leaves
Flowers
A
B
C
D
E
F
G
H
I
J
NH$_4^+$ (μmol g^{-1} FW)
NO$_3^-$ (μmol g^{-1} FW)
Aa (μmol g^{-1} FW)
Prot (mg g^{-1} FW)
Chl (mg g^{-1} FW)
0 10 20 30 40
0 20 40 60 80 100 120 140 160
0 50 100 150 200 250 300
0 2 4 6 8 10
0 1 2 3 4
Ac Ac Ac Ac Ab Ac Bbc Aab Bb Bb Bab Ba Ba Ba
Ad Ad Ad Ad Ac Ac Ac Abc Ab Ab Aab Aa Aa Aa
Ac Ac Ac Ac Aa Ac Abc Aa Bb Bb Aa Ab Aa Aa
Ac Ac Ac Ac Ab Ac Ac Ab Ab Ab Ab Aa Ba Aa
Ac Ac Ac Ac Ac Abc Abc Abc Aab Bb Aab Aa Ba Aa
Bc Bc Bbc Bc Bc Bb Ab Bbc Aa Aa Bb Aa Aa Aa
Ac Ab Ab Ab Aa Aa Aa Aa Aa Aa Aa Aa Aa Aa
Ab Ab Ab Aa Aa Aa Aa Aa Ba Aa Aa Ba Ba Aa
Chlorophyll a
Chlorophyll b
Ac Ab Ab Aab Aab Aa Aa Aa Aab Aa
Ac Ab Aab Aab Aa Aa Bab Bab Bb Bab
0 20 50 125 312.5
N dose (mg dm^{-3})

Figure 7. Nitrogen metabolism related metabolites in two *C. chinense* accessions, Biquinho (left) and Habanero (right), grown on different nitrogen doses. The analyses were performed on sink leaves (light gray bars), source leaves (dark gray bars) and flowers (blank bars) 24h before the fourth fertilization (59 DAF). Contents in NH_4^+ (A and B), NO_3^- (C and D), total amino acids (E and F) and protein (G and H). Chlorophylls *a* and *b* are shown in source leaves (I and J). Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

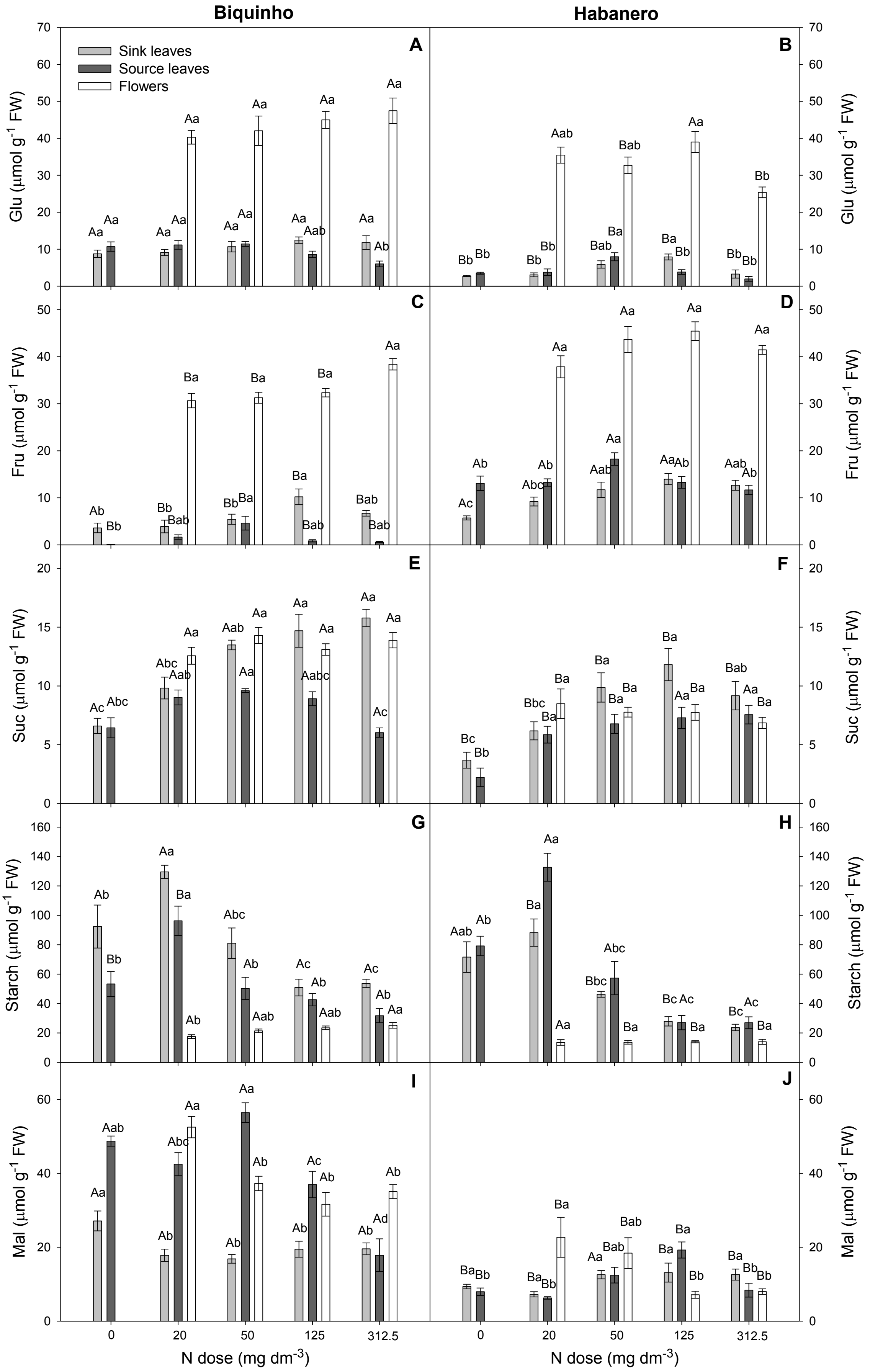
Biquinho
Habanero
Sink leaves
Source leaves
Flowers
A
B
C
D
E
F
G
H
I
J
Glu (µmol g-1 FW)
Fru (µmol g-1 FW)
Suc (µmol g-1 FW)
Starch (µmol g-1 FW)
Mal (µmol g-1 FW)
N dose (mg dm-3)
0
20
50
125
312.5
Aa Aa Aa Aa Aa Aa Aa Aa Aa Aa Aab Aa Aa Ab Aa
Bb Bb Bb Bb Aab Bab Ba Bab Ba Bb Aa Bb Bb Bb
Ab Bb Bb Bab Ba Bb Ba Ba Ba Ba Bab Ba Bab Bab Aa
Ac Ab Abc Ab Aa Aab Aa Aa Aa Ab Aa Aab Ab Aa
Ac Abc Abc Aab Aa Aab Aa Aa Aa Aabc Aa Aa Ac Aa
Bc Bb Bbc Ba Ba Ba Ba Ba Ba Aa Ba Bab Aa Ba
Ab Bb Aa Ba Ab Abc Ab Aab Ac Ab Aab Ac Ab Aa
Aab Ab Ba Aa Aa Bbc Abc Ba Bc Ac Ba Bc Ac Ba
Aa Aab Ab Abc Aa Ab Aa Ab Ab Ac Ab Ab Ad Ab
Ba Bb Ba Bb Ba Aa Bab Bab Ba Ba Bb Ba Bb Bb

Figure 8. Carbon metabolism related metabolites in two *C. chinense* accessions, Biquinho (left) and Habanero (right), submitted to different doses of nitrogen. The analyses were performed on sink leaves (light gray bars), source leaves (dark gray bars) and flowers (blank bars) 24h before the fourth fertilization (59 DAF). The content of glucose (A and B), fructose (C and D), sucrose (E and F), starch (G and H) and malate (I and J). Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

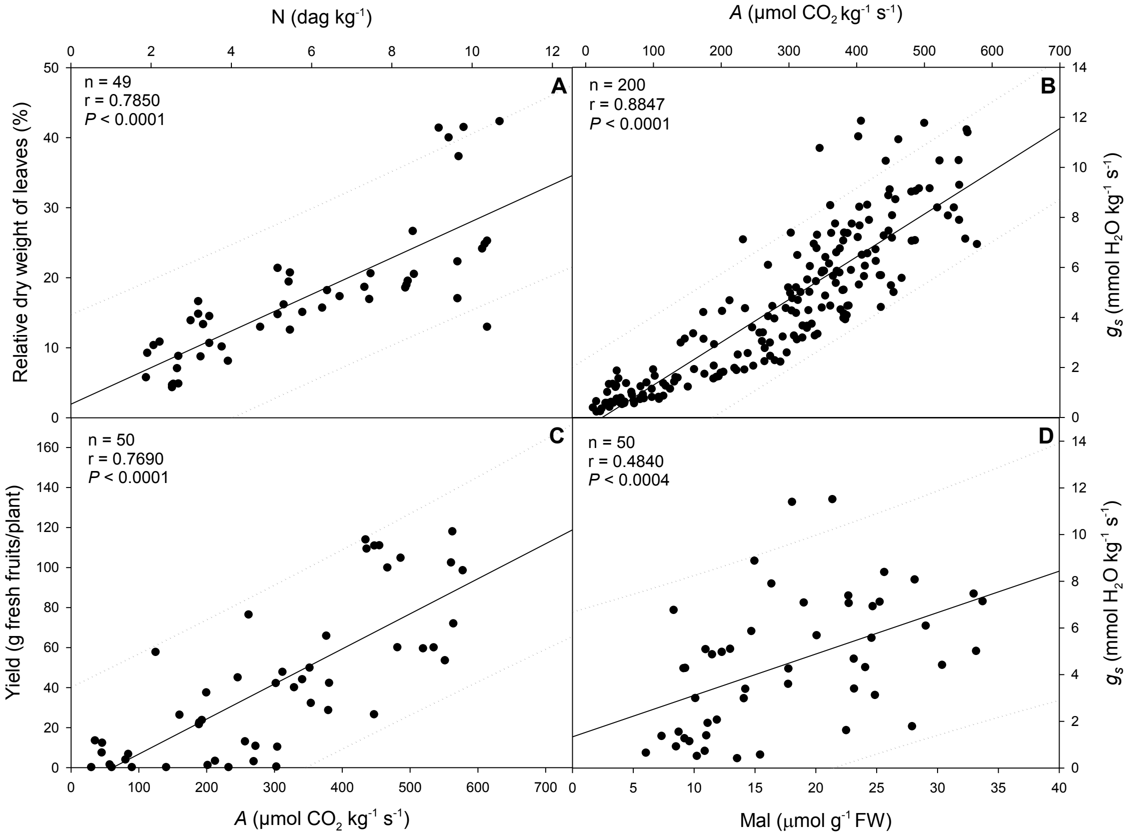

Figure 9. Pearson correlation of relative dry weight of leaves and N content in upper leaves (A), net carbon assimilation rate and stomatal conductance at 25, 45, 65 and 85 days after flowering (B), yield of fresh fruits and net carbon assimilation rate at 65 days after flowering (C), malate content in source leaves at 59 days after flowering and stomatal conductance at 65 days after flowering (D). The correlation was performed using two *C. chinense* accessions, Biquinho and Habanero, submitted to different doses of nitrogen. The number of observations (n), Pearson's correlation coefficient (r), P-value, regression line (black line) and prediction line (gray dotted line) are indicated in each graph.

SUPPLEMENTAL MATERIAL

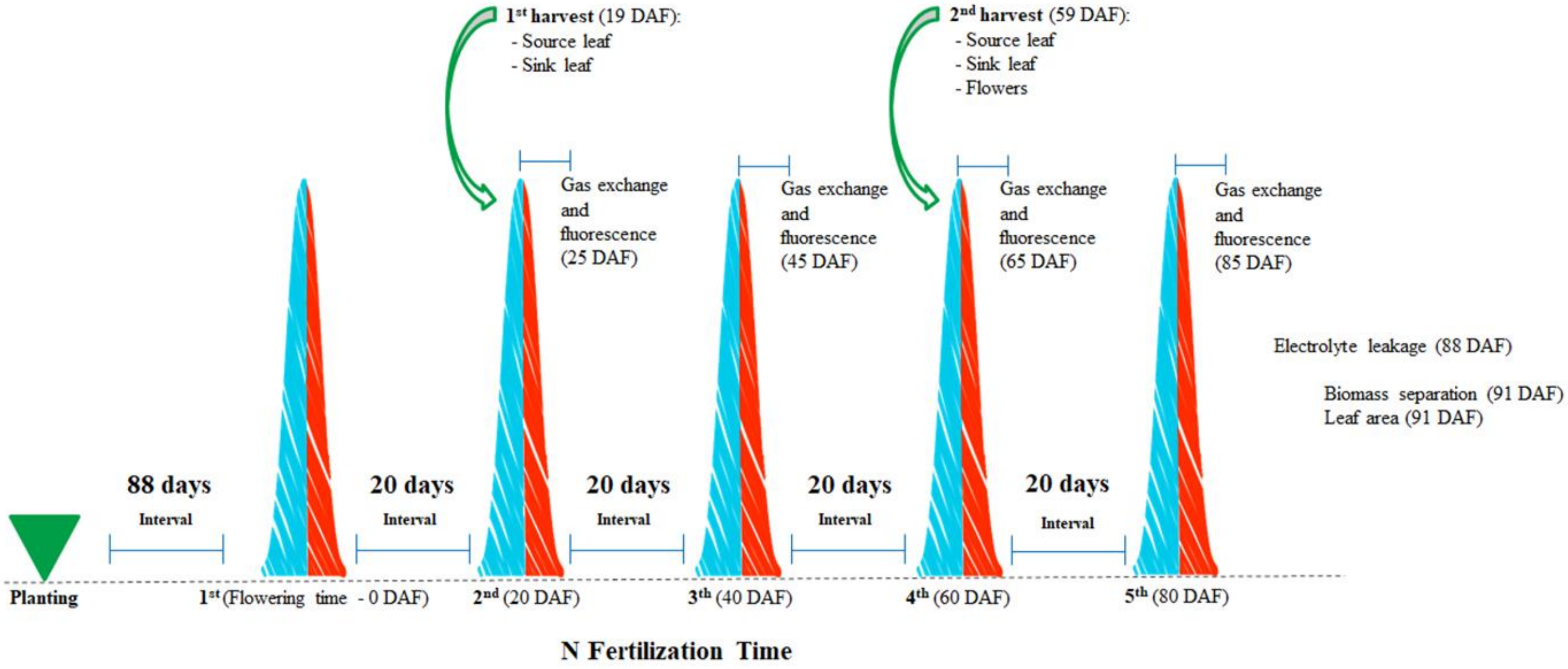

Figure S1. Scheme of the experiment evaluation dates based on the flowering period of each cultivar. The first N fertilization occurred concomitant with the flowering time.

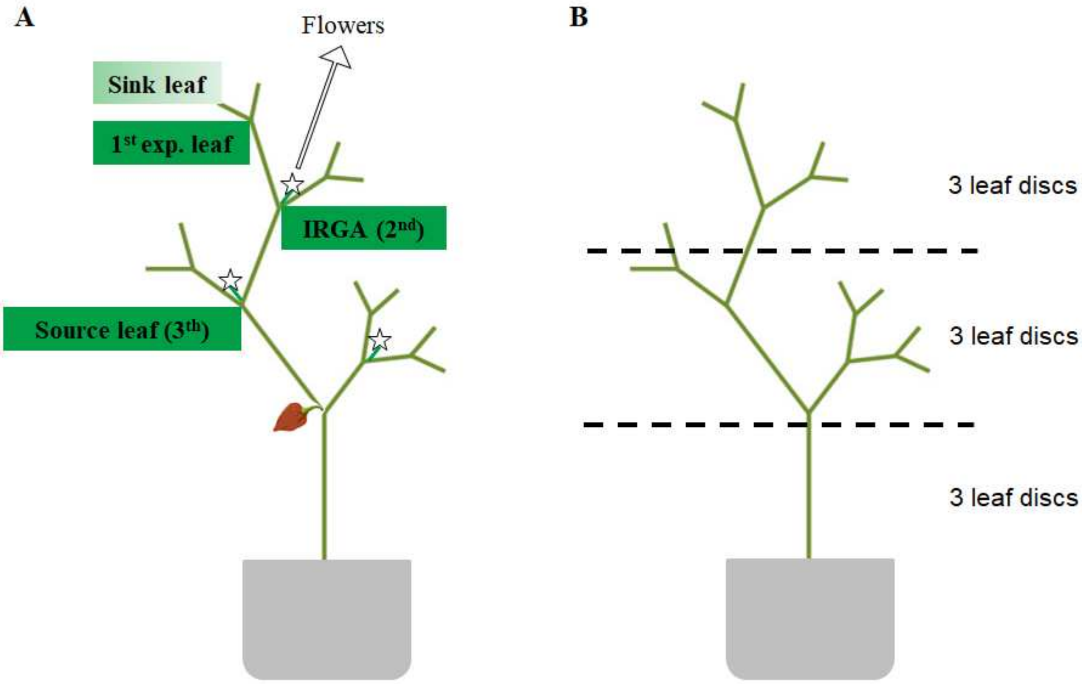

Figure S2. A) Scheme of the position of the sink leaves, source leaves and leaves for analysis of gas exchange and fluorescence (IRGA in the figure). Source leaves and sink leaves were harvested for subsequent metabolic analyzes 24 hours before the second and fourth nitrogen fertilization, in the middle of the light period. Flowers were also harvested in the middle of the light period, just before the fourth fertilization. For source leaves, completely expanded leaves were harvested in the third sympodial unit from the apex. For sink leaves were harvested the younger leaves which were not yet fully expanded; the flowers were collected about two days after the anthesis. **B)** Leaf discs for electrolyte leakage analysis were collected 88 days after flowering (DAF). Nine discs of 1.03 cm^2 each were collected from nine source leaves lamina distributed in the upper, middle and lower third of the plant.

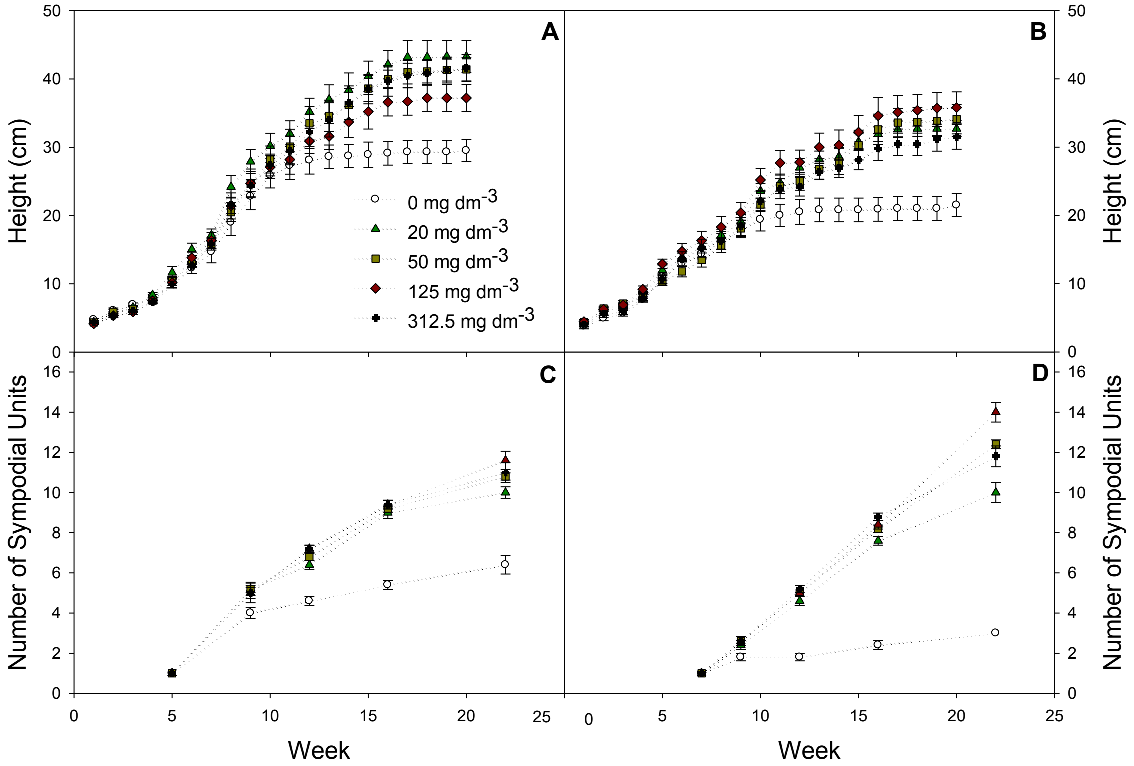

Figure S3. Height and number of sympodial units in two *C. chinense* accessions, Biquinho (left) and Habanero (right), submitted to different doses of nitrogen. In the sequence is presented height (A and B) and a number of sympodial units (C and D). Values are presented as means ± SE of five individual plants per cultivar.

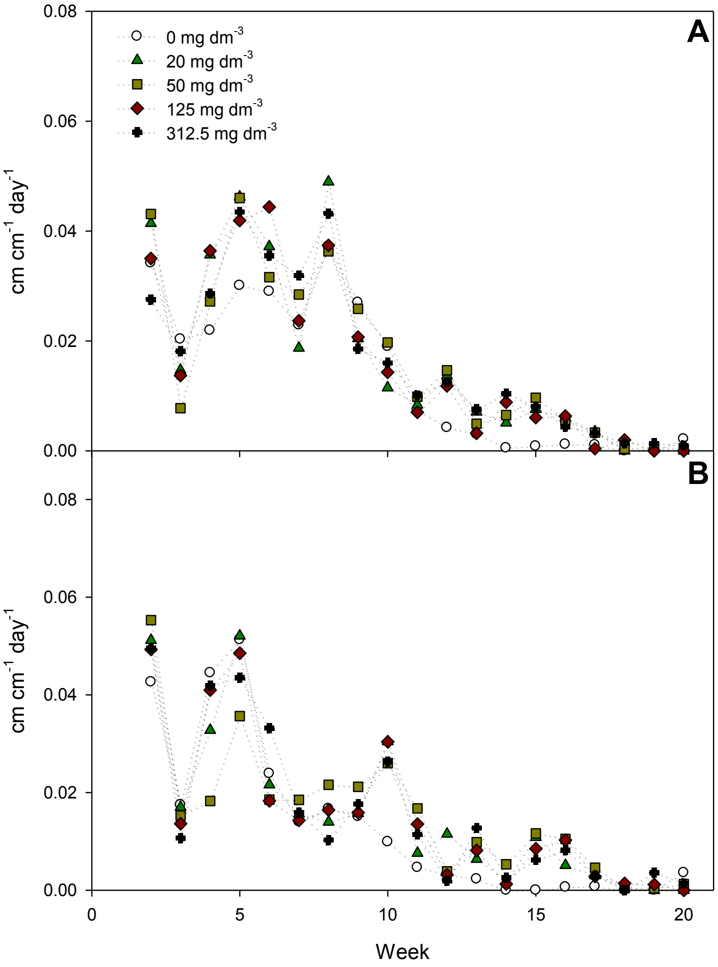

Figure S4. Relative growth rate per week (means) in two *C. chinense* accessions, Biquinho (A) and Habanero (B), submitted to different doses of nitrogen.

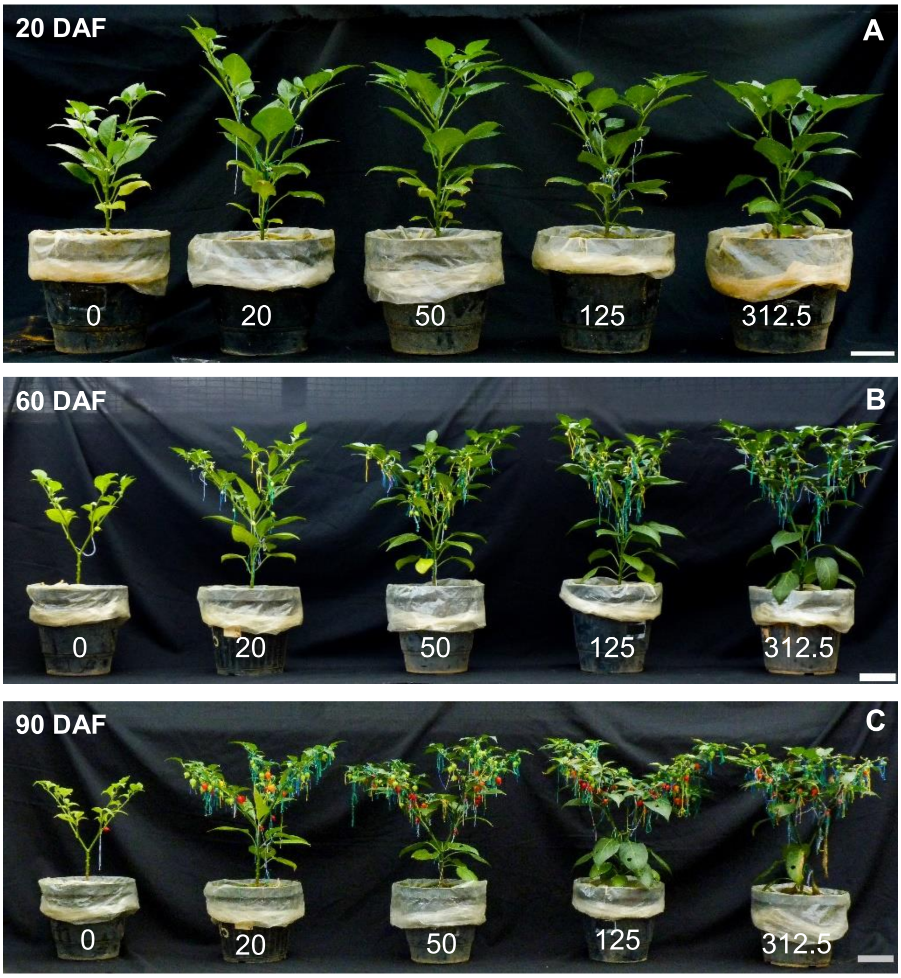

Figure S5. Pictures of Biquinho 20 (A), 60 (B) and 90 DAF (C). The scale on the pictures represents 7.5 cm.

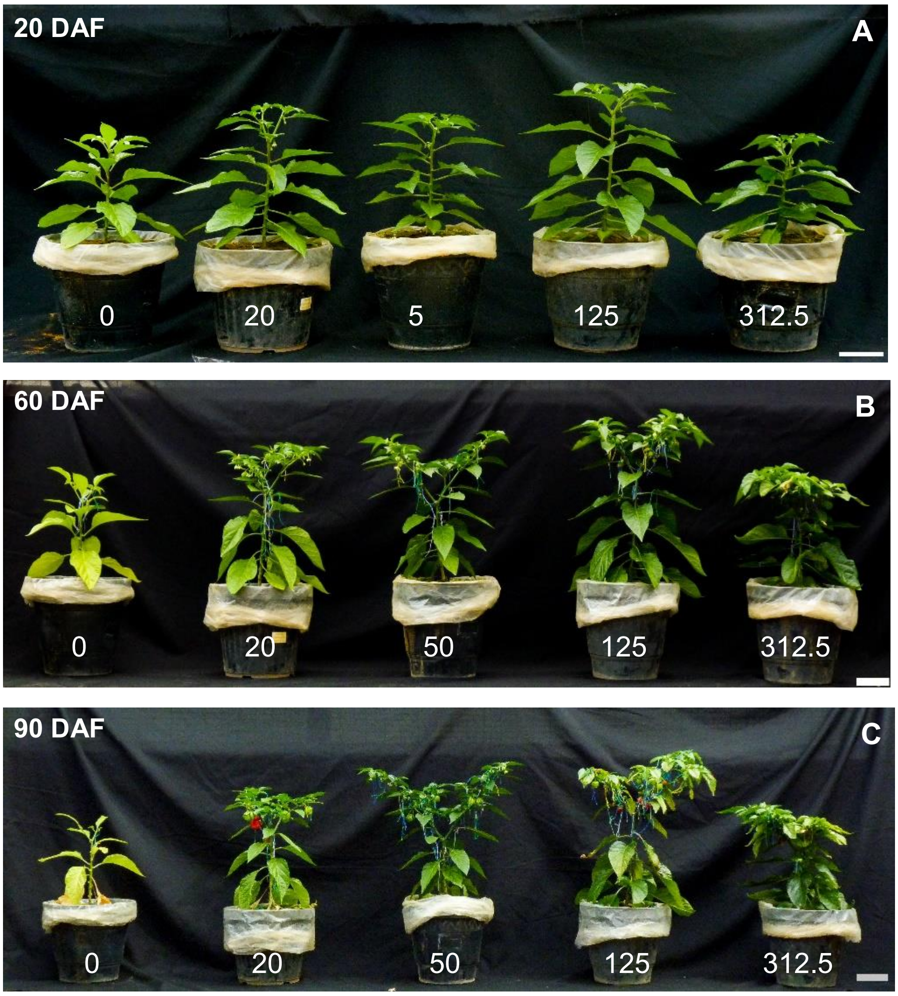

Figure S6. Pictures of Habanero 20 (A), 60 (B) and 90 DAF (C). The scale on the pictures represents 7.5 cm.

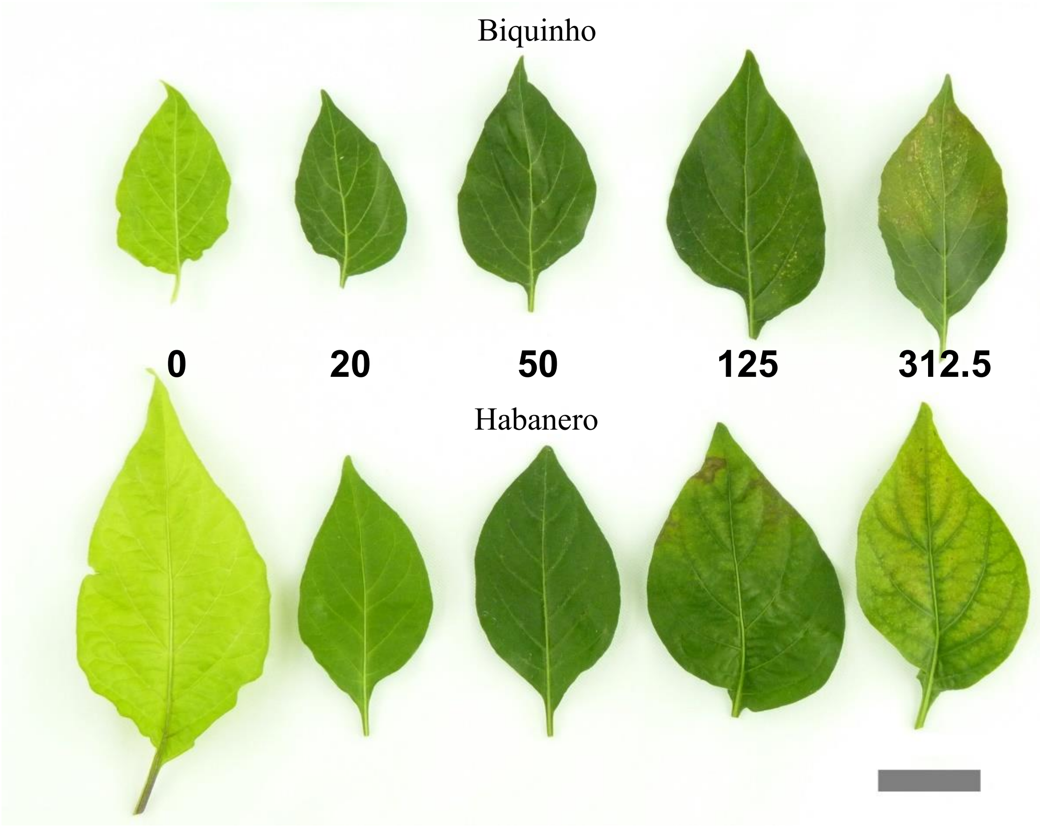

Figure S7. Pictures of leaves of the third sympodial unit from the apex of Biquinho (upper) and Habanero (lower) 90 DAF. Exception for the Habanero leaf under no N supply, which was a leaf below the first sympodial unit (due to the low availability of leaves above the first sympodial unit at that accession under no N supply). The scale on the pictures represents 2.5 cm.

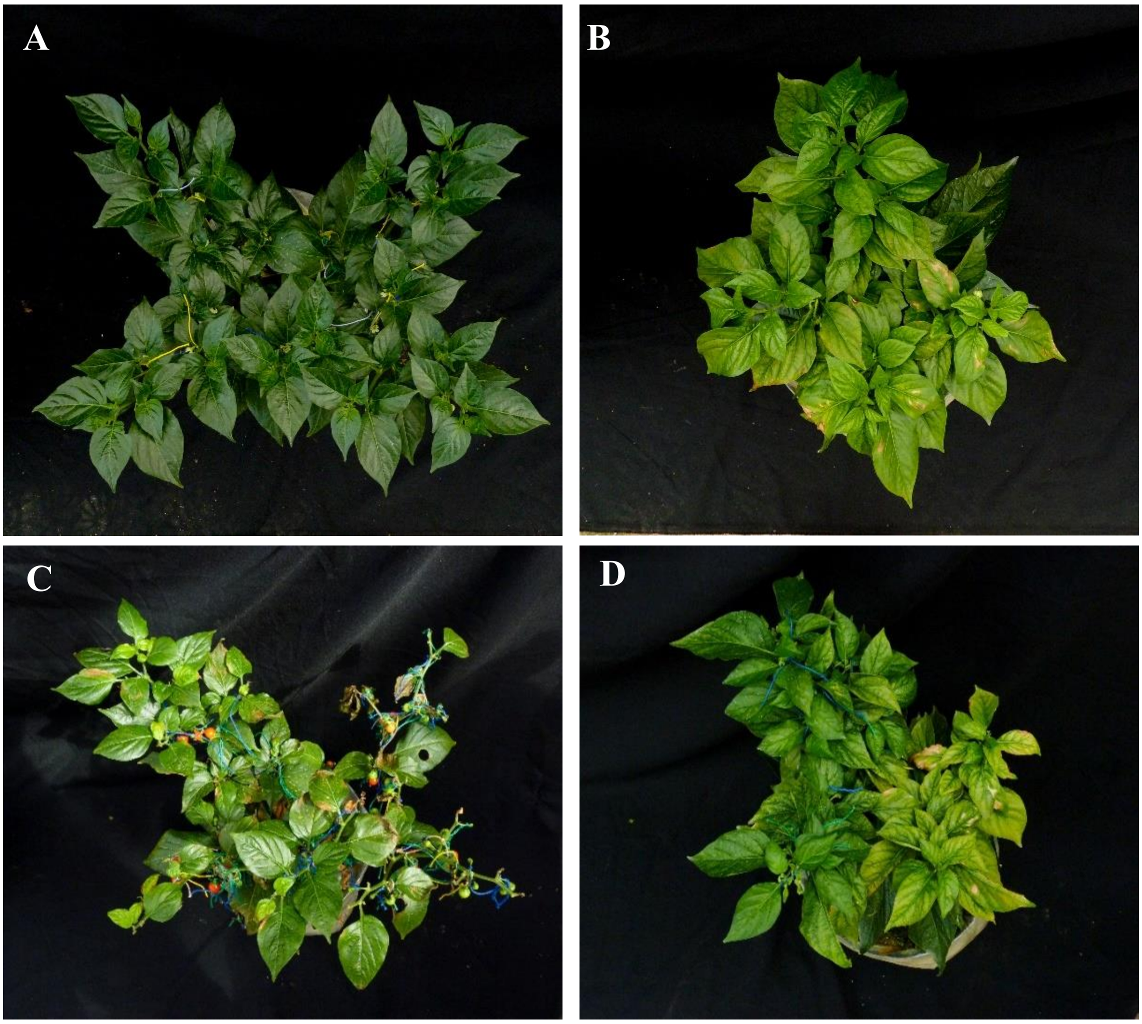

Figure S8. Superior picture of Biquinho (A and C) and Habanero (B and D) under 312.5 mg dm^{-3} at 60 (above) and 90 DAF (below). After the 5th fertilization, the access Biquinho showed a high drop of the upper leaves and symptoms of toxicity.

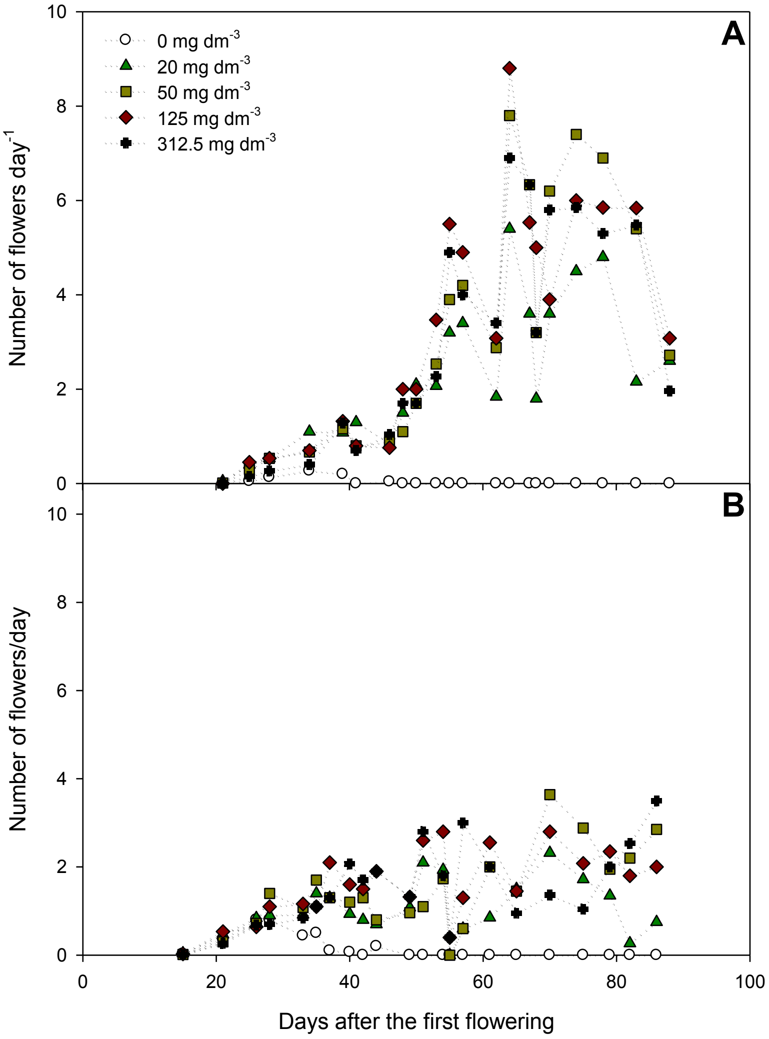

Figure S9. Number of flowers per day (means) in two *C. chinense* accessions, Biquinho (A) and Habanero (B), submitted to different doses of nitrogen.

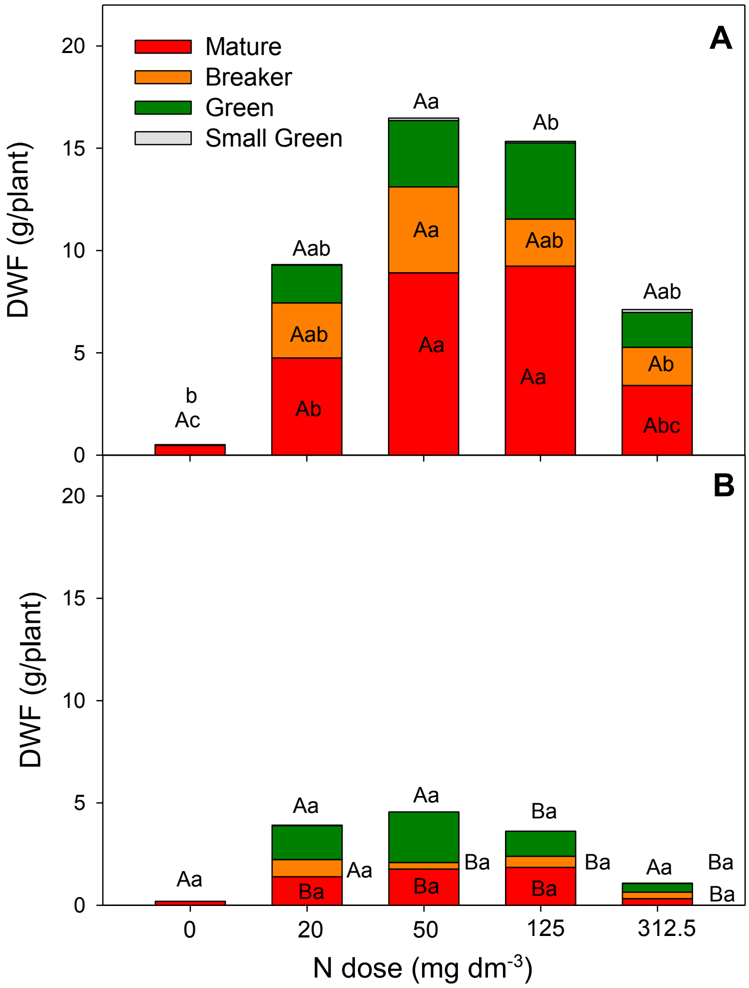

Figure S10. Dry weight of fruits in two *C. chinense* accessions, Biquinho (A) and Habanero (B), submitted to different doses of nitrogen. The fruits were classified according to the maturity stage. Values are presented as means of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

Table S1. Equations and statistical analysis of the regressions. The regression model was tested by F-test and the coefficients by *t*-test. For the quadratic models we used the N doses from 0 to 125 mg dm^{-3} due to the best adjustment. For the linear model we used the N doses from 20 to 312.5 mg dm^{-3} due to the absence of fruits in plants under N deprivation. Differentiation was used to analyse the properties such as intervals of increase, decrease and local maximum of quadratic functions (X_{max} and Y_{max}).

Yield parameter	**Biquinho**									
	r^2 (1)		**β_2x^2 (2)**		**β_1x (2)**		**β_0 (2)**		**X_{max}**	**Y_{max}**
Number of flowers (flowers/plant)	0.9664	**	-0.0375	**	6.4644	**	18.3836		86.19	296.97
Number of fruits (fruits/plant)	0.9601	**	-0.0216	**	3.9362	**	-0.5039		91.12	178.82
Fruit set (%)	0.9608	**	-0.0103	**	1.7706	**	3.2458		85.95	79.34
Fresh weight of fruits (g/plant)	0.9414	**	-0.0200	**	2.9900	**	3.6900		74.75	115.44
Dry weight of fruits (g/plant)	0.9408	**	-0.0028	**	0.4620	**	0.5877		82.50	19.65
Harvest index	0.8632	**	-5.61E-05	**	0.0093	**	0.0571		82.86	0.44
Individual fresh fruit weight (g/fruit)	0.8273	**			-0.0017	**	0.8854	**		

Yield parameter	**Habanero**									
	r^2 (1)		**β_2x^2 (2)**		**β_1x (2)**		**β_0 (2)**		**X_{max}**	**Y_{max}**
Number of flowers (flowers/plant)	0.7444	**	-0.0164	**	2.8542	**	18.1927		87.02	142.38
Number of fruits (fruits/plant)	0.7193	**	-0.0030	**	0.4718	**	0.3261		78.63	18.88
Fruit set (%)	0.6709	**	-0.0029	**	0.4328	**	1.2093		74.62	17.36
Fresh weight of fruits (g/plant)	0.4968	**	-0.0100	**	1.0300	**	4.7800		51.50	31.30
Dry weight of fruits (g/plant)	0.4865	**	-0.0009	**	0.1320	**	0.6715		73.33	5.51
Harvest index	0.4727	**	-2.45E-05	**	0.0037	**	0.0170		75.48	0.16
Individual fresh fruit weight (g/fruit)	0.3798	**			-0.0066	**	3.2913	**		

(1) F-test at $P \leq 0.01$

(2) t-test at $P \leq 0.01$

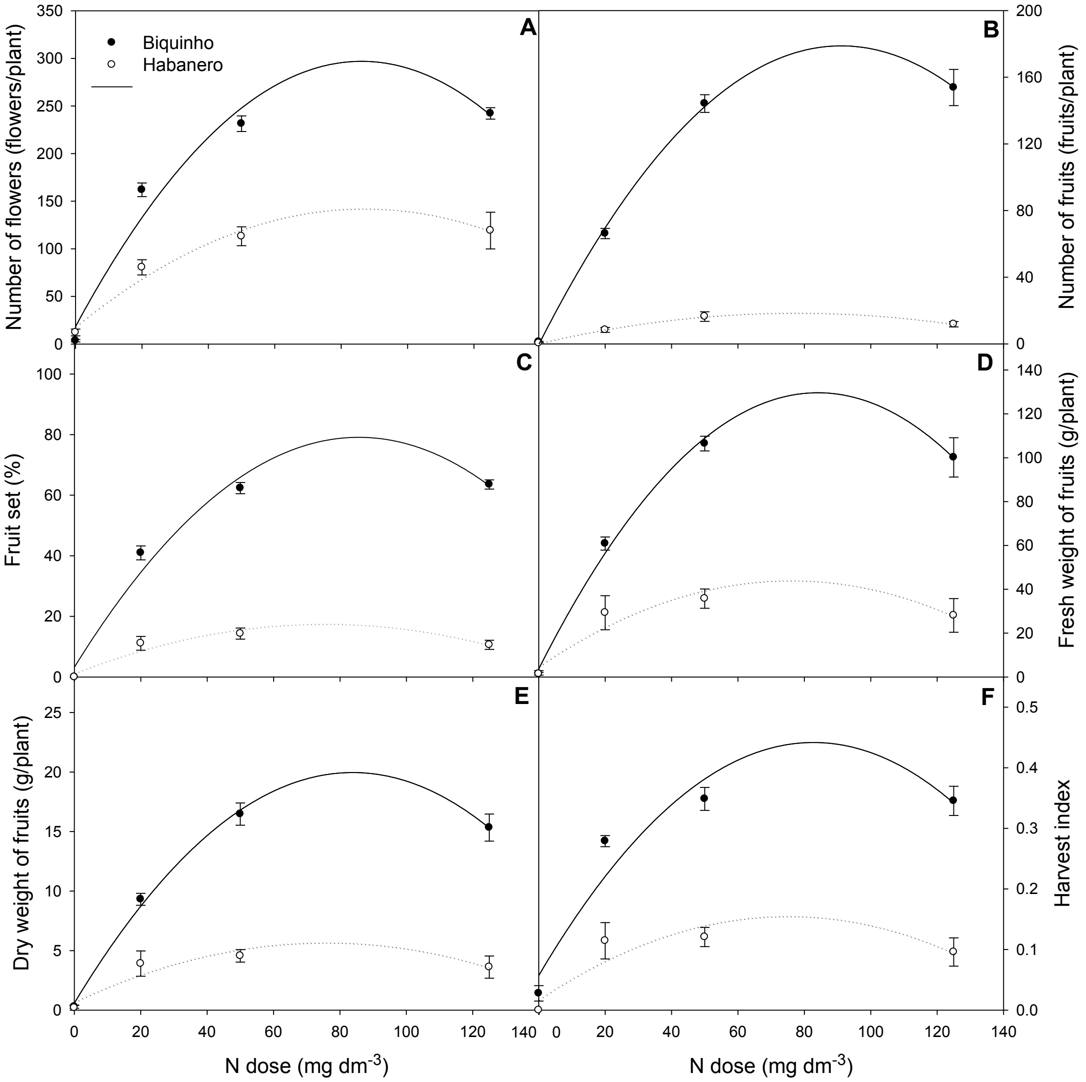

Figure S11. Quadratic regressions of yield parameters. We used the N doses from 0 to 125 mg dm^{-3}.

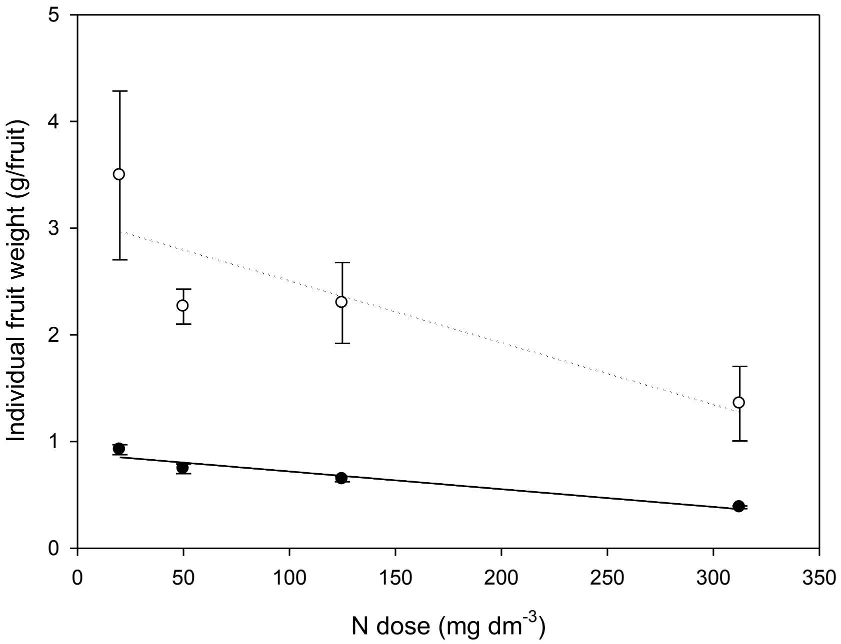

Figure 12. Linear regression to individual fruit weight. We used the N doses from 20 to 312.5 mg dm^{-3}.

Table S2. Values and statistical analysis of gas exchange data and fluorescence parameters. Uppercase letters indicate significant differences between cultivars for each dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test. The analysis was performed for each time.

A (μmol CO$_2$ kg^{-1} s^{-1})

	Days after flowering			
	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	393.23 Abc	64.70 Bb	186.76 Ac	64.83 Bc
20	472.26 Aab	324.30 Ba	450.13 Aa	327.80 Bb
50	485.85 Aa	288.68 Ba	388.50 Aa	415.78 Aa
125	409.18 Aab	342.00 Ba	272.55 Ab	308.44 Ab
312.5	311.56 Ac	116.52 Bb	205.08 Abc	32.57 Bc
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	244.30 Ac	73.62 Bc	141.62 Ac	40.75 Bb
20	530.68 Aa	310.21 Ba	381.07 Ab	348.86 Aa
50	525.16 Aa	383.14 Ba	442.36 Aa	368.50 Ba
125	436.41 Ab	184.45 Bb	327.42 Ab	274.81 Bb
312.5	242.99 Ac	61.21 Bc	56.14 Ad	45.34 Ab

g_s (mol H$_2$O kg^{-1} s^{-1})

	Days after flowering			
	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	9.42 Aa	0.84 Bb	2.61 Abc	1.21 Abc
20	8.53 Aa	5.88 Ba	8.95 Aa	5.34 Ba
50	7.82 Aa	3.29 Bab	4.46 Ab	5.84 Aa
125	7.51 Aab	3.55 Bab	2.54 Abc	2.98 Ab
312.5	4.73 Ab	0.87 Bb	1.90 Ac	0.42 Bc
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	5.89 Ab	1.49 Bb	2.91 Ac	1.41 Bb
20	8.55 Aa	4.64 Ba	7.68 Aa	6.70 Aa
50	7.40 Aab	5.94 Aa	7.66 Aa	5.95 Ba
125	5.59 Ab	2.00 Bb	5.17 Ab	1.07 Bb
312.5	2.69 Ac	0.64 Bb	0.74 Ad	0.49 Ab

Fv’/Fm’

	Days after flowering			
	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	0.48 Ab	0.34 Bc	0.41 Ab	0.31 Bb
20	0.53 Aab	0.50 Aab	0.56 Aa	0.57 Ab

50	0.59 Aa	0.52 Bab	0.54 Aa	0.59 Aa
125	0.58 Aa	0.56 Aa	0.50 Aab	0.54 Aa
312.5	0.54 Aa	0.47 Bb	0.49 Aab	0.39 Bb
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	0.42 Ac	0.26 Bd	0.39 Ab	0.24 Bc
20	0.55 Aab	0.53 Ab	0.51 Aa	0.53 Aa
50	0.59 Aa	0.61 Aa	0.54 Aa	0.58 Aa
125	0.59 Aa	0.45 Bc	0.54 Aa	0.43 Bb
312.5	0.51 Ab	0.40 Bc	0.37 Bb	0.44 Ab

Φ_{PSII}

Days after flowering

	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	0.25 Ab	0.08 Bc	0.13 Ab	0.10 Ac
20	0.31 Aa	0.28 Ab	0.24 Aa	0.28 Ab
50	0.34 Aa	0.29 Bb	0.30 Ba	0.37 Aa
125	0.35 Aa	0.38 Aa	0.30 Ba	0.35 Aa
312.5	0.33 Aa	0.25 Bb	0.28 Aa	0.14 Bc
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	0.18 Ac	0.07 Bd	0.11 Ac	0.05 Be
20	0.32 Aab	0.25 Bb	0.22 Bb	0.26 Ab
50	0.37 Aab	0.34 Aa	0.30 Ba	0.33 Aa
125	0.37 Aa	0.23 Bb	0.28 Aa	0.17 Bc
312.5	0.32 Ab	0.17 Bc	0.13 Ac	0.13 Ad

qP

Days after flowering

	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	0.52 Ab	0.23 Bc	0.31 Ac	0.28 Ac
20	0.58 Aab	0.55 Ab	0.44 Ab	0.50 Ab
50	0.58 Aab	0.56 Ab	0.55 Ba	0.63 Aa
125	0.61 Ba	0.68 Aa	0.61 Aa	0.66 Aa
312.5	0.61 Aa	0.53 Bb	0.56 Aa	0.36 Bc
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	0.43 Ab	0.27 Bc	0.28 Ac	0.23 Bd
20	0.59 Aa	0.48 Bab	0.44 Bb	0.50 Ab
50	0.63 Aa	0.55 Ba	0.55 Aa	0.58 Aa
125	0.63 Aa	0.51 Bab	0.52 Aa	0.40 Bc
312.5	0.63 Aa	0.43 Bb	0.34 Ac	0.29 Bd

ETR (umol e m^{-2} s^{-1})

	Days after flowering			
	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	108.26 Ab	34.74 Bc	56.00 Ab	42.92 Ac
20	135.02 Aa	121.82 Ab	107.24 Aa	121.85 Ab
50	150.20 Aa	125.70 Bb	130.44 Ba	163.35 Aa
125	154.14 Aa	167.52 Aa	132.06 Ba	154.12 Aa
312.5	144.13 Aa	108.67 Bb	120.79 Aa	60.58 Bc
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	78.21 Ac	30.62 Bd	49.19 Ac	23.77 Be
20	140.97 Aab	111.11 Bb	98.28 Bb	115.96 Ab
50	161.14 Aab	147.89 Aa	131.46 Ba	147.18 Aa
125	161.33 Aa	101.31 Bb	122.20 Aa	113.23 Bc
312.5	138.89 Ab	75.47 Bc	55.75 Ac	55.21 Ad

***E* (mmol H_2O kg^{-1} s^{-1})**

	Days after flowering			
	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	159.28 Aa	18.40 Bb	46.00 Abc	26.84 Ac
20	159.40 Aa	98.54 Ba	123.43 Aa	108.71 Aa
50	148.79 Aa	64.12 Ba	71.65 Bb	108.27 Aa
125	132.19 Aa	67.95 Ba	45.29 Bbc	71.54 Ab
312.5	86.95 Ab	18.98 Bb	31.86 Ac	9.98 Ac
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	119.61 Ab	38.11 Bb	36.79 Ac	24.75 Ab
20	170.10 Aa	102.65 Ba	86.56 Aa	88.25 Aa
50	137.33 Aab	115.14 Aa	86.58 Aa	76.36 Aa
125	111.06 Ab	50.72 Bb	61.19 Ab	20.47 Bb
312.5	57.24 Ac	17.86 Bb	9.79 Ad	8.65 Ab

C_i/C_a

	Days after flowering			
	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	0.78 Aa	.653 Bab	0.67 Bab	0.75 Aa
20	0.73 Aa	0.74 Aa	0.76 Aa	0.70 Aa
50	0.68 Aa	0.60 Bb	0.61 Abc	0.65 Aa
125	0.73 Aa	0.57 Bb	0.52 Ac	0.51 Ab
312.5	0.68 Aa	0.44 Bc	0.53 Bc	0.67 Aa
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero

0	0.79 Aa	0.76 Aa	0.77 Ba	0.86 Aa
20	0.70 Aab	0.67 Aab	0.77 Aa	0.76 Aab
50	0.64 Abc	0.69 Aa	0.73 Aa	0.70 Abc
125	0.63 Abc	0.56 Ac	0.71 Aa	0.60 Bc
312.5	0.56 Ac	0.58 Abc	0.67 Aa	0.61 Ac

NPQ

	Days after flowering			
	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	1.74 Ba	2.870 Aa	2.13 Ba	3.30 Aa
20	1.44 Bab	1.92 Ab	1.12 Bb	1.73 Ab
50	1.09 Bb	1.77 Abc	1.42 Aab	1.23 Ab
125	1.14 Ab	1.35 Ac	1.62 Aab	1.36 Ab
312.5	1.23 Bb	2.12 Ab	1.52 Aab	1.83 Ab
	65		85	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	2.18 Ba	4.88 Aa	2.85 Ba	4.07 Aa
20	1.13 Aab	1.63 Ab	1.58 Ab	1.61 Ab
50	0.96 Ab	1.07 Ab	1.42 Ab	1.38 Ab
125	0.83 Ab	1.43 Ab	1.46 Ab	1.38 Ab
312.5	1.46 Aab	1.97 Ab	2.40 Aa	1.79 Bb

R_d (µmol CO$_2$ kg^{-1} s^{-1})

	Days after flowering			
	25		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	29.39 Aab	30.450 Aa	27.15 Ab	21.89 Ab
20	29.61 Aa	32.58 Aa	37.98 Aa	33.75 Aa
50	25.97 Aab	29.38 Aa	31.41 Aab	31.54 Aa
125	25.38 Bab	31.93 Aa	27.36 Bb	33.46 Aa
312.5	22.64 Bb	28.25 Aa	26.07 Ab	26.00 Aab
	65		45	
N dose (mg dm^{-3})	Biquinho	Habanero	Biquinho	Habanero
0	29.57 Aa	32.02 Ab	14.75 Aa	17.30 Ac
20	37.11 Aa	42.26 Ab	19.83 Ba	27.84 Ab
50	38.44 Aa	37.24 Ab	17.55 Ba	29.93 Ab
125	32.04 Ba	58.05 Aa	17.64 Ba	45.56 Aa
312.5	29.92 Ba	41.00 Ab	17.09 Ba	28.54 Ab

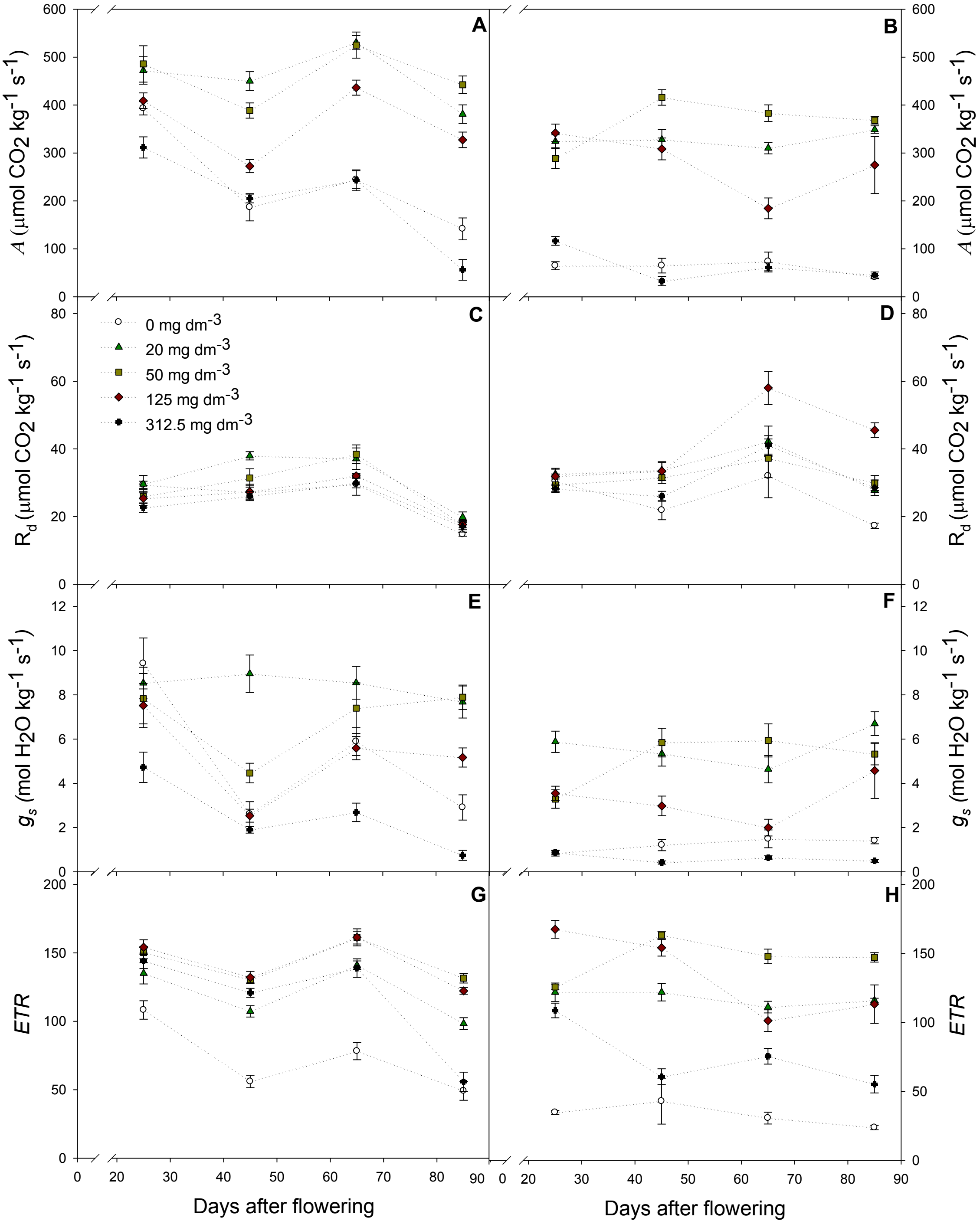

Figure S13. Gas exchange and fluorescence parameters of two *C. chinense* accessions, Biquinho (left) and Habanero (right), submitted to different N doses. Net CO_2 assimilation rate (A and B), dark respiration rate (C and D), stomatal conductance (E and F) and electron transport rate (G and H) were determined in fully expanded leaves from the second sympodial unit. The determinations were made 25, 55, 65 and 85 after the first flowering. Values are presented as means ± SE of five individual plants per cultivar. The statistical data are displayed in Table S2.

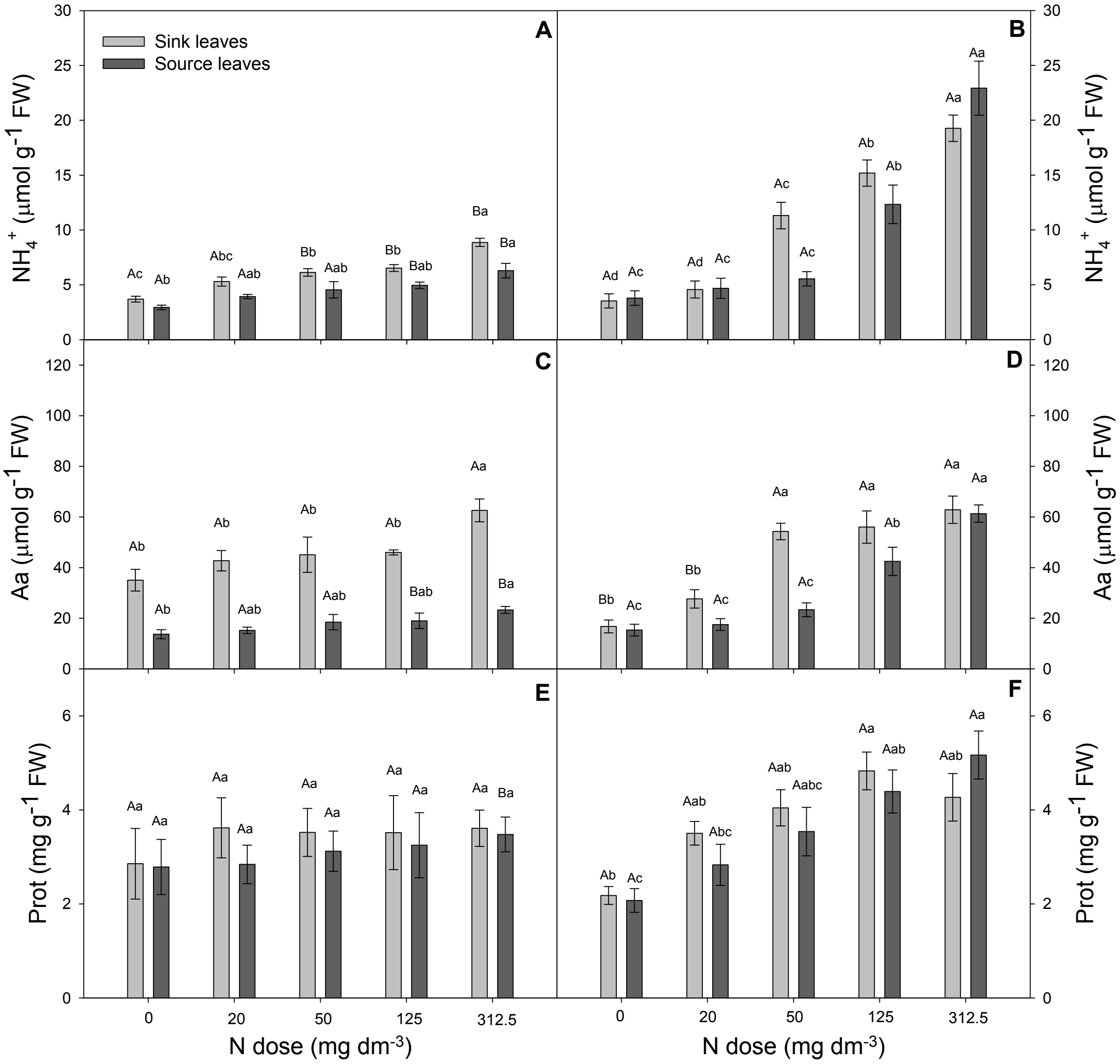

Figure S14. Metabolites content in two *C. chinense* accessions, Biquinho (left) and Habanero (right), submitted to different doses of nitrogen. The analyses were performed on sink leaves (light gray bars), source leaves (dark gray bars) and flowers (blank bars) before (24h) the second fertilization (19 DAF). In the sequence is presented the content of NH$_4^+$ (A and B), the total amount of amino acids (C and D) and the total amount of proteins (E and F). Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

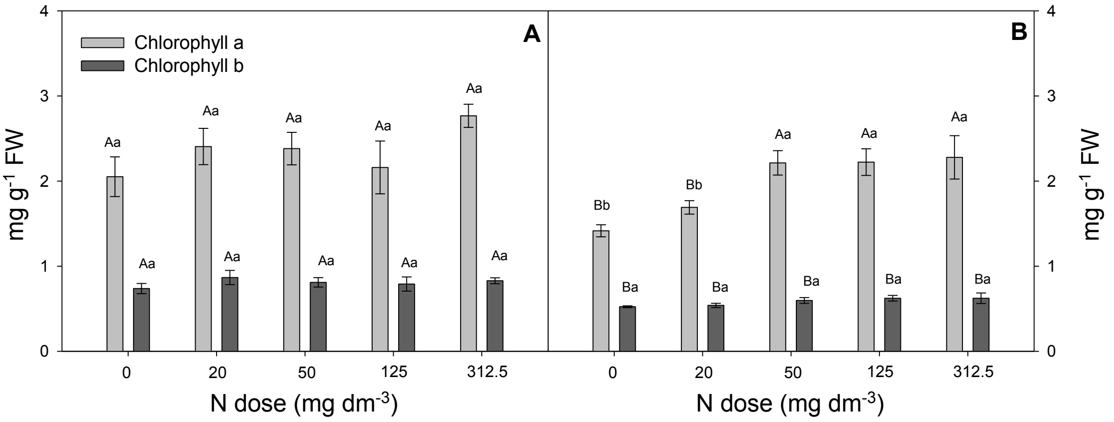

Figure S15. Chlorophyll *a* and *b* content in two *C. chinense* accessions, Biquinho (left) and Habanero (right), submitted to different doses of nitrogen. The analyses were performed on source leaves before (24h) the second fertilization (19 DAF). Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

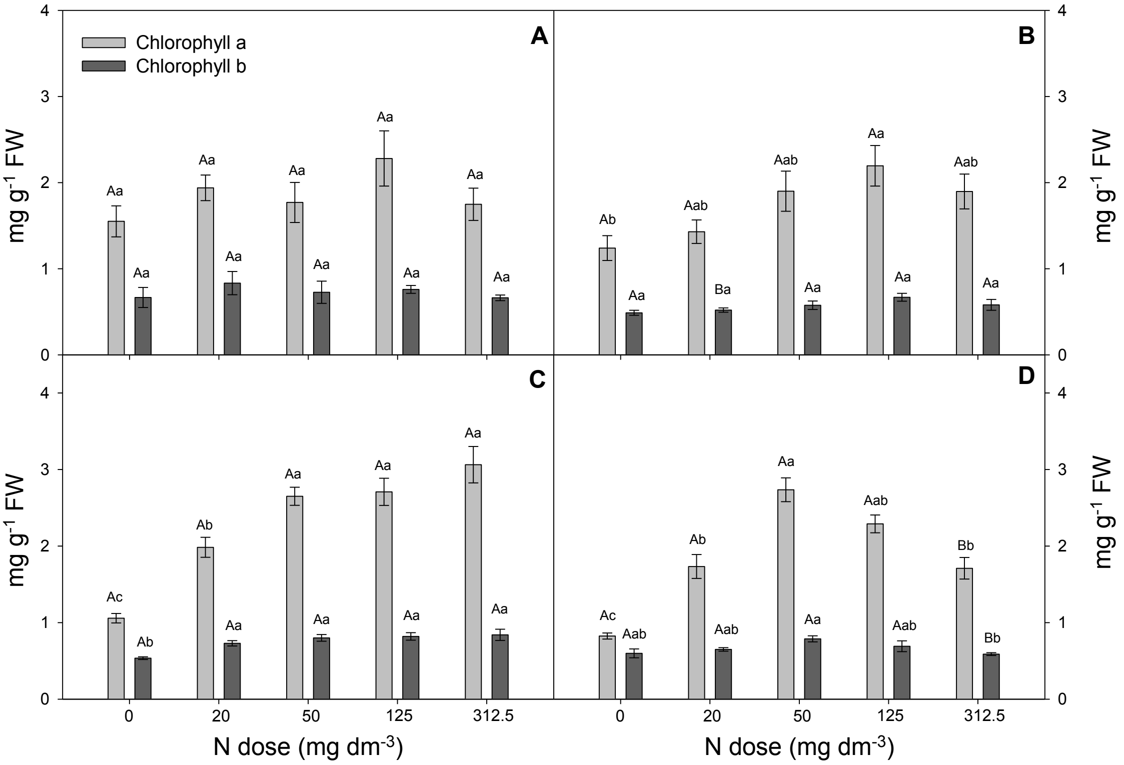

Figure S16. Chlorophyll *a* and *b* content in two *C. chinense* accessions, Biquinho (left) and Habanero (right), submitted to different doses of nitrogen. The analyses were performed on sink leaves before (24h) the second fertilization (upper graphs) and fourth fertilization (lower graphs), 19 and 59 DAF, respectively. Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

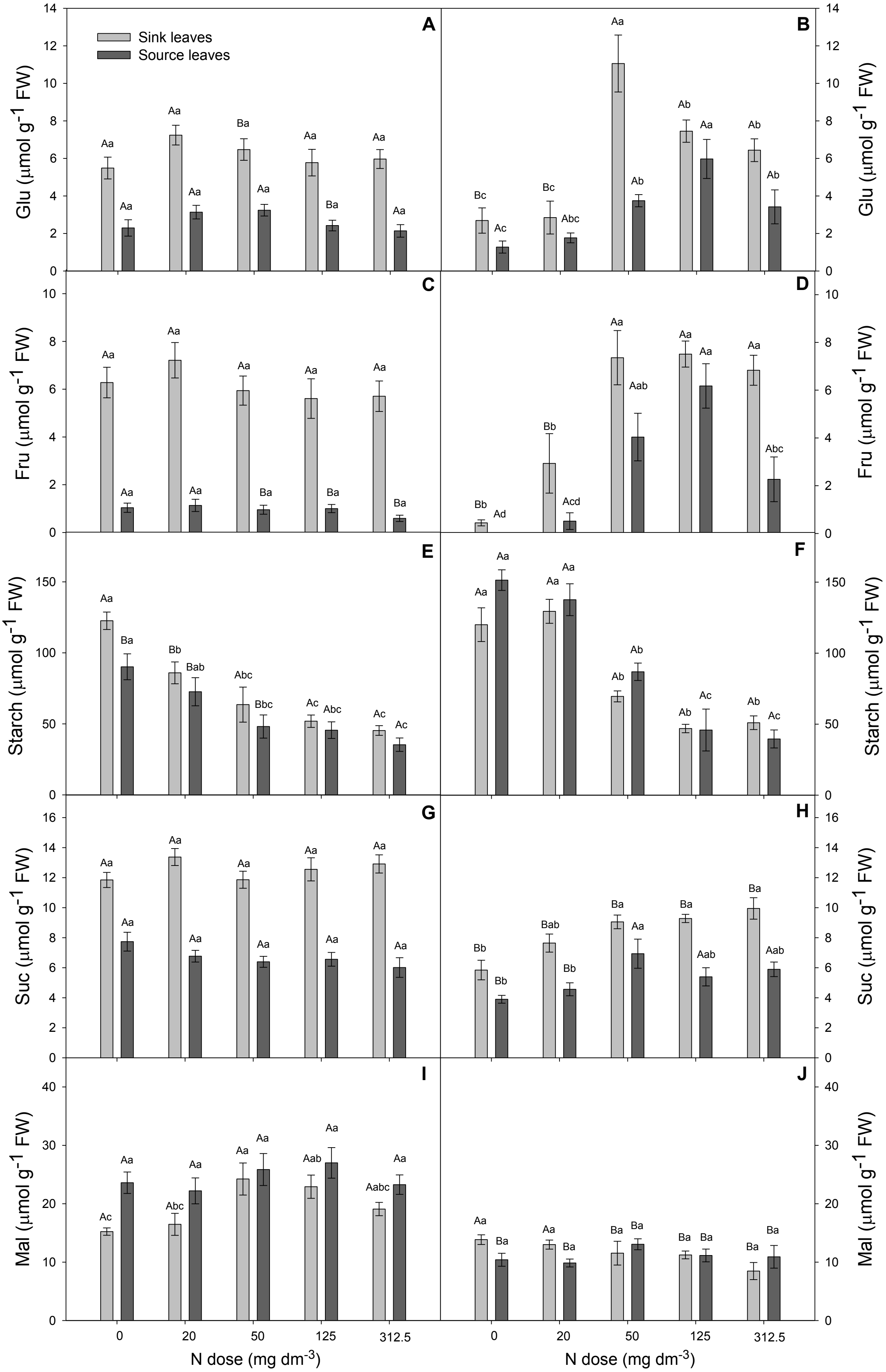
A
B
C
D
E
F
G
H
I
J
Sink leaves
Source leaves
Glu (µmol g^{-1} FW)
Fru (µmol g^{-1} FW)
Starch (µmol g^{-1} FW)
Suc (µmol g^{-1} FW)
Mal (µmol g^{-1} FW)
N dose (mg dm^{-3})
0
20
50
125
312.5
Aa Aa Aa Aa Ba Aa Aa Ba Aa Aa
Bc Ac Bc Abc Aa Ab Ab Aa Ab Ab
Aa Aa Aa Aa Aa Ba Aa Ba Aa Ba
Bb Ad Bb Acd Aa Aab Aa Aa Aa Abc
Aa Ba Bb Bab Abc Bbc Ac Abc Ac Ac
Aa Aa Aa Aa Ab Ab Ab Ac Ab Ac
Aa Aa Aa Aa Aa Aa Aa Aa Aa Aa
Bb Bb Bab Bb Ba Aa Ba Aab Ba Aab
Ac Aa Abc Aa Aa Aa Aab Aa Aabc Aa
Aa Ba Aa Ba Ba Ba Ba Ba Ba Ba

Figure S17. Metabolites content in two *C. chinense* accessions, Biquinho (left) and Habanero (right), submitted to different doses of nitrogen. The analyses were performed on sink leaves (light gray bars), source leaves (dark gray bars) before (24h) the second fertilization (19 DAF). In the sequence is presented the content of starch (A and B), sucrose (C and D) and malate (E and F). Values are presented as means ± SE of five individual plants per cultivar. Uppercase letters indicate significant differences between cultivars for each dose and lowercase letters indicate significant differences between N fertilization for the same cultivar at $P \leq 0.05$ according to Tukey's multiple comparison test.

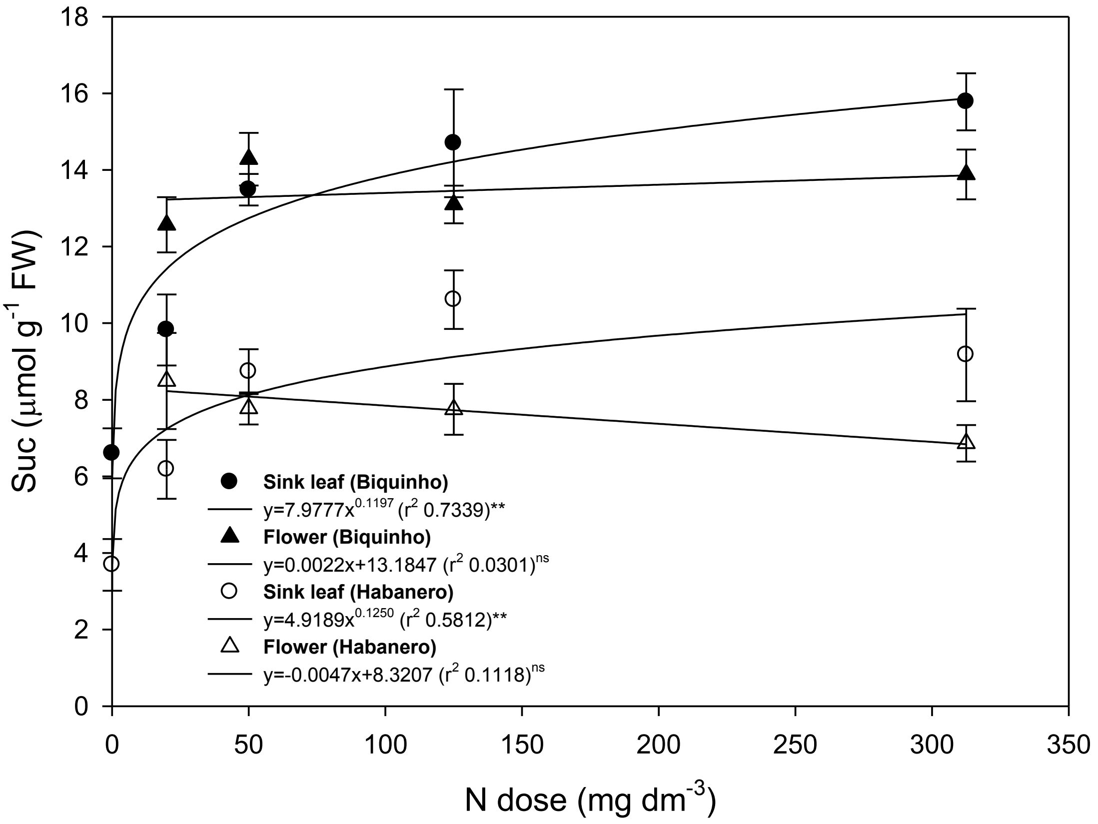

Figure S18. Graphs, equations and statistical analysis of the regressions to sink leaf (circle) and flower (triangle) of Biquinho (black) and Habanero (white). The regression models were tested by F-test and the coefficients by *t*-test at $P \leq 0.05$. For the power models we used the N doses from 0 to 312.5 mg dm^{-3}. For the linear model we used the N doses from 20 to 312.5 mg dm^{-3} due to no production of flowers in plants under N deprivation. The point of intersection indicates the N dose where the sucrose supply is the same for both organs at 59 DAF.

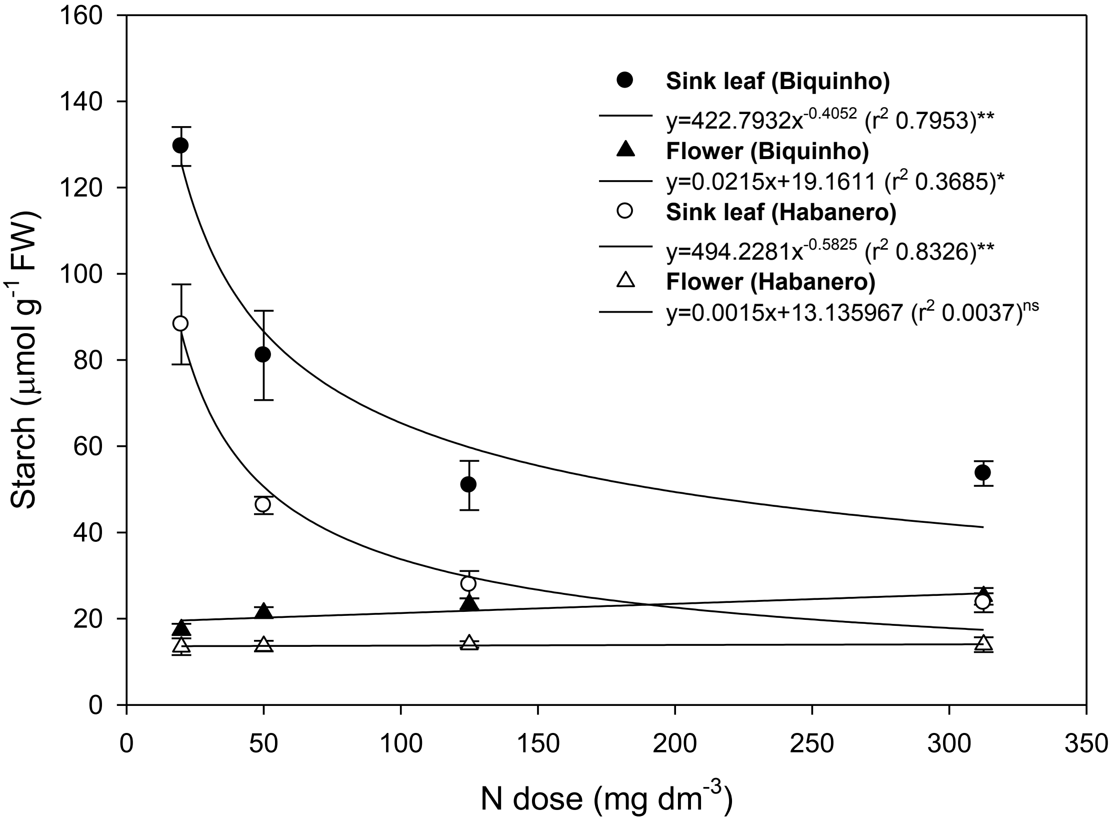

Figure S19. Graphs, equations and statistical analysis of the regressions to sink leaf (circle) and flower (triangle) of Biquinho (black) and Habanero (white). The regression models were tested by F-test and the coefficients by *t*-test at $P \leq 0.05$. For the power and linear models were used the N doses from 20 to 312.5 mg dm^{-3}.

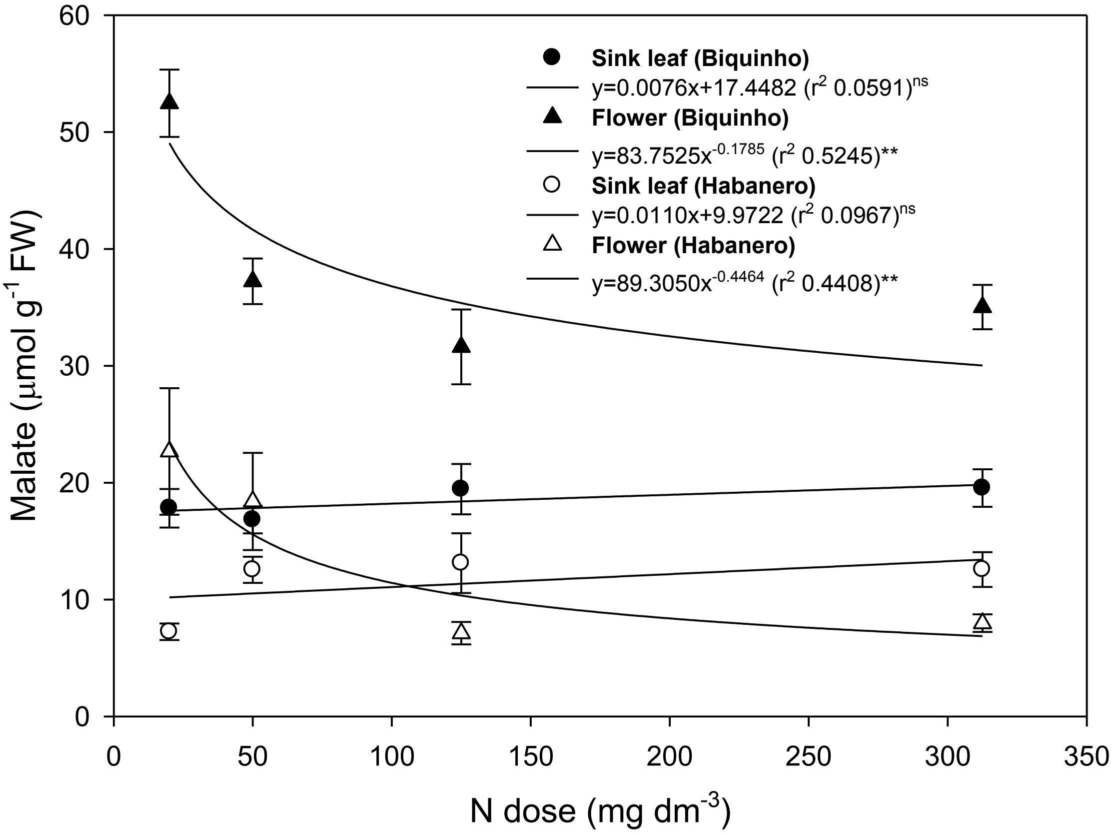

Figure S20. Graphs, equations and statistical analysis of the regressions to sink leaf (circle) and flower (triangle) of Biquinho (black) and Habanero (white). The regression models were tested by F-test and the coefficients by *t*-test at $P \leq 0.05$. For the power and linear models were used the N doses from 20 to 312.5 mg dm^{-3}.

CHAPTER 2

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SOURCE STRENGTH MODULATES FRUIT SET BY STARCH TURNOVER AND EXPORT OF BOTH SUCROSE AND AMINO ACIDS IN PEPPER

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Running title: Metabolic shifts influencing fruit set in pepper

HIGHLIGHT

- Primary metabolites in source leaves and flowers influence fruit set;
- Source strength modulates fruit set by starch turnover and export of both sucrose and amino acids in pepper.

ABSTRACT

Fruit set is an important yield-related parameter which varies drastically due to genetic and environmental factors. Here, two commercial cultivars of *Capsicum chinense* (Biquinho and Habanero) were evaluated in response to light intensity (unshaded and shaded) and N supply (deficiency and sufficiency) to understand the role of source strength on fruit set at the metabolic level. We assessed the metabolic balance of primary metabolites in source leaves during the flowering period. Furthermore, we investigated the metabolic balance of the same metabolites in flowers to gain more insights into their influence on fruit set. Genotype and N supply had a strong effect on fruit set and the levels of primary metabolites, while light intensity had a moderate effect. Higher fruit set was mainly related to the export of both sucrose and amino acids from source leaves to flowers. Additionally, starch turnover in source leaves, but not in flowers, had a central role on the sucrose supply to sink organs at night. In flowers, our results not only confirmed the role of daily supply of carbohydrates on fruit set but also indicated a potential role of the balance of amino acids and malate.

Keywords: pepper; metabolism; flowers; shade; nitrogen; yield

List of abbreviations

ATP – adenosine triphosphate
DAF – days after flowering
RGR – relative growth rate
SEM – structural equation modeling
TCA – tricarboxylic acid

Introduction

The interaction between light intensity and nitrogen metabolism has been explored for a wide range of crops (Abbate et al. 1995; Aloni et al. 1994; Chan and Mackenzie 1972; Grashoff and D'Antuono 1997; Tabatabaei et al. 2008). Plants can tolerate or avoid shade by using different mechanisms, e.g. via alterations in leaf physiology, biochemistry, anatomy, morphology, and plant architecture (Roig-Villanova and Martínez-García 2016; Valladares and Niinemets 2008). Low growth rates, thinner leaves, reduced apical dominance (increased branching) and low elongation are common responses in shade tolerant plants (Casal 2012; Gommers et al. 2013; Martínez-García et al. 2010; Roig-Villanova and Martínez-García 2016). On the other hand, increases in plant height, horizontal leaves, reduced branching, decreased leaf blade area, and early flowering are usually observed in shade-avoiding plants (Casal 2012; Smith and Whitelam 1997). Crop species often have a limited production of photoassimilates under shade and consequently show a deficient plant immunity as well as exhibit a reduced reproductive development and yield (Ballaré 2014; Casal 2013; Kebrom and Brutnell 2007). Both carbon (C) and nitrogen (N) metabolisms are affected by a decrease in light intensity and modifications in these metabolisms are associated with shade tolerance (Valladares and Niinemets 2008). The amount of ATP consumed for protein synthesis and carbohydrate export from source leaves to the sinks can be critical for a positive C balance under shaded environments (Noguchi et al. 2001). In addition, the variability in leaf N concentration in relation to light intensity has a key role in shade tolerant species (Niinemets 1997), mainly due to the modulation of the light harvesting capacity. Indeed, N supply can influence biosynthesis of essential biomolecules, such as chlorophylls, nucleotides, and proteins.

During crop domestication and the continuous breeding process, several important traits related to crop survival during biotic and abiotic stresses were lost (Kraft et al. 2014; Perry et al. 2007; Pickersgill 1971, 1969; Zsögön et al. 2017). Despite that, landrace accessions of Capsicum can still grow under a wide range of shade conditions (Tewksbury et al. 1999; Tewksbury and Nabhan 2001). Thus, Capsicum accessions are particularly interesting for studies of the interaction between the light intensity and N supply because they are currently farmed in a wide range of conditions, in field under high light intensities, in glasshouse with lower light intensities and under different plant spacing and N supply (Díaz-Pérez 2014, 2013; Pinto et al. 2006a, 2006b; Roberts and Anderson 1994; Rylski et al. 1994; Rylski and Spigelman 1986). As a consequence of these variations in light intensity and N supply, yield-related traits, such as fruit set, are affected (Aloni et al. 1996; Johnson and Decoteau 1996; Marcelis et al. 2004; Medina-Lara et al. 2008; Stroehlein and Oebker 1979). Fruit set is limited by flower abscission, which has been partly attributed to a decrease in carbohydrate supply (Aloni et al. 1997, 1996; Turner and Wien, 1994a). Thus, it can be influenced by low light intensity and/or variation in N supply (Aloni et al. 1997, 1996; Marcelis et al. 2004; Stroehlein and Oebker 1979). In Capsicum, flower abscission has been concisely explained by an unbalanced demand/supply for assimilates due to the source-sink relation (Heuvelink et al. 2004; Marcelis et al. 2004; Wubs et al. 2009b).

The molecular control of the source-sink relationship is strongly associated with the synthesis and transport of sucrose and amino acids (Lalonde et al. 2004). Sucrose transporters (*SUC*s/*SUT*s and *SWEET*s) influence the sucrose efflux, whereas cell wall (*CWIN)*, cytoplasmic (*CIN)* and vacuolar (*VIN*) invertases influence sucrose influx and consequently impact fruit set and yield (Bihmidine et al. 2013; Chang and Zhu 2017; Chen et al. 2012; Ruan et al. 2010, 2012). Furthermore, it has also been shown that many sucrose and amino acids transporters are present in floral tissues (Frommer et al. 1995; Okumoto et al. 2002; Dietrich et al. 2004; Su et al. 2004; Grallath et al. 2005; Hammes et al. 2006; Hirner et al. 2006; Meyer et al. 2006; Tegeder and Rentsch 2010; Borghi and Fernie 2017). Flowers and seeds represent the major N sink during reproductive growth (Tegeder and Rentsch 2010). In Capsicum, flower buds are able to import approximately 150 µg of sucrose h^{-1}, assuming that 90% of the imported assimilates are in the form of sucrose (Aloni et al. 1991), which represent a considerable amount of carbohydrates necessary for the flower maintenance. This massive amount of C is provided by mature leaves, which are considered as the major C- and N-sources to sink organs (Chang and Zhu 2017; Tegeder 2014; Tegeder and Hammes 2018; Tegeder and Masclaux-Daubresse 2018) and thus the metabolism of source leaves represent a key factor for fruit set (Wubs et al. 2009a).

Source strength manipulated by shade has been associated with the fruit set via the amount of sucrose taken up by flowers of *C. annuum* (Aloni et al. 1997, 1996). In addition, changes in light intensity, via modification of the plant density or leaf pruning, also resulted in altered flower and fruit abortion in pepper plants

(Heuvelink et al. 2004; Marcelis et al. 2004). Despite its importance for Capsicum yield, our current knowledge of the (i) metabolic regulation of the interactions between N and light intensity and (ii) how variations in day / night source strength can determine fruit set remains rather limited. In this study, two commercial *C. chinense* cultivars, Biquinho and Habanero, contrasting in terms of fruit set and fruit size were cultivated under distinct light intensities and N supply. Metabolic traits were evaluated in both source leaves and flowers. Importantly, the levels of primary metabolites were analyzed at the end of the day (dusk) and at the end of the night (dawn), thus allowing to identify diurnal variations in metabolite supply to the reproductive organs and assess separately the potential importance of C and N supply at daytime and night on the fruit set and other yield related traits.

Results

Fruit set and fruit yield of C. chinense are moderately influenced by light intensity but markedly by the N supply and cultivars

Biquinho and Habanero are commercial cultivars of *C. chinense* with a noticeable difference in fruit set (Table S1). We observed a moderate effect of light intensity on fruit set, where unshaded plants exhibited a higher fruit set than shaded plants (Table 1 and 2). On the other hand, there was a considerable effect of the N supply and cultivars on fruit set (Table 1). Biquinho displayed higher fruit set than Habanero under any N supply (Figure 1A). In addition, both cultivars exhibited higher fruit set under 125 than 20 mg of N dm^{-3} (Figure 1A).

Concerning the fruit yield (g/plant), there was an interaction effect between the light intensity and N supply (Table 1). Plants under 125 mg of N dm^{-3} displayed higher fruit yield than plants under 20 mg of N dm^{-3} under any light intensity (Figure 1B). Furthermore, there was also an interaction effect between the N supply and cultivars on fruit yield (Table 1). Biquinho had higher fruit yield than Habanero under any N supply, showing the major effect of cultivar (Figure 1C). Additionally, both cultivars showed higher fruit yield under 125 compared to 20 mg of N dm^{-3} (Figure 1C). Overall, the factors cultivar and N supply displayed a large influence on fruit set and fruit yield of *C. chinense,* while there was only a moderate effect of the light intensity.

Balance of C and N metabolism-related metabolites in source leaves

After determining the metabolite levels, we used the difference between the metabolite content at dusk and dawn to calculate the balance of each metabolite in source leaves (see Tables S2 and S3 for raw data). Our results revealed that there is no effect of the evaluated factors on glucose balance (Table 1). In contrast, fructose levels accumulated at daytime in plants under 125 mg of N dm^{-3} or in the cultivar Habanero; on the other hand, there was a slightly depletion of fructose at daytime in plants under 20 mg of N dm^{-3} or in Biquinho (Table 1 and 2). Habanero also displayed higher sucrose accumulation than Biquinho at daytime (Table 1 and 2).

The malate balance in source leaves showed a triple interaction between the light intensity, N supply and cultivars (Table 1). Shaded Biquinho plants under 125 mg of N dm^{-3} exhibited higher malate balance than other combinations of factors (Table S4). Also, it is worthy to note that Biquinho showed in average about 2.4 times more malate in source leaves than Habanero (Table S2 for raw data). Concerning the starch turnover, there was an interaction effect between the level of shade and N supply (Table 1). According to this interaction, the starch turnover was higher in shaded plants when they were supplied with 125 instead of 20 mg of N dm^{-3}. Interestingly, the N supply did not alter the starch turnover of unshaded plants (Figure 1D). Additionally, there was an interaction effect between the light intensity and cultivar on the starch turnover (Table 1). In this case, Biquinho exhibited higher starch turnover than Habanero in unshaded condition, without differences between cultivars under shade (Figure 1E). These results show that Biquinho has higher starch turnover under unshaded condition and that N supply has a major effect on starch turnover in plants under shade.

The total amino acids balance varied according to the N supply. Amino acids accumulated in source leaves of plants grown under high N during the daytime, but decreased under low N (Table 1 and 2). In addition, the total amino acids levels decreased during daytime in Biquinho leaves and in unshaded plants, while they accumulated in Habanero and in shaded plants. Furthermore, the cultivars differed in terms of protein balance. Biquinho exhibited a slight decrease of about 8.96% in the levels of protein during daytime whereas Habanero showed a slight accumulation of about 11.62% (Table 1 and 2). Similar to proteins, NO_3^- levels decreased during daytime in Biquinho but accumulated in Habanero (Table 1 and 2). An interaction effect between the N supply and cultivars was observed for the balance of NH_4^+ (Table 1). Interestingly, there was a higher NH_4^+ accumulation in Habanero than

Biquinho under any N supply during daytime (Figure 1F). Together, these results suggest that the genetic differences between cultivars and N supply are responsible for large daily variations in the primary metabolism of source leaves in *C. chinense*. On the other hand, variation in the light intensity influenced the balance of amino acids and the starch turnover according to the interactions between light intensity and N supply as well as light intensity and cultivar.

C and N metabolism-related metabolites in flowers

In order to calculate the balance of each metabolite in flowers, we also evaluated the difference between the metabolite levels at dusk and dawn (see Tables S5 and S6 for raw data). There was no significant effect of the evaluated factors on fructose balance (Table 1). On the other hand, there was a significant crossover interaction between N supply and cultivars on glucose balance in flowers, indicating that the differences in means of cultivars were opposite under different N supplies (Table 1 and Figure 1G). Nevertheless, no significant difference was found between main effects. Similar result was found for light intensity and N supply effect (Table 1 and Figure 1H). In addition, there was also an interaction effect between the N supply and cultivars on sucrose balance (Table 1), wherein Biquinho exhibited higher sucrose accumulation in flowers during daytime than Habanero under 125 mg of N dm^{-3} (Figure 1I).

Concerning the malate balance in flowers, there was an interaction between the light intensity and cultivars. Malate accumulated in Habanero during the daytime but decreased in Biquinho under any N supply (Table 1 and Figure 1J). Furthermore, Habanero flowers exhibited larger starch accumulation than Biquinho at daytime (Table 1 and 2). Altogether, these results suggest a higher sucrose import by Biquinho flowers, mainly when the plants were grown under optimal N supply. On the other hand, Habanero exhibited higher starch and malate accumulation in flowers in relation to Biquinho.

In relation to the balance of total amino acids, high N led to an increase in the levels of amino acids in flowers at daytime (Table 1 and 2). In addition, amino acids decreased in Biquinho flowers during daytime (Table 1 and 2). Similarly, there was a larger depletion of protein content in Biquinho flowers in comparison with Habanero during daytime (Table 1 and 2). Furthermore, we observed a crossover interaction effect between the light intensity and N supply on proteins balance, however, there was no significant difference between the mains effects (Table 1 and Figure 1K). The flowers of Biquinho showed higher decrease in NO_3^- and NH_4^+ levels during daytime than Habanero flowers (Table 1 and 2). In general, Biquinho had a higher depletion of the N related metabolites at daytime compared to Habanero. Thus, these results indicate that changes in flower metabolite contents are primarily explained by a cultivar effect and that environmental variations (N supply and light intensity) have a lower impact.

Pearson correlation and Structural Equation Modeling (SEM) analysis reveal the key metabolites modulating fruit set

After the univariate analysis, we investigated whether the balance of metabolites correlate with fruit set and between each other. Thus, we performed a Pearson's correlation analysis between each trait in source leaves and flowers (Figure 2), and here we will highlight the significant correlations ($P \leq 0.01$). As expected, fruit set correlated positively with fruit yield. In source leaves, starch turnover showed a positive correlation with fruit set while the balance of sucrose, NH_4^+ and amino acids correlated negatively with this trait. In contrast, sucrose balance in flowers displayed a positive correlation with fruit set while the balance of NH_4^+, amino acids, malate and proteins in flowers correlated negatively with it.

Subsequently, we applied a SEM, a statistical approach for the analysis of complex datasets with multiple mutually intercorrelated dependent and independent variables (Grace, 2006; Grace et al., 2010; Kline, 2011; Lamb et al., 2011), considering the correlations between the balance of all metabolites in each organ (Figure 3, Table S7 and S8). According to our initial path model (Figure 3A), the metabolite balance in flowers can influence directly the fruit set while the metabolite balance in source leaves can affect fruit set indirectly. There were positive correlations between the balance of sucrose and fructose, sucrose and proteins, sucrose and amino acids, fructose and amino acids, and proteins and amino acids in source leaves (Figure 3B and Table S7). Only the correlation between the balance of sucrose and starch turnover was negative in source leaves (Figure 3B and Table S7). In flowers, there were positive correlations between the balance of fructose and glucose, starch and malate, starch and amino acids, malate and proteins, malate and amino acids, and proteins and amino acids (Figure 3B and Table S7). On the other hand, the balance of sucrose and malate, sucrose and proteins, sucrose and amino acids were negatively correlated in flowers (Figure 3B and Table S7). Interestingly, 86.6% of the correlations realized by the SEM were also significant by Pearson's correlation at $P \leq 0.01$. The exceptions were the positive correlations between the balance of sucrose and fructose in source leaves besides proteins and amino acids in flowers, that were correlated at $P \leq 0.05$.

Among the metabolites in flowers, the balance of amino acids, malate, and starch turnover displayed significant regression weights, explaining 66% of the fruit set (Figure 3 and Table S7). In addition, fruit set explained 45% of the fruit yield. Regressions instead of correlations showed to be more appropriate to explain the link of sucrose and amino acids between source leaves and flowers (Figure 3B and Table S8). The initial path of the complementary model was confirmed, where the balance of sucrose in source leaves influenced the balance of sucrose and amino acids in flowers. Also, the balance of amino acids in source leaves influenced the balance of amino acids in flowers (Figure 3B and Table S8). Moreover, the balance of sucrose and amino acids in flowers were also directly explaining 54% of the fruit set in the complementary model (Figure 3B and Table S8).

Together, these results confirmed the starch turnover and the balance of amino acids and sucrose in source leaves and flowers as the major metabolites regulating fruit set in Capsicum. In source leaves we highlighted the role of the starch turnover and export of sucrose and amino acids on fruit set. Regression analysis confirmed sucrose and amino acids as linking metabolites between source leaves and flowers. Interestingly, both Pearson's correlation and SEM approaches highlighted a possible role of the malate depletion in flowers at daytime on fruit set.

Discussion

Source strength indirectly impacts fruit set at the metabolic level

Fruit set is limited by the abscission / abortion of flower buds, flowers, and fruits in a wide range of crops, such as *Cucumis sativus*, *Juglans regia*, *Vicia faba*, *Phaseolus vulgaris*, *Glycine max*, *Vigna radiata*, *Vigna unguiculate,* and *Citrus* spp. (Ascough et al. 2005; Bacci et al. 2006; van Doorn and Stead 1997). Flower abortion and fruit set seem to be indirectly affected by the source strength on *Cucumis sativus*, *Prunus persica,* and *Actinidia deliciosa* (Marcelis 1993; Marini and Sowers 1990; Tombesi et al. 1993). The fruit set of *C. annuum* is also influenced by source strength and vary due to cultivar and environmental factors, such as light intensity, photoperiod, CO_2 concentration, temperature, relative air humidity, water and nutrient supply (Aloni et al. 1996; Marcelis et al. 2004; Turner and Wien 1994b; Wubs et al. 2009a). In our study, we varied the shading levels to manipulate the source strength of *C. chinense* cultivars. Previous studies have suggested a large influence of shade conditions on fruit set of *C. annuum* (Aloni et al. 1996; Marcelis et al. 2004; Wien et al. 1989). In contrast, in *C. chinense,* we only observed moderate effects of shade on fruit set. Moreover, the variation in shading levels led to only moderate changes on the levels of primary metabolites in source leaves. On the other hand, the variation in the N supply and the cultivar effect led to remarkable alterations on the levels of primary metabolites and a strong effect on fruit set.

In our study, we estimated the amount of the main primary metabolites at dusk and dawn, in flowers and matures leaves. This allowed us to calculate the balance of compounds during daytime and nighttime in source and sink tissues. The balance of sucrose and amino acids was largely influenced by the genotypes. Our results suggest that the cultivars display different behavior, Biquinho having a higher export of sucrose and amino acids from source leaves to flowers than Habanero at daytime. We observed that Habanero plants exhibit lower photosynthetic rate than Biquinho (411.21 ± 40.46 and 556.52 ± 27.13 μmol CO_2 kg^{-1} dry weight s^{-1}, respectively). The lower photosynthesis in Habanero plants might be related with the accumulation at daytime of sucrose observed in source leaves (Table 1 and 2) leading to feedback inhibition of the Calvin-Benson cycle (Iglesias et al. 2002; Stitt et al. 1990). Indeed, both cultivars started the day with sucrose content about 30 μmol g^{-1} but only in Habanero there was an increase at dusk (about 50 μmol g^{-1}) (Table S2). Additionally, if our data suggest higher amino acids export in Biquinho, this cultivar showed in average 9.5% more amino acids and especially 17.5% more protein in source leaves than Habanero (Table S3). These two traits can be related with the higher photosynthesis and sucrose transport to sinks (Perchlik and Tegeder 2018; Zhang et al. 2010). Moreover, the correlation between the balance of sucrose and amino acid levels observed in this study (Figure 2) suggest that the export of amino acids from source leaves to flowers can be related to the role of sucrose as the main driver of phloem sap flow (Jensen et al. 2013; Peuke et al. 2015; Tegeder and Hammes 2018). The balance of sucrose and amino acids in source leaves negatively correlated with the fruit set, suggesting that (1) higher export at daytime of these metabolites and / or (2) preferential storage of carbohydrates as starch in source leaves during daytime for sucrose export at night, associated with higher fruit set in *C. chinense* (Figure 2). In agreement with a storage of carbohydrates for night export, the starch turnover in source leaves correlates positively with the fruit set (Figure 2) and represent 85.5 and 42.0% of the total carbon turnover in Biquinho and Habanero, respectively.

Sugars (mainly sucrose) and amino acids (such as glutamine, glutamate, aspartate, and asparagine in most of the species) are the main C- and N-sources transported forms from photosynthetic active leaves to heterotrophic tissues (Braun et al. 2014; Knoblauch et al. 2016; Lalonde et al. 2003; Lemoine et al. 2013; Tegeder and Hammes 2018; Tegeder and Rentsch 2010). Besides the crucial significance of the sucrose for maintenance of flower buds (Aloni et al. 1997, 1996, 1991), the results presented in this study also highlighted the importance of amino acids for *C. chinense* fruit setting.

Transport of N containing compounds to newly developing flowers is an important determinant of flower set and growth (Gifford et al. 1984; Pate 1980; Patrick and Stoddard 2010). Biquinho, the cultivar displaying higher fruit set, exhibited a higher depletion of amino acids during daytime concomitant with increased NO_3^- reduction and NH_4^+ assimilation in comparison with Habanero plants. Moreover, the decrease in protein content of about 8.96% during daytime in Biquinho leaves suggested a possible protein hydrolysis at daytime to ensure an amino acids supply to the long-distance transport path (Figure 2 and 3) (Barneix and Causin 1996; Tegeder and Hammes 2018). Together with the cost of carbohydrate export from leaves, the ATP consumed for protein synthesis is a major issue for a positive carbon balance under shaded environments (Noguchi et al. 2001). It has been shown that up to a quarter of the decrease in relative growth rate (RGR) between large and small Arabidopsis accessions is attributed to ATP consumption in protein synthesis associated with protein turnover (Ishihara et al. 2017). However, the energy consumed can be compensated, at least partially, by a higher photosynthetic rate in Biquinho compared to Habanero, ensuring higher production of energy for maintenance and C skeletons necessary to support development and growth of reproductive organs. In addition, it has been shown that a large amount of enzymes related with photosynthetic apparatus are present in excess in leaves and that a decrease in their amount can be compensated via probably postranslational regulation mechanisms (Stitt et al. 2010). In fact, Biquinho plants under low N supply were able to maintain high photosynthesis (530.68 ± 14.33 in comparison with 525.16 ± 27.29 µmol CO_2 kg^{-1} s^{-1}) despite almost 50% of reduction in the total protein amounts in their source leaves as compared to adequate N doses (2.94 ± 0.31 and 5.59 ± 0.54 mg g^{-1} fresh weight, respectively).

As expected, plants under higher N supply contained more amino acids at daytime than plants under lower N supply. Accordingly, both cultivars displayed higher fruit set under optimal N (Table 1 and Figure 1A). There was a negative correlation between the balance of amino acids and fruit set (Figure 2) as well as a positive correlation between the amino acids level at dawn and fruit set (Figure S2). Furthermore, the role of amino acids export at daytime on fruit set was clearer because the amino acids balance was the only metabolite's balance affected by the light intensity without interactions with other factors (Table 1). Consequently, unshaded plants had higher fruit set and also a decrease of amino acids levels at daytime in comparison with shaded plants (Table 2). Altogether, it seems reasonable to suggest that the amino acids export is an important trait to achieve higher fruit set. Then, an adequate N nutrition can avoid a sharp decrease of N compounds in the source leaves and ensure adequate production of sucrose to export.

The starch turnover was influenced by interactions between the light intensity and N supply as well as light intensity and cultivar (Table 1 and Figure 1). Starch is the major storage metabolite in many plant species and leaf starch is often transitory, being in many growth conditions synthesized and degraded almost completely within day/night cycle (Caspar et al. 1985; Lloyd and Kossmann 2015; Schulze et al. 1991; Stitt and Zeeman 2012). Starch has been identified as a key metabolite in the regulation of plant growth (Sulpice et al. 2009). The RGR in Arabidopsis correlated with the starch degradation rate (Gibon et al. 2009; Sulpice et al. 2014). In maize plants, starchless *agps-ml* mutants show a reduced growth and yield, demonstrating the importance of normal starch levels in source leaves to achieve high yields (Schlosser et al. 2012). Sucrose supply during the night is closely related to the starch turnover. Sucrose-phosphate synthase double mutant (*spsa1/spsc*) was strongly impaired in growth and accumulated high levels of starch due to an impaired starch mobilization during the night (Volkert et al. 2014). The negative correlation between starch turnover and sucrose balance as well as the positive correlation between starch turnover and fruit set suggest the role of the transitory starch to supply sucrose to flowers at nighttime (Figure 2 and 3). Considering there is a higher positive balance of C and higher starch turnover in flowers of Habanero but a higher fruit set and a higher starch turnover in source leaves of Biquinho, we can assume than starch degradation in leaves and its export to flowers at night under the form of sucrose is very important to avoid starvation of the flowers at night, mainly for the cultivar Biquinho. Thus, the starch turnover and the export of sucrose and amino acids can be considered important players on the metabolic mechanism to explain how source strength can indirectly influence the fruit set.

New insights into flower metabolism to understand fruit set in C. chinense

While the source strength displays an indirect influence on fruit set (Marcelis 1993; Marini and Sowers 1990; Tombesi et al. 1993), studies on *C. annuum* indicated a direct effect of the photoassimilate allocation to the flower and its metabolism within this organ on fruit set (Aloni et al. 1997, 1996, 1991; Turner and Wien 1994b). With exception of sepals and young petals, other floral parts are dependent of photoassimilates import (Müller et al. 2010). Sucrose and amino acids (e.g., glutamine, glutamate, aspartate, and asparagine) are the main metabolites imported by heterotrophic floral parts (Braun et al. 2014; Ho and Nichols 1975; Knoblauch et al. 2016; Lalonde et al. 2003; Müller et al. 2010; Tegeder and Hammes 2018; Tegeder and Rentsch 2010). The regression analysis performed in this study suggests an important role for sucrose and amino acids as transport metabolites between source leaves and flowers (Figure 3B and Table S8). After sugar uptake, the flower capacity to accumulate sugars and starch at daytime until pollination is considered an important factor for flower retention and fruit set of *C. annuum* (Aloni et al. 1997, 1996, 1991). In terms of balance of sugar at daytime, the flower of Biquinho, a cultivar with higher fruit set, accumulated about 2.00 mg of C g^{-1} fresh weight, while Habanero, a cultivar with lower fruit set, accumulated about 1.01 mg of C g^{-1} fresh weight in its flowers. However, besides the sucrose balance in flowers were positively correlated with fruit set in *C. chinense* (Figure 2) and the role of the starch turnover in flowers was suggested by regression analysis by SEM (Figure 3B and Table S7), the balance of all metabolites analysed at daytime represented about 0.81 and 1.09 mg of C g^{-1} fresh weight in Biquinho and Habanero, thus suggesting than the resources at night in Biquinho were largely coming from the source leaves, and in particular starch (Table 2). In addition, the C accumulation at dusk was about 12.44 and 15.76 mg of C g^{-1} fresh weight in Biquinho and Habanero flowers (Table

S5), respectively, highlighting a possible role of the C balance and accumulation in source leaves on fruit set. Thus, the importance of starch in flowers can be further questioned in relation to the starch in source leaves because Habanero showed a three times higher turnover of starch in its flowers compared to Biquinho, despite a much lower fruit set.

The balance of total amount of amino acids in flowers have an important role on fruit set of *C. chinense* and a remarkable contrast between the two cultivars was observed (Table 1 and 2; Figure 2 and 3). Biquinho, the cultivar with higher fruit set, exhibited a depletion of amino acids at daytime while Habanero, the cultivar with lower fruit set, exhibited an accumulation. In flowers, amino acids can be utilized in the synthesis of enzymes and structural proteins or as precursors of N-containing secondary metabolites and signaling molecules (Borghi and Fernie 2017). Together with the depletion in the protein content at daytime in flowers, the results suggest a *de novo* synthesis of amino acids to the formation of secondary metabolites (Maeda et al. 2011, 2010; Oliva et al. 2017; Spitzer-Rimon et al. 2010; Widhalm et al. 2015). Indeed, genes related to secondary metabolism, fatty acid catabolism, and amino acid transport are up-regulated during petal development of Snapdragon flowers (Muhlemann et al. 2012). Thus, the results suggest that the depletion of amino acids in Biquinho can be related with the development of floral parts or the formation of secondary metabolites.

The malate balance in flowers was also highlighted by its negative correlation with fruit set (Figure 2 and 3). Malate is known as a carbon source related to growth (Finkemeier and Sweetlove 2009; Lehmann et al. 2015). As a sink organ, the accumulation of malate in flowers might indicate lower TCA cycle turnover and energy limitation, once malate is an important C-source to the anapleurotic flux replenishing intermediates of the TCA cycle (Lehmann et al. 2015). Biquinho displayed during daytime a depletion of malate of about 0.25 mg of C g^{-1} fresh weight while Habanero displayed an accumulation of about 0.17 mg of C g^{-1} fresh weight. These results suggest that malate can be used as a C-source for Biquinho at daytime, probably because flowers of cultivars with smaller fruits and higher fruit set are always competing with fast-growing fruits (Marcelis et al. 2004). In this case, the ability of the flowers to use an alternative C-source at daytime may be an important strategy to flower and fruit set.

Hence, the results indicated a possible role of the balance of sugars as well as of the balance of amino acids and malate on fruit set of *C. chinense*. However, the role of starch in source tissues for the supply of C at night has been highlighted, its importance in flowers being more questionable. Further experiments can confirm the role of the starch turnover in flowers and leaves mainly comparing more cultivars with bigger and smaller fruits. In addition, since strong floral sinks are vital for the establishment of new fruits (Bihmidine et al. 2013), further efforts are required to increase our comprehension on the role of individual amino acids and organic acids on floral development and thus fruit set. This could be approached via the investigation of more genotypes harboring contrasting fruit sets, and also via metabolic flux analysis, to the identification of metabolic pathways involved in fruit set. Key genes could then be investigated by virus-induced gene silencing (VIGS). A better understanding of the relationship between the genetic base of fruit set and metabolism has potential applications in Capsicum breeding.

Material and methods

Plant growth conditions and fruit set determination

Seeds from Biquinho and Habanero plants were obtained from TopSeed® (Agristar do Brasil Ltd), germinated and grown on a commercial substrate (Tropstrato® HT; Vida Verde Ltd). Afterward, 46 days old plantlets were transplanted to pots with 5 dm^3 of a Yellow-Red Oxisol soil, ensuring no restriction to the root development. Before transplantation, the soil used for cultivation was sieved, homogenized and its acidity neutralized by applying $CaCO_3$ and $MgCO_3$ (molar ratio of 3.5:1). Then nutrients were added according to Novais et al. (1991), with the exception of N. The total amount of nutrients was 300, 150, 40, 0.81, 1.33, 1.55, 3.66, 0.15 and 4.00 mg dm^{-3} for P, K, S, B, Cu, Fe, Mn, Mo, and Zn, respectively. Due to the low N content of the original soil, 5 mg dm^{-3} of this nutrient was applied as NH_4NO_3 to ensure the initial development of the plants after transplantation. To avoid leaching of N from the soil, plastic bags were used to cover the base of the pots.

The experiment was performed in a greenhouse with a temperature of 27 ± 5 °C and an average irradiance of 800 µmol photons m^{-2} s^{-1}. When the plants developed the first sympodial unit, which corresponds to the reproductive differentiation, half of the plants were submitted to 50% shading while the others were maintained without shading. Concomitantly, we applied 20 or 125 mg of N dm^{-3}, using NH_4NO_3 as N source, to obtain plants growing under deficient and optimal amounts of N, respectively. The N was supplied in the same concentrations every twenty days, for a total of four supplementations until the final harvest (for details see Figure S1). All experimental analyses were performed between February and April of 2017 with an average photoperiod of 12h11mins daylight / 11h49mins night (source: Astronomical Applications Dept., U. S. Naval Observatory, Washington, DC). Water was supplied daily to maintain the soil at constant field capacity over the course of the experiment.

The flowering period was determined by labeling all flowers two days after their anthesis. This allowed to determine the fruit set as previously described (Rosado-Souza et al., 2015). At 74 days after flowering (DAF) the fruits were harvested, and the number and weight of fruits were determined.

Metabolite analyses

For metabolite analyses, source leaf and flower samples were harvested 5 days after the second N fertilization (25 DAF), at dawn (between 5 and 6 am) and dusk (between 5 and 6 pm). Samples from two to three days old flowers (after the anthesis) were collected. At the same time, fully expanded leaves, in the third sympodial unit from the apex, were considered as source leaves and collected at the same time points. All samples were snap frozen in liquid nitrogen and stored at -80 °C until further analyses.

Metabolite extraction was performed by grinding the tissues in liquid nitrogen followed by the determination of the sample fresh weight and then ethanol extraction (Cross et al. 2006). In the ethanol extracts were determined, sucrose, fructose, glucose (Fernie et al. 2001), malate (Nunes-Nesi et al. 2007), total amino acid (Cross et al. 2006), NH_4^+ (Sarasketa et al. 2014) and nitrate (Sienkiewicz-Porzucek et al. 2010). Starch and protein levels were determined in the ethanol insoluble fraction, as previously described (Cross et al. 2006). The effect of the shade, N supply, and cultivar on the metabolite balance during daytime and nighttime was determined using the difference between the metabolite content at dusk and dawn, as proposed by Aloni et al. (1996) (Equation 1).

$$\Delta Metabolite = Metabolite\ content\ at\ dusk - Metabolite\ content\ at\ dawn$$

Eq. 1

Statistical analysis

The experiment was performed in a split-split-plot design (Steel et al. 1997), with three factors: light intensity (unshaded and shaded plants) set up as the main plot, N supply (20 and 125 mg of N dm^{-3}) set up as subplot and cultivars (Biquinho and Habanero) set up as sub-subplot. Six replicates were used for each treatment. The normality of the data was verified by the Shapiro-Wilk test and after the variables were analyzed by three-way ANOVA. The separation of significant mean differences was achieved by F-test ($P \leq 0.05$) for each factor effect and *t*-test ($P \leq 0.05$) for factor splitting. These analyses were performed using the PROC GLM software from SAS University Edition. Afterward, a Pearson's correlation was performed using the R ($P \leq 0.05$). Lastly, we used Structural Equation Modeling (SEM) with observed variables to separate the putative direct and indirect effects of source leaves and flowers on fruit set and yield. The SEM was performed using the IBM SPSS Amos 25 software (Amos Development Corporation, Crawfordville, FL, USA) (Arbuckle 2013). For this we used a dataset of 136 datapoints, exceeding the recommended minimums of 100 samples or 5 times the number of model parameters (Grace 2006; Kline 2011). The model fit was tested by χ^2 test ($P > 0.05$) and significant differences were identified by *t*-test ($P \leq 0.05$).

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FIGURES AND TABLES

Table 1. Split-split plot ANOVA performed to indicate the effect of light intensity (unshaded or shade plants), N supply (20 or 125 mg of N dm^{-3}), cultivar (Biquinho or Habanero) and their interactions on fruit set, yield and balance of metabolites in source leaves and flowers. * and ** indicate the statistical significance at $P \leq 0.05$ and $P \leq 0.01$ by F-test, respectively. The data are expressed as F-value.

	ANOVA (F test) - Fruit set and metabolite's balance in source leaves									
Factors	**Fruit set**	**ΔGlucose**	**ΔFrutose**	**ΔSucrose**	**ΔStarch**	**ΔMalate**	**ΔAmino acids**	**ΔProtein**	**ΔNH_4^+**	**ΔNO_3^-**
Light	6.58 *	0.77	1.57	0.00	0.90	4.63	13.65 **	0.00	5.98	0.02
N	186.49 **	1.22	25.37 **	2.39	42.30 **	0.30	36.01 **	0.01	12.03 **	2.20
Cultivar	771.55 **	0.21	20.34 **	189.89 **	63.37 **	12.79 **	89.42 **	8.89 **	93.24 **	5.31
Light x N	0.08	1.09	3.69	2.54	5.16 *	10.49 **	2.54	1.44	0.73	1.71
Light x Cultivar	0.50	2.52	1.62	1.45	29.30 **	6.66 *	3.47	0.03	3.19	0.29
N x Cultivar	31.92 **	0.05	2.18	0.00	4.09	34.96 **	0.20	3.06	10.24 **	2.06
Light x N x Cultivar	0.42	0.00	0.09	0.07	1.03	9.42 **	1.64	2.09	1.79	0.29

	ANOVA (F test) - Yield and metabolite's balance in flowers									
Factors	**Yield**	**ΔGlucose**	**ΔFrutose**	**ΔSucrose**	**ΔStarch**	**ΔMalate**	**ΔAmino acids**	**ΔProtein**	**ΔNH_4^+**	**ΔNO_3^-**
Light	14.64 **	0.01	0.00	3.86	1.26	0.75	0.26	0.36	3.73	2.13
N	1454.61 **	0.78	0.04	1.60	1.93	1.66	8.76 *	0.97	3.17	0.06
Cultivar	149.49 **	1.42	0.05	63.31 **	8.86 **	107.86 **	162.98 **	10.84 *	123.76 **	6.63
Light x N	23.06 **	0.06	0.08	8.45 **	0.22	0.49	1.92	5.35 *	1.40	0.78
Light x Cultivar	0.03	1.95	1.06	2.58	0.04	5.13 *	1.02	0.00	0.34	0.03
N x Cultivar	4.42 *	5.72 *	1.12	12.60 **	0.36	1.16	1.53	0.24	1.75	3.66
Light x N x Cultivar	0.04	2.34	2.43	0.51	0.40	4.27	0.53	2.27	0.11	0.11

Table 2. Mean and significance of each factor, light intensity (unshaded or shaded plants), N supply (20 or 125 mg of N dm^{-3}) and cultivar (Biquinho or Habanero), on fruit set, yield and balance of metabolites in source leaves and flowers according the difference between the content at dusk and dawn. Means followed by stars are statistical different by F-test at $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

	Factor								
	Light			**N**			**Cultivar**		
	Unshaded	*Shaded*		*20*	*125*		*Biq*	*Hab*	
Fruit set (%)	34.36	30.73	*	22.88	42.21	**	52.21	12.88	**
Yield (g/plant)	22.57	20.06	**	8.85	33.78	**	25.31	17.32	**
Source leaves (µmol g^{-1} FW or [a]mg g^{-1} FW)									
ΔGlucose	-0.29	-0.10		-0.33	-0.06		-0.12	-0.27	
ΔFructose	0.23	0.51		-0.11	0.85	**	-0.24	0.98	**
ΔSucrose	10.42	10.42		11.49	9.35		0.92	19.92	**
ΔStarch	54.33	59.17		40.17	73.33	**	77.05	36.45	**
ΔMalate	1.09	2.79		1.78	2.10		2.82	1.06	**
ΔAmino acids	-1.98	0.74	**	-2.83	1.59	**	-4.10	2.87	**
ΔProteins[a]	0.03	0.05		0.01	0.07		-0.77	0.85	**
ΔNH_4^+	0.29	0.36		0.17	0.47	**	-0.06	0.71	**
ΔNO_3^-	-1.48	-1.23		0.02	-2.72		-3.82	1.12	*
Average:									
ΔC[a]	5.36	6.00		4.44	6.92		5.13	6.22	
ΔN[a]	-0.07	0.02		-0.07	0.02		-0.29	0.24	
Flowers (µmol g^{-1} FW or [a]mg g^{-1} FW)									
ΔGlucose	4.69	4.83		4.16	5.37		5.41	4.11	
ΔFructose	4.71	4.65		4.53	4.84		4.55	4.82	
ΔSucrose	2.06	-0.65		1.57	-0.17		6.19	-4.78	**
ΔStarch	11.84	8.34		7.93	12.25		5.45	14.72	**
ΔMalate	-0.34	-1.24		-1.25	-0.33		-5.17	3.59	**
ΔAmino acids	0.90	1.32		0.58	1.64	*	-3.26	5.49	**
ΔProteins[a]	-1.03	-0.90		-0.86	-1.07		-1.41	-0.51	*
ΔNH_4^+	0.35	0.44		0.46	0.33		0.01	0.79	**
ΔNO_3^-	-3.00	-1.18		-1.86	-2.31		-3.35	-0.82	*
Average:									
ΔC[a]	1.32	0.73		0.94	1.11		0.81	1.09	
ΔN[a]	-0.18	-0.12		-0.14	-0.15		-0.36	0.00	

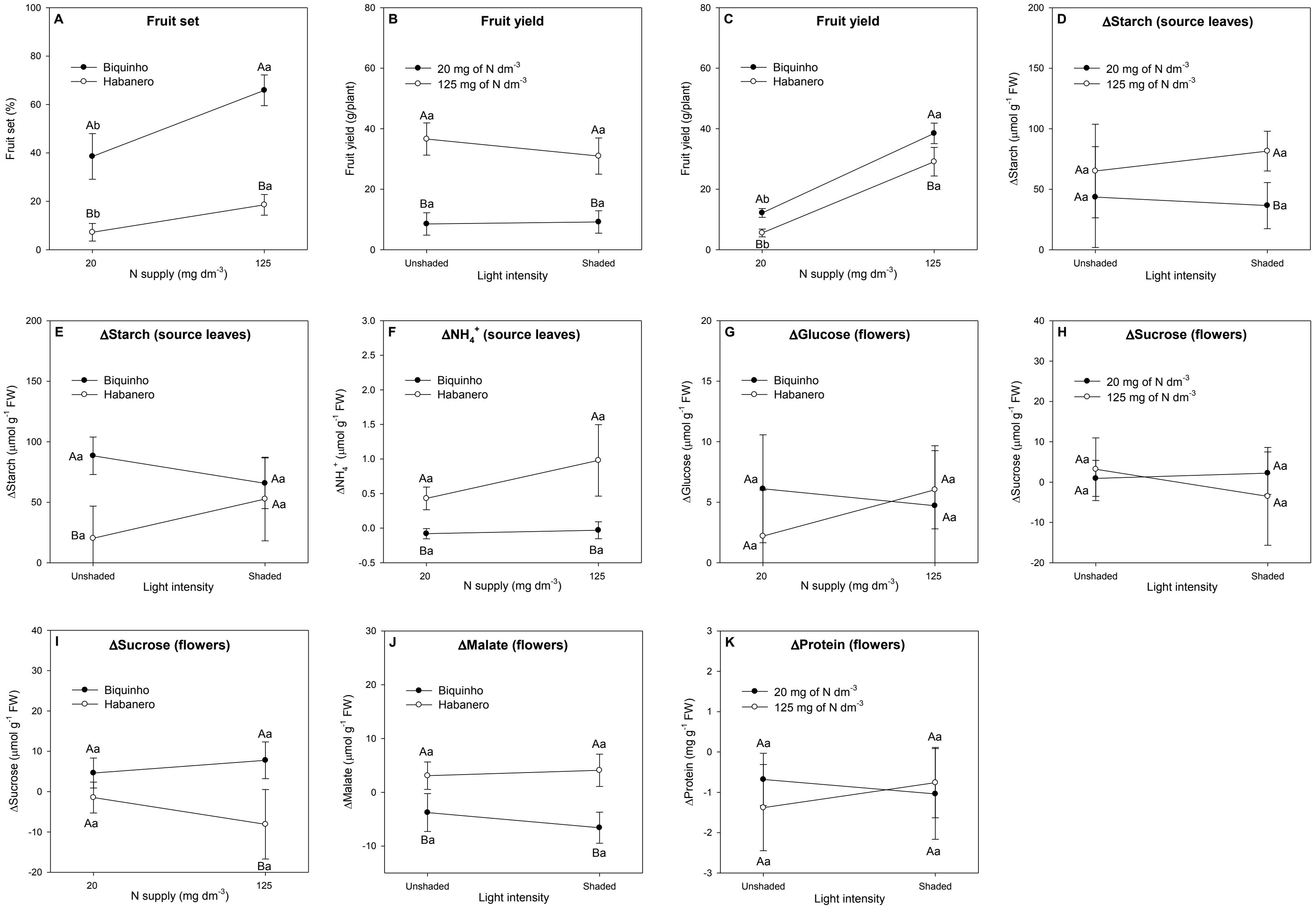
A
Fruit set
Biquinho
Habanero
Fruit set (%)
N supply (mg dm^{-3})
20
125
Ab
Aa
Bb
Ba
B
Fruit yield
20 mg of N dm^{-3}
125 mg of N dm^{-3}
Fruit yield (g/plant)
Light intensity
Unshaded
Shaded
C
Fruit yield
D
ΔStarch (source leaves)
ΔStarch (μmol g^{-1} FW)
E
ΔStarch (source leaves)
F
ΔNH$_4^+$ (source leaves)
ΔNH$_4^+$ (μmol g^{-1} FW)
G
ΔGlucose (flowers)
ΔGlucose (μmol g^{-1} FW)
H
ΔSucrose (flowers)
ΔSucrose (μmol g^{-1} FW)
I
ΔSucrose (flowers)
J
ΔMalate (flowers)
ΔMalate (μmol g^{-1} FW)
K
ΔProtein (flowers)
ΔProtein (mg g^{-1} FW)

Figure 1. Interaction plots (mean ± SD) between the factors light intensity (unshaded or shaded plants), N supply (20 or 125 mg of N dm^{-3}) or cultivar (Biquinho or Habanero) as indicated by ANOVA for fruit set (A), fruit yield (B and C) as well as the balance of metabolites, as starch in source leaves (D and E), NH_4^+ in source leaves (F), glucose in flowers (G), sucrose in flowers (H and I), malate in flowers (J) and protein in flowers (K). Uppercase letters compare the main factor (*x*-axis) for each secondary factor (as indicated in the legend) and lowercase letters compare the secondary factor for each main factor at $P \leq 0.05$ according to *t*-test.

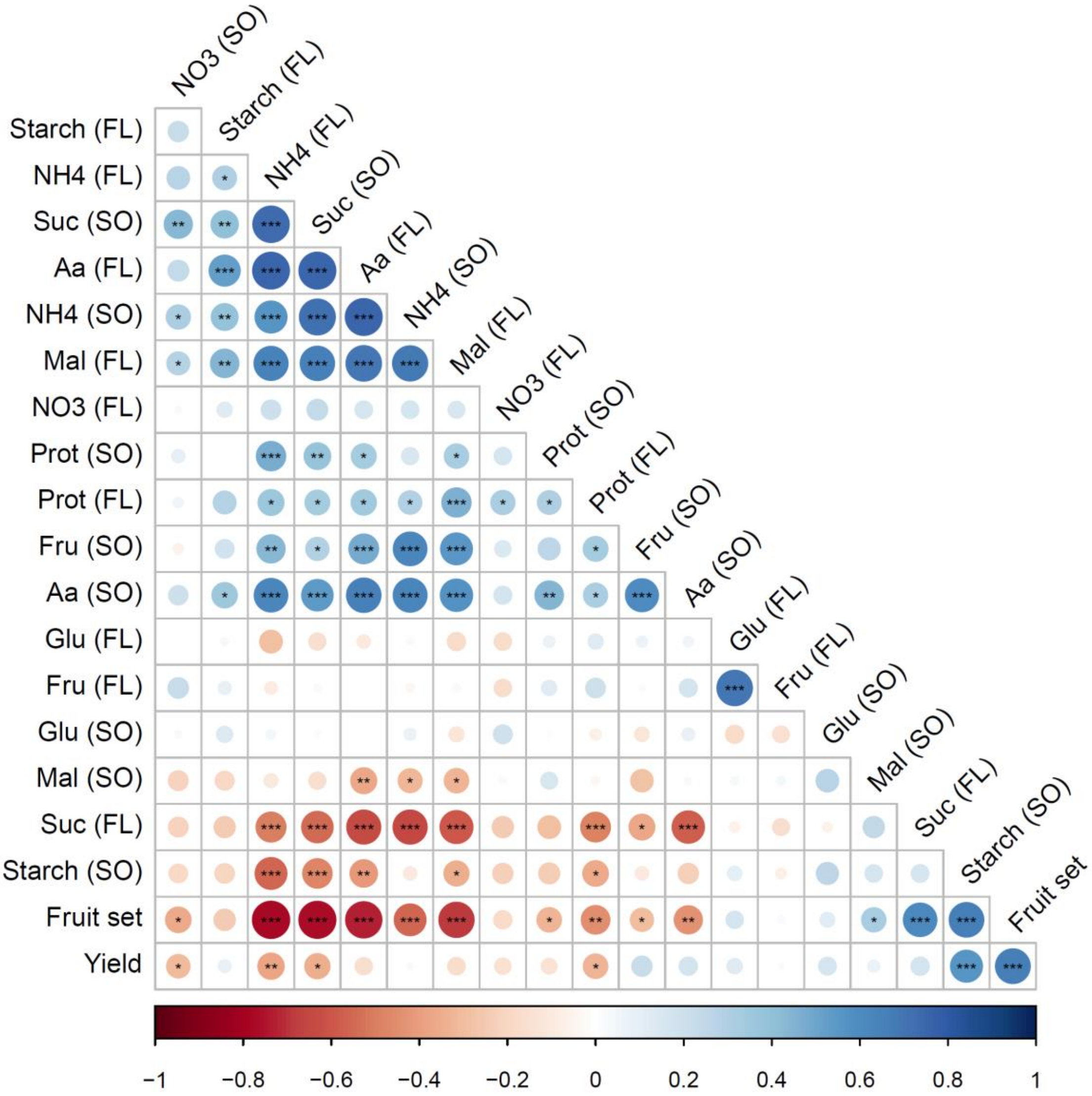

Figure 2. Pearson correlation between fruit set, yield and balance of metabolites in source leaves (SO) and flowers (FL) of two contrasting cultivars of *C. chinense* (Biquinho and Habanero) submitted to light (unshaded or shade plants) and N (20 or 125 mg of N dm^{-3}) variation. Blue and red circles represent positive and negative correlations at $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Abbreviations: glucose (Glu), fructose (Fru), sucrose (Suc), malate (Mal), amino acids (Aa), proteins (Prot), NH$_4^+$ (NH4) and NO$_3^-$ (NO3).

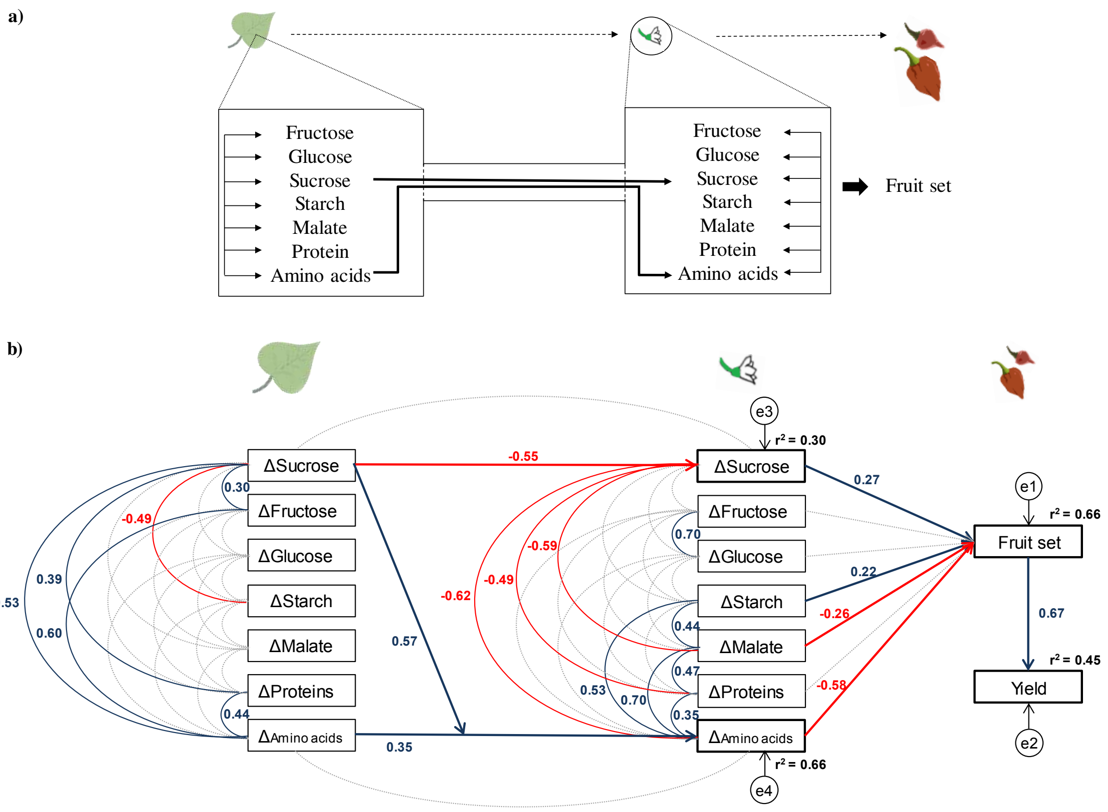

Figure 3. (A) Initial path model considering the correlations between the balance of all metabolites in each organ and the balance of sucrose and amino acids as links between source leaves and flowers ('Δ' means the difference of the metabolite content at dusk and dawn). The balance of metabolites in source leaves and flowers are capable to indirectly and directly influence the fruit set, respectively. (B) Structured equation model fitted with observed variables based on *t*-test ($P \leq 0.05$) (Table S7 and S8). Single-headed arrows indicate a causal relationship where a change in the variable at the tail is a direct cause of changes in the variable at the head. Concave lines indicate an unresolved covariance between two variables. The values in concave lines and arrows represent significant correlations and standardized regressions weight, respectively. Standardized path coefficients are displayed for significant paths as are r^2 values for the four endogenous variables. Regressions instead of correlations explained the link between source leaves and flowers by the export of sucrose and amino acids.

SUPPLEMENTAL MATERIAL

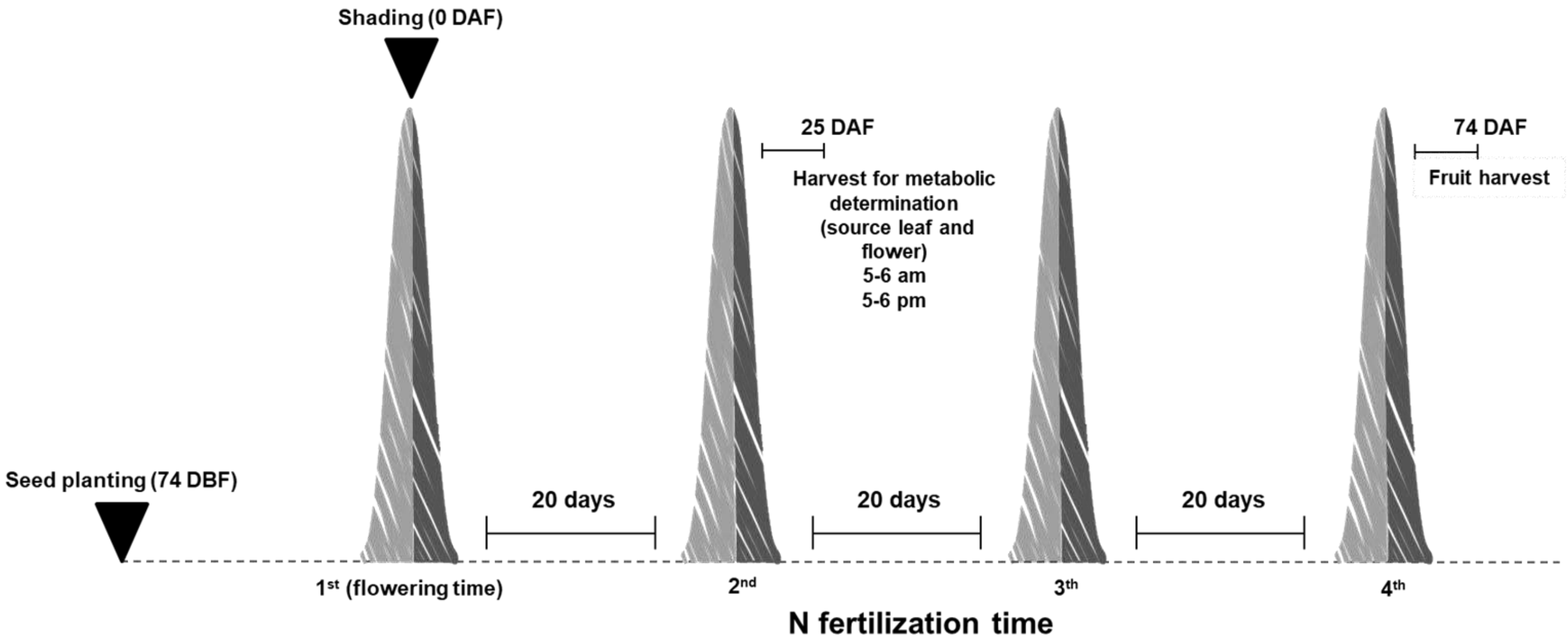

Figure S1. Scheme of the experiment evaluation dates based on the flowering period of each cultivar. The first N fertilization and shading occurred concomitant with the flowering time.

Table S1. Fruit set and yield considering the three factors: light intensity (unshaded or shade plants), N supply (20 or 125 mg N dm^{-3}) and cultivar (Biquinho or Habanero). The data are expressed as means.

Fruit set (%)							
Biquinho				**Habanero**			
20	*20 shaded*	*125*	*125 shaded*	*20*	*20 shaded*	*125*	*125 shaded*
41.11	35.97	67.93	63.82	7.87	6.56	20.52	16.57

Yield (g of dry fruits/plant)							
Biquinho				**Habanero**			
20	*20 shaded*	*125*	*125 shaded*	*20*	*20 shaded*	*125*	*125 shaded*
11.96	12.35	41.28	35.66	5.10	5.98	31.93	26.27

Table S2. Carbon metabolism related metabolites in source leaves considering the three factors: light intensity (unshaded or shade plants), N supply (20 or 125 mg N dm^{-3}) and cultivar (Biquinho or Habanero). The source leaves were harvested at dawn (5-6 am) and dusk (5-6 am), the difference of the metabolite content between dusk and dawn were statistically analyzed. The data are expressed as means.

Carbon metabolism related metabolites in source leaves (µmol g^{-1} FW)

Biquinho

Metabolite	**20**			**20 shaded**			**125**			**125 shaded**		
	Dawn	*Dusk*	$\Delta_{(dusk\text{-}dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk\text{-}dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk\text{-}dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk\text{-}dawn)}$
Glucose	2.73	2.73	0.00	2.25	1.68	-0.57	2.09	2.16	0.07	1.15	1.16	0.01
Fructose	2.02	1.62	-0.41	1.64	0.88	-0.76	0.74	0.73	-0.01	0.45	0.67	0.22
Sucrose	31.08	30.97	-0.12	30.04	34.15	4.11	32.66	32.95	0.29	32.66	32.04	-0.62
Starch	60.26	140.47	80.21	84.36	135.41	51.04	34.96	131.60	96.65	15.84	96.13	80.29
Malate	13.98	15.25	1.27	13.45	14.59	1.15	10.60	11.99	1.39	8.69	16.15	7.46

Habanero

Metabolite	**20**			**20 shaded**			**125**			**125 shaded**		
	Dawn	*Dusk*	$\Delta_{(dusk\text{-}dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk\text{-}dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk\text{-}dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk\text{-}dawn)}$
Glucose	1.99	1.40	-0.60	1.65	1.49	-0.16	2.45	1.82	-0.63	1.45	1.77	0.32
Fructose	0.96	1.22	0.26	0.72	1.16	0.44	1.28	2.35	1.07	0.61	2.76	2.14
Sucrose	32.01	52.90	20.89	31.39	52.44	21.06	31.28	51.90	20.62	34.48	51.61	17.12
Starch	157.38	164.26	6.88	140.93	163.48	22.55	121.37	154.96	33.59	61.30	144.10	82.80
Malate	4.15	6.32	2.17	5.59	8.13	2.53	5.94	5.45	-0.49	4.30	4.33	0.03

Table S3. Nitrogen metabolism related metabolites in source leaves considering the three factors: light intensity (unshaded or shade plants), N supply (20 or 125 mg N dm^{-3}) and cultivar (Biquinho or Habanero). The source leaves were harvested at dawn (5-6 am) and dusk (5-6 am), the difference of the metabolite content between dusk and dawn were statistically analyzed. The data are expressed as means.

Nitrogen metabolism related metabolites in source leaves (µmol g^{-1} FW)

Metabolite	**Biquinho**											
	20			**20 shaded**			**125**			**125 shaded**		
	Dawn	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$
Amino acids	14.31	7.17	-7.14	12.31	7.15	-5.16	22.34	17.17	-5.17	13.99	15.04	1.05
Protein	7.02	6.41	-0.62	7.09	5.15	-1.94	12.49	11.44	-1.05	9.32	9.84	0.51
NH_4^+	0.90	0.85	-0.06	0.72	0.61	-0.10	1.29	1.31	0.02	0.98	0.89	-0.09
NO_3^-	20.97	17.57	-3.40	19.06	20.63	1.57	42.84	37.19	-5.66	40.02	32.20	-7.81

Metabolite	**Habanero**											
	20			**20 shaded**			**125**			**125 shaded**		
	Dawn	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$
Amino acids	7.42	7.35	-0.07	6.81	7.85	1.05	17.21	21.66	4.46	12.81	18.84	6.04
Protein	3.32	4.59	1.27	4.64	5.97	1.33	11.13	11.63	0.51	8.46	8.76	0.30
NH_4^+	0.83	1.24	0.42	0.73	1.17	0.44	1.62	2.41	0.79	1.22	2.39	1.17
NO_3^-	14.02	14.79	0.77	13.72	14.86	1.14	31.23	33.61	2.38	31.31	31.51	0.20

Table S4. Comparison of the significant triple interactions between the factors light intensity (unshaded or shade plants), N supply (20 or 125 mg of N dm^{-3}) and cultivar (Biquinho or Habanero) on malate balance in source leaves. The letters compare the means by *t*-test at $P \leq 0.05$.

Light	**Unshaded**				**Shaded**			
N	*20*		*125*		*20*		*125*	
Cultivar	Biq	Hab	Biq	Hab	Biq	Hab	Biq	Hab
Mean	1.27 b	2.17 b	1.39 b	-0.49 b	1.15 b	2.53 b	7.46 a	0.03 b

Table S5. Carbon metabolism related metabolites in flowers considering the three factors: light intensity (unshaded or shade plants), N supply (20 or 125 mg N dm^{-3}) and cultivar (Biquinho or Habanero). The flowers were harvested at dawn (5-6 am) and dusk (5-6 am), the difference of the metabolite content between dusk and dawn were statistically analyzed. The data are expressed as means.

Carbon metabolism related metabolites in flowers (µmol g^{-1} FW)

Biquinho

Metabolite	**20**			**20 shaded**			**125**			**125 shaded**		
	Dawn	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$
Glucose	17.76	24.05	6.28	16.93	22.88	5.94	21.02	23.90	2.87	19.63	26.18	6.55
Fructose	20.44	26.08	5.64	22.47	26.94	4.47	28.66	30.90	2.24	26.08	31.92	5.85
Sucrose	27.00	30.35	3.35	26.92	32.80	5.88	23.28	32.81	9.52	23.23	29.24	6.01
Starch	10.89	16.81	5.92	11.11	13.64	2.53	23.22	31.11	7.89	21.36	26.85	5.48
Malate	27.22	24.57	-2.65	30.11	22.41	-7.70	17.73	12.85	-4.88	18.53	13.07	-5.46

Habanero

Metabolite	**20**			**20 shaded**			**125**			**125 shaded**		
	Dawn	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$
Glucose	12.93	15.15	2.22	13.04	15.21	2.17	15.11	22.49	7.38	13.18	17.85	4.67
Fructose	19.68	23.61	3.93	20.14	24.22	4.08	29.56	36.61	7.04	29.25	33.46	4.21
Sucrose	51.12	49.62	-1.50	55.48	54.05	-1.43	50.06	46.92	-3.14	55.65	42.59	-13.06
Starch	16.71	28.68	11.97	15.84	27.14	11.30	32.88	54.46	21.58	30.84	44.88	14.04
Malate	14.97	16.52	1.55	20.26	24.07	3.81	7.58	12.21	4.62	7.71	12.08	4.38

Table S6. Nitrogen metabolism related metabolites in flowers considering the three factors: light intensity (unshaded or shaded plants), N supply (20 or 125 mg N dm^{-3}) and cultivar (Biquinho or Habanero). The flowers were harvested at dawn (5-6 am) and dusk (5-6 am), the difference of the metabolite content between dusk and dawn were statistically analyzed. The data are expressed as means.

Nitrogen metabolism related metabolites in flowers (μmol g^{-1} FW)												
	Biquinho											
Metabolite	**20**			**20 shaded**			**125**			**125 shaded**		
	Dawn	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$
Amino acids	10.95	7.22	-3.73	9.02	6.00	-3.01	14.57	12.05	-2.52	14.42	10.63	-3.79
Protein	2.13	1.29	-0.84	3.31	1.67	-1.63	4.97	2.88	-2.09	3.50	2.43	-1.07
NH_4^+	0.89	0.93	0.03	1.06	1.08	0.02	1.63	1.56	-0.07	1.44	1.48	0.04
NO_3^-	32.41	28.18	-4.23	28.25	24.36	-3.90	41.73	37.61	-4.12	38.30	37.15	-1.15
	Habanero											
Metabolite	**20**			**20 shaded**			**125**			**125 shaded**		
	Dawn	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$	*Dawn*	*Dusk*	$\Delta_{(dusk-dawn)}$
Amino acids	6.02	10.00	3.98	6.74	11.83	5.09	12.17	18.06	5.89	7.58	14.57	7.00
Protein	1.98	1.48	-0.51	2.36	1.91	-0.44	2.79	2.12	-0.67	2.62	2.18	-0.44
NH_4^+	0.94	1.83	0.89	0.98	1.89	0.91	1.94	2.49	0.55	1.43	2.23	0.80
NO_3^-	10.57	10.90	0.33	9.23	9.58	0.36	17.89	13.92	-3.96	15.69	15.68	-0.01

Table S7. Main structural equation model proposed to explain the effect of primary metabolism on fruit set in *C. chinense*. In this model, sucrose and amino acids in source leaves (SO) and flowers (FL) were considered non-independent variables. Regression weights, stardardized regression weights, covariances and correlations were estimated by maximum likelihood and tested by *t*-test ($P \leq 0.05$). Unstandardized path coefficients represent the effect of a change in one variable on the other in absolute terms. Standardized path coefficients are in standard deviation units and are primarily used to compare the relative strengths of paths within a given model.

			Regression Weights (RW)			**Standardized RW**
			Estimate	S.E.	*P*-value	Estimate
Aa (FL)	→	Fruit set	-2.5	0.66	< 0.001	-0.53
Prot (FL)	→	Fruit set	-3.96	2.53	0.12	-0.16
Malate (FL)	→	Fruit set	-1.11	0.58	0.05	-0.26
Starch (FL)	→	Fruit set	0.46	0.22	0.04	0.22
Suc (FL)	→	Fruit set	0.29	0.36	0.42	0.1
Fru (FL)	→	Fruit set	-0.08	0.63	0.9	-0.02
Glu (FL)	→	Fruit set	0.51	0.66	0.44	0.09
Fruit set	→	Yield	0.39	0.06	< 0.001	0.67

			Covariances			**Correlations**
			Estimate	S.E.	*P*-value	Estimate
Suc (SO)	↔	Fru (SO)	3.76	1.92	0.05	0.3
Suc (SO)	↔	Glu (SO)	-0.35	1.62	0.83	-0.03
Suc (SO)	↔	Starch (SO)	-187.48	61.92	< 0.001	-0.49
Suc (SO)	↔	Malate (SO)	-5.03	4.81	0.29	-0.15
Suc (SO)	↔	Prot (SO)	8.03	3.2	0.01	0.39
Suc (SO)	↔	Aa (SO)	28.73	8.97	< 0.001	0.53
Fru (SO)	↔	Glu (SO)	-0.13	0.17	0.44	-0.11
Fru (SO)	↔	Starch (SO)	-3.9	5.83	0.5	-0.1
Fru (SO)	↔	Malate (SO)	-0.93	0.51	0.07	-0.27
Fru (SO)	↔	Prot (SO)	0.56	0.32	0.08	0.26
Fru (SO)	↔	Aa (SO)	3.4	0.96	< 0.001	0.6
Glu (SO)	↔	Starch (SO)	9.21	5.28	0.08	0.26
Glu (SO)	↔	Malate (SO)	0.83	0.45	0.07	0.28
Glu (SO)	↔	Prot (SO)	0.04	0.27	0.89	0.02
Glu (SO)	↔	Aa (SO)	0.43	0.73	0.56	0.09
Starch (SO)	↔	Malate (SO)	16.73	15.16	0.27	0.16
Starch (SO)	↔	Prot (SO)	-15.38	9.63	0.11	-0.24
Starch (SO)	↔	Aa (SO)	-36.77	25.51	0.15	-0.21
Malate (SO)	↔	Prot (SO)	0.84	0.81	0.3	0.15
Malate (SO)	↔	Aa (SO)	0.48	2.13	0.82	0.03
Prot (SO)	↔	Aa (SO)	4.05	1.46	0.01	0.44
Suc (FL)	↔	Fru (FL)	-5.92	5.26	0.26	-0.17
Suc (FL)	↔	Glu (FL)	-2.43	5	0.63	-0.07
Suc (FL)	↔	Starch (FL)	-20.22	12.95	0.12	-0.23

Suc (FL)	↔	Malate (FL)	-25.13	7.22	< 0.001	-0.59
Suc (FL)	↔	Prot (FL)	-3.68	1.23	< 0.001	-0.49
Suc (FL)	↔	Aa (FL)	-24.17	6.68	< 0.001	-0.62
Fru (FL)	↔	Glu (FL)	13.33	3.4	< 0.001	0.7
Fru (FL)	↔	Starch (FL)	4.34	7.08	0.54	0.09
Fru (FL)	↔	Malate (FL)	-0.66	3.48	0.85	-0.03
Fru (FL)	↔	Prot (FL)	0.88	0.63	0.16	0.21
Fru (FL)	↔	Aa (FL)	-0.24	3.17	0.94	-0.01
Glu (FL)	↔	Starch (FL)	1.47	6.79	0.83	0.03
Glu (FL)	↔	Malate (FL)	-4.05	3.4	0.23	-0.18
Glu (FL)	↔	Prot (FL)	0.5	0.6	0.4	0.12
Glu (FL)	↔	Aa (FL)	-2.09	3.07	0.5	-0.1
Starch (FL)	↔	Malate (FL)	25.65	9.25	0.01	0.44
Starch (FL)	↔	Prot (FL)	2.9	1.56	0.06	0.28
Starch (FL)	↔	Aa (FL)	27.88	8.72	< 0.001	0.53
Malate (FL)	↔	Prot (FL)	2.38	0.82	< 0.001	0.47
Malate (FL)	↔	Aa (FL)	18.24	4.65	< 0.001	0.7
Prot (FL)	↔	Aa (FL)	1.63	0.71	0.02	0.35
Suc (SO)	↔	Suc (FL)	-3.73	6.53	0.57	-0.04
Aa (SO)	↔	Aa (FL)	0.43	1.42	0.76	0.02

Table S8. Complementary structural equation model proposed to explain the effect of primary metabolism on fruit set in *C. chinense*. In this model, sucrose and amino acids in source leaves (SO) and flowers (FL) were considered independent variables. Regression weights, stardardized regression weights, covariances and correlations were estimated by maximum likelihood and tested by *t*-test ($P \leq 0.05$). Unstandardized path coefficients represent the effect of a change in one variable on the other in absolute terms. Standardized path coefficients are in standard deviation units and are primarily used to compare the relative strengths of paths within a given model.

			Regression Weights			**Standardized RW**
			Estimate	S.E.	*P*-value	Estimate
Suc (SO)	→	Suc (FL)	-0.4	0.09	< 0.001	-0.55
Aa (SO)	→	Aa (FL)	0.34	0.1	< 0.001	0.35
Suc (SO)	→	Aa (FL)	0.25	0.05	< 0.001	0.57
Aa (FL)	→	Fruit set	-2.64	0.49	< 0.001	-0.58
Suc (FL)	→	Fruit set	0.74	0.3	0.01	0.27
Fruit set	→	Yield	0.39	0.07	< 0.001	0.66

			Covariances			**Correlations**
			Estimate	S.E.	*P*-value	Estimate
Suc (SO)	↔	Fru (SO)	3.71	1.92	0.05	0.29
Suc (SO)	↔	Glu (SO)	-0.19	1.62	0.91	-0.02
Suc (SO)	↔	Starch (SO)	-182.19	61.68	< 0.001	-0.48
Suc (SO)	↔	Malate (SO)	-5.11	4.82	0.29	-0.16
Suc (SO)	↔	Prot (SO)	7.94	3.2	0.01	0.39
Suc (SO)	↔	Aa (SO)	29.85	9.1	< 0.001	0.54
Fru (SO)	↔	Glu (SO)	-0.13	0.17	0.44	-0.11
Fru (SO)	↔	Starch (SO)	-3.9	5.83	0.5	-0.1
Fru (SO)	↔	Malate (SO)	-0.93	0.51	0.07	-0.27
Fru (SO)	↔	Prot (SO)	0.56	0.32	0.08	0.26
Fru (SO)	↔	Aa (SO)	3.43	0.97	< 0.001	0.6
Glu (SO)	↔	Starch (SO)	9.21	5.28	0.08	0.26
Glu (SO)	↔	Malate (SO)	0.83	0.45	0.07	0.28
Glu (SO)	↔	Prot (SO)	0.04	0.27	0.89	0.02
Glu (SO)	↔	Aa (SO)	0.42	0.74	0.57	0.08
Starch (SO)	↔	Malate (SO)	16.73	15.16	0.27	0.16
Starch (SO)	↔	Prot (SO)	-15.38	9.63	0.11	-0.24
Starch (SO)	↔	Aa (SO)	-38.34	25.73	0.14	-0.22
Malate (SO)	↔	Prot (SO)	0.84	0.81	0.3	0.15
Malate (SO)	↔	Aa (SO)	0.41	2.15	0.85	0.03
Prot (SO)	↔	Aa (SO)	4.11	1.47	0.01	0.45

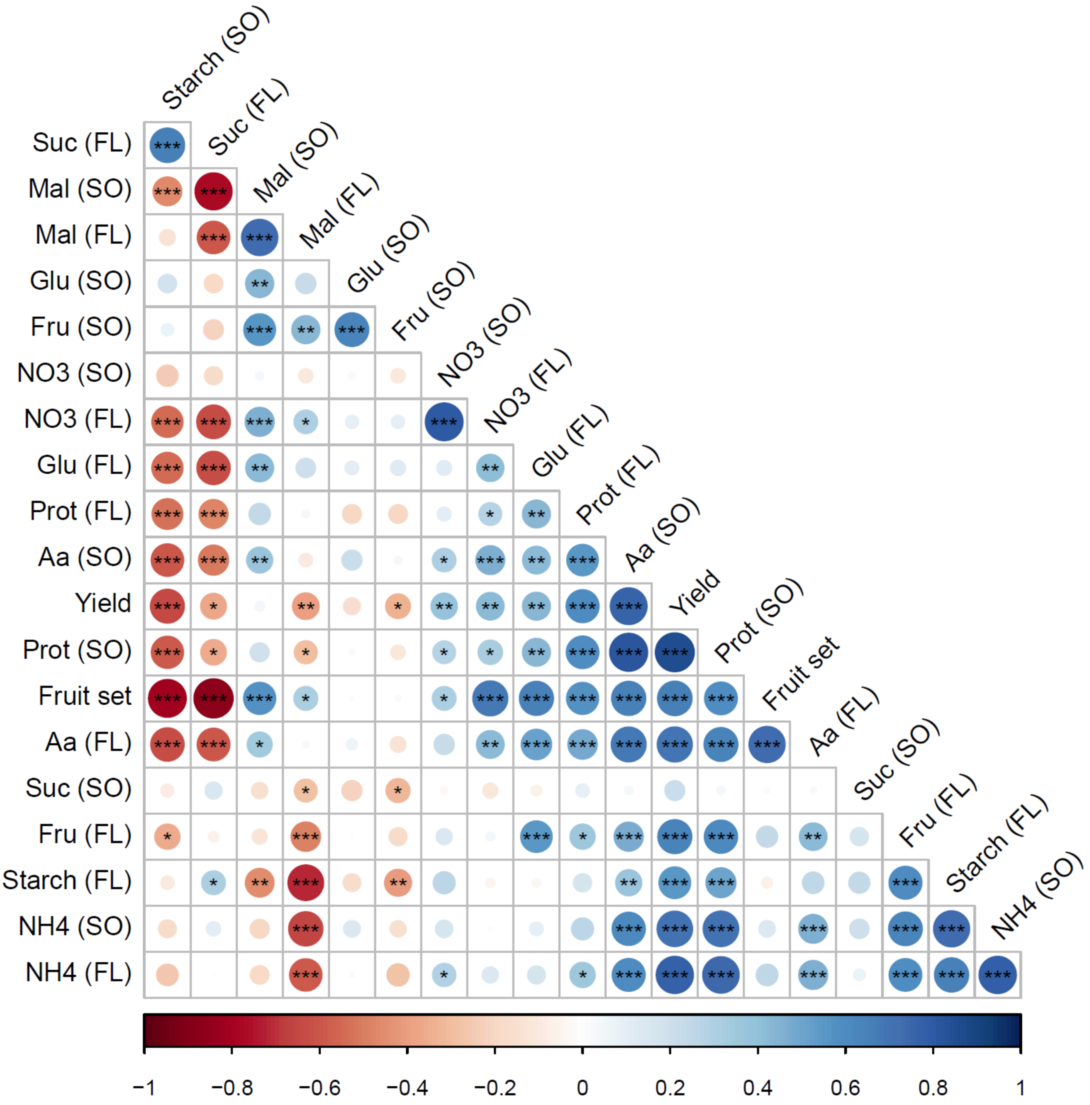

Figure S2. Pearson correlation between fruit set, yield and metabolites level at dawn (5-6 am) in source leaves (SO) and flowers (FL) of two contrasting cultivars of *C. chinense* (Biquinho and Habanero) submitted to light (unshaded or shade plants) and N (20 or 125 mg of N dm^{-3}) variation. Blue and red circles represent positive and negative correlations at $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Abbreviations: glucose (Glu), fructose (Fru), sucrose (Suc), malate (Mal), amino acids (Aa), proteins (Prot), NH_4^+ (NH4) and NO_3^- (NO3).

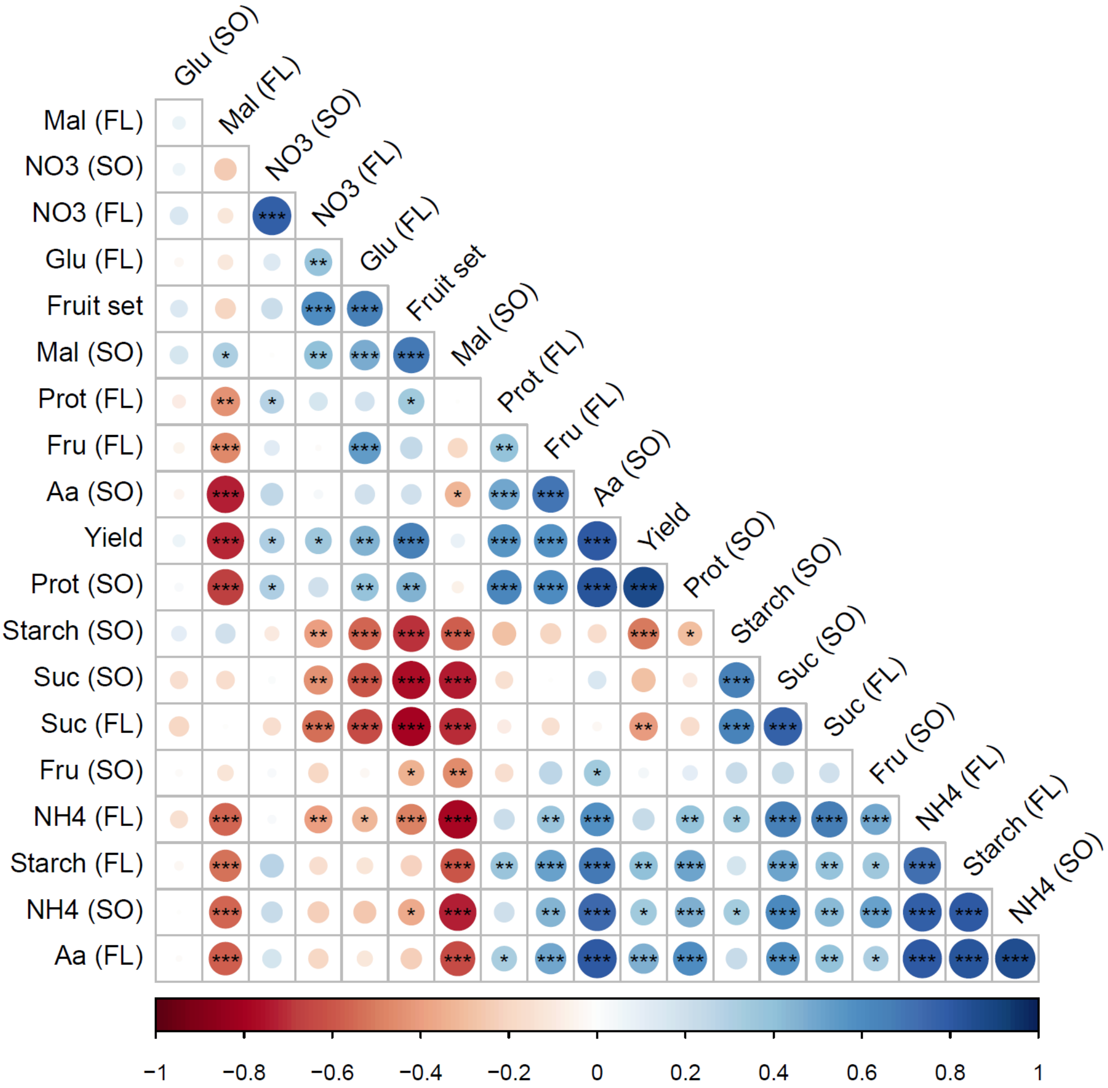

Figure S3. Pearson correlation between fruit set, yield and metabolites level at dusk (5-6 pm) in source leaves (SO) and flowers (FL) of two contrasting cultivars of *C. chinense* (Biquinho and Habanero) submitted to light (unshaded or shade plants) and N (20 or 125 mg of N dm^{-3}) variation. Blue and red circles represent positive and negative correlations at $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Abbreviations: glucose (Glu), fructose (Fru), sucrose (Suc), malate (Mal), amino acids (Aa), proteins (Prot), NH_4^+ (NH4) and NO_3^- (NO3).

CHAPTER 3

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SPECIFIC LEAF AREA IS AFFECTED BY NITROGEN VIA CHANGES IN PRIMARY METABOLITES AND PARENCHYMAL THICKNESS IN PEPPER

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ABSTRACT

Specific leaf area (SLA) is a key trait influencing light interception and light use efficiency that correlates with whole plant growth. SLA has been used to explain the ecological differences between slow- and fast-growing species under different environments. Both light and nitrogen (N) supply are important determinants of SLA. To better understand the effect of irradiance level and N on SLA of a fruit species we evaluated the primary metabolism and anatomy traits of two genotypes of *Capsicum chinense*, cultivars Biquinho and Habanero in response to changes in both parameters. Overall, both genotypes showed an increase in SLA with shading and a decrease in SLA according to the increase of N supply. Our results indicate that the decrease in SLA in response to increased N supply was mostly explained by amino acids, protein and starch level, influencing the leaf density. However, in the range of moderate N deficiency to N sufficiency, the genotypes exhibited a differential SLA response to N supply. Biquinho and Habanero displayed alterations on palisade and spongy parenchyma, respectively.

Keywords: *Capsicum*, leaf anatomy, shade, chili pepper

1. INTRODUCTION

Specific leaf area (SLA) is the leaf area to leaf mass ratio (Coombe, 1960), the inverse of leaf mass per area (LMA) (Poorter et al., 2009). SLA has been positively correlated with photosynthetic capacity in different non-woody and woody species growing in a growth chamber or natural environments (Poorter et al., 1990; Reich et al., 1994; Reich and Walters, 1994). Indeed, SLA can affect photosynthetic capacity (Dijkstra and Lambers, 1989; Xiong et al., 2016) given its influence on light capture and CO_2 diffusion to the carboxylation site (Hanba et al., 1999; Lloyd et al., 1992; Terashima and Hikosaka, 1995). Furthermore, SLA is an important parameter related to plant growth (Mooney et al., 1978; Poorter et al., 2009; Poorter and Remkes, 1990; Potter and Jones, 1977; Witkowski and Lamont, 1991).

SLA has been used to understand and explain ecological differences in growth of distinct species (Garnier et al., 1997; Poorter et al., 2009; Poorter and Pothmann, 1992; Poorter and Remkes, 1990). However, in addition to the variation of SLA between species (Poorter and Remkes, 1990), many environmental cues such as light quantity and quality, UVB, CO_2 concentration, ozone, soil compaction, water stress, temperature, salinity and nutrient availability can affect the relation between the leaf area and leaf mass (Poorter et al., 2009; Tian et al., 2016). Among nutrients, N content in both soil and leaf is an important factor regulating leaf morphology and anatomical traits (Liu et al., 2017; Tian et al., 2016).

Several studies reported higher SLA (or decreased LMA) of diverse species when increasing the leaf content of N (An and Shangguan, 2008; Jinwen et al., 2009; Knops and Reinhart, 2000; Poorter et al., 2009; van Arendonk et al., 1997; Witkowski and Lamont, 1991; Wright et al., 2004). Most of them implied in ecological studies often performed in field/natural environments, where is harder to isolate the possible N supply effect on SLA from the self-shading effect due to the higher leaf area according to the increment in the leaf content of N. On the other hand, the response of LMA to N supply varied across rice cultivars pot grown (Xiong et al., 2016).

The variation in SLA derives from independent changes in leaf density and/or thickness (Witkowski and Lamont, 1991). Leaf density and thickness explained 80% and 20% of the differences observed in LMA, respectively, across a wide range of plant species and conditions from many studies integrated by meta-analysis (Poorter et al., 2009). In agreement, rice leaf density was the major cause of the variation in LMA across varieties and N treatments (Xiong et al., 2016).

Few studies, however, have explored SLA behavior among cultivars of crop species (Jinwen et al., 2009; van Den Boogaard et al., 1997; Xiong et al., 2016). Surprisingly, the effects of N availability on SLA of fruit species, or even crops in general, are still not well resolved. Due to its vast phenotypic diversity and natural genetic variation (Finger et al., 2010; Moreira et al., 2018; Rosado-Souza et al., 2015), we selected *Capsicum chinense* as a potential fruit species to investigate the role of N supply on SLA. Thus, we assessed the influence of N on the SLA of two *C. chinense* commercial varieties, cv Biquinho and cv Habanero, which vary in fruit set, fruit size, and leaf morphology. First, we submitted both genotypes to a range of N between 0 and 312.5 mg dm^{-3} to investigate the SLA behavior and its relationship to metabolic changes under different N supply. This preliminary analysis set the ground for determining the N deficiency and N sufficiency limits (20 to 125 mg of N dm^{-3}). The genotypes showed different SLA behavior under the range of moderate N deficiency to N sufficiency. We further zoomed in on the N effect at the anatomical level. Moreover, we compared the N effect with the well-known shade effect on SLA and leaf thickness.

2. MATERIAL AND METHODS

2.1.Plant growth conditions

Seeds from Biquinho and Habanero (TopSeed®, Agristar do Brasil Ltd) were germinated and grown on a commercial substrate (Tropstrato® HT; Vida Verde Ltd) and 45 days old plantlets were transplanted to pots with 5 dm^3 of a Yellow-Red Oxisol soil, ensuring no restriction to the root system development. Before transplantation, the soil used for cultivation was sieved, homogenized and its acidity neutralized by applying $CaCO_3$ and $MgCO_3$ (molar ratio of 3.5:1). Afterwards, it was prepared and fertilized with nutrients according to Novais, Neves & Barros (1991), with the exception of N. The total amount of nutrients was 300, 150, 40, 0.81, 1.33, 1.55, 3.66, 0.15 and 4.00 mg dm^{-3} for P, K, S, B, Cu, Fe, Mn, Mo, and Zn, respectively. Due to the low N content in the soil, 5 mg dm^{-3} of this nutrient was applied as NH_4NO_3 to ensure the initial development of the plants after transplantation. To avoid leaching of N from the soil, plastic bags were used to cover the base of the pots. The first N fertilization, using NH_4NO_3 as N source, occurred at the development of the first sympodial unit, which corresponds to the reproductive differentiation. Water was supplied daily maintaining the soil at constant field capacity over the course of the experiments.

In the first experiment 0, 20, 50, 125 and 312.5 mg dm^{-3} of N were applied every twenty days for a total of five equal supplementations. The experiment was performed in a greenhouse with temperature of 25 ± 5 °C and an average irradiance of 500 µmol photons m^{-2} s^{-1}. The plants were cultivated for 91 days after flowering (DAF). All experimental analyses were performed between July and October of 2016 with an average photoperiod of 11h30mins day light / 12h30mins night (source: Astronomical Applications Dept., U. S. Naval Observatory, Washington, DC).

The second experiment was performed with a temperature of 27 ± 5 °C and an average irradiance of 800 µmol photons m^{-2} s^{-1}. When the plants developed the first sympodial unit, half of the plants were submitted to 50% shading and the others continued without shading. Concomitantly, we applied 20 and 125 mg of N dm^{-3} of N every twenty days for a total of four equal supplementations. The plants were cultivated for 74 DAF. All experimental analyses were performed between February and April of 2017 with an average photoperiod of 12h11mins day light / 11h49mins night (source: Astronomical Applications Dept., U. S. Naval Observatory, Washington, DC).

2.2. Metabolite analyses and N content determination

Fully expanded leaves of the third sympodial unit from the apex were harvested in the middle of the light period 24 h before the fourth N fertilization in the first experiment (59 DAF) for metabolite analyses. All samples were snap frozen in liquid nitrogen and stored at -80 °C until further analyses. Metabolite extraction was performed by grinding the tissues in liquid nitrogen followed by the determination of the sample fresh weight and then ethanol extraction (Cross et al., 2006). In the ethanol extracts were determined, sucrose, fructose, glucose (Fernie et al. 2001), malate (Nunes-Nesi et al., 2007), total amino acid (Cross et al., 2006), NH_4^+ (Sarasketa et al., 2014), NO_3^- (Sienkiewicz-Porzucek et al., 2010), chlorophyll *a* and *b* (Porra et al., 1989). Starch and protein levels were determined in the ethanol insoluble fraction (Bradford, 1976; Fernie et al., 2001). The N content was determined in leaf samples (Kalra, 1998) harvested at the end of each experiment.

2.3.Specific leaf area estimation and anatomical traits

SLA was estimated in both experiments according to Cavatte et al. (2012). In the first experiment, nine discs of 1.03 cm^2 were collected from the blade of mature leaves distributed throughout the plant at 88 DAF. In the second experiment, six discs were collected at 10, 30, 50 and 70 DAF throughout the plant. Furthermore, the leaf area from each treatment was measured using a planimeter (Li 3100C Li-Cor, Inc., Lincoln, NE, USA) at 72 DAF. Leaves were then oven-dried at 80 °C to reach a constant weight. The SLA was also calculated as the ratio of leaf area to dry mass (m^{-2} kg^{-1}).

Anatomical analyses were performed at 70 DAF (second experiment). For this, fully expanded leaves of the third sympodial unit from the apex were collected in the middle of the light period, fixed in FAA_{50} (Johansen, 1940) for 48 h under vacuum and stored in 70% ethanol. Three samples from each treatment were selected at random, dehydrated in ethanolic series and embedded in glycol methacrylate (Leica® Embedding Kit) according to the manufacturer's recommendations. Transverse sections, 8 µm thick, were made in rotary microtome (model RM2155, Leica Microsystems, Deerfield, IL) stained with Toluidine Blue O (O'Brien and McCully, 1981) for 10 min washed with water and dried at room temperature. The slides were mounted in synthetic resin (Permount, Fisher) for observation and documentation in a light microscope (AX70–Olympus). The analysis of the leaf histometry were performed with the Image Pro Plus program (MediaCybernetics). Histometric data were obtained from photomicrographs. The total leaf, palisade and spongy parenchyma thickness were evaluated.

2.4.Statistical analysis

The first experiment was performed in a completely randomized block design with five replicates, considering the factorial genotype and N supply. The normality of the data was verified by the Shapiro-Wilk test after the variables were analyzed by two-way ANOVA. Separation of significant mean differences between genotypes was achieved by Tukey's test at $P \leq 0.05$ and regression models was adjusted according to the N supply. The second experiment was performed in a split-split-plot design (Steel et al., 1997), with three factors: shading (sun and shade plants) set up as the main plot, N supply (20 and 125 mg of N dm^{-3}) set up as subplot and genotypes (Biquinho and Habanero) set up as sub-subplot. Six replicates were used for each treatment. Only leaf anatomical and histological analyses were based on the analysis of three samples selected at random for each treatment. Three histological slides were prepared with average nine sections from each sample. One field were documented for slide and three measures were taken from each field. The normality of the data was verified by the Shapiro-Wilk test and after the variables were analyzed by three-way ANOVA. The separation of significant mean differences was achieved by F-test ($P \leq 0.05$) for each factor effect and Tukey's test ($P \leq 0.05$) for factor splitting. The graphs, comparison of means, regression analyses, and Pearson's correlations were performed using SigmaPlot v.11.0 (Systat Software, San Jose, CA, USA) and R.

3. RESULTS

3.1. SLA is altered by N supply

Overall, decreased SLA was observed in both genotypes under increasing N supply (Figure 1A). Regression models were adjusted to explain the SLA behavior according the N supply for each genotype (Figure 1A). Biquinho exhibited higher SLA than Habanero in plants under 20 and 50 mg of N dm^{-3} (Figure 1A).

In the second experiment we investigated the SLA of both genotypes under moderate N deficiency and N sufficiency (20 and 125 mg of N dm^{-3}) combined with different shading conditions. A significant triple interaction between shading, N supply and genotypes on SLA was observed (P = 0.041, Table A.1). Thus, there were differences in the mean values among the different levels of shading or N supply according to the genotype (Figure 1B). The SLA of both genotypes increased under shade but only Biquinho displayed higher SLA under 20 mg of N dm^{-3} (Figure 1B).

We further assessed the SLA dividing the total leaf area per plant by the total dry weight of leaves per plant. This analysis confirmed the shade effect increasing SLA in both genotypes. The SLA varied from 19.36 ± 0.75 to 27.49 ± 0.88 m^2 kg^{-1} under sun and shaded conditions respectively (Table A.1). Moreover, we observed an interaction between N supply and genotype on SLA (Table A.1). Biquinho leaves showed higher SLA under 20 mg of N dm^{-3}, no N effect was observed on the SLA of Habanero (Figure 2A).

3.2. N supply influences leaf and parenchyma thickness distinctly in the genotypes

Shading affected anatomical traits independently of N supply or genotype (Table A.1). Both genotypes showed thinner leaves under shade (191.55 ± 8.74 µm) than sun (217.71 ± 8.48 µm) conditions (Table A.1). In agreement with the shade effect on leaf thickness, there were thinner palisade and spongy parenchyma in plants under shade (Table A.1). The palisade parenchyma thickness was 69.86 ± 2.64 µm under shade and 80.76 ± 4.84 µm under sun conditions (Table A.1). In addition, spongy parenchyma thickness was 92.89 ± 6.37 µm under shade and 105.89 ± 5.94 µm under sun conditions (Table A.1).

Different from the independence of shade effect on anatomical traits, the N supply effect was genotype-dependent. Habanero showed thicker leaves under 20 mg of N dm^{-3} while there was no N supply effect on the leaf thickness of Biquinho (Figure 2B). Furthermore, Biquinho showed thicker palisade parenchyma under 125 mg of N dm^{-3} while there was no N supply effect on the palisade parenchymal thickness of Habanero (Figure 2C). On the other hand, Habanero showed thicker spongy parenchyma under 20 mg of N dm^{-3} while there was no N supply effect on the same trait of Biquinho (Figure 2D). In addition, Habanero showed thicker spongy parenchyma than Biquinho under 20 but not under 125 mg of N dm^{-3} (Figure 2D). These results suggest Biquinho mainly displays alterations in the palisade parenchyma while Habanero alters the spongy parenchyma according to the N supply (Figure 3).

3.3. N content and N metabolism-related metabolites correlate with SLA

C and N metabolism-related primary metabolites were analyzed according to the N supply (Figure A.1). Next, we performed a Pearson correlation analysis between SLA, N content, C and N metabolism-related metabolites (Figure 4). Positive correlations between the N content and N metabolism-related metabolites such as amino acids, protein, NH_4^+ and NO_3^- levels were observed. On the other hand, N content and all N metabolism-related metabolites negatively correlated with SLA (Figure 4). Among the C metabolism-related metabolites, starch, glucose and malate levels positively correlated with SLA (Figure 4).

4. DISCUSSION

Two different genotypes of *C. chinense* in terms of fruit set, fruit size, and leaf morphology were cultivated to understand the effect of light and N on SLA of a fruit species. We independently evaluated traits that can affect both leaf density and thickness, modulators of leaf area to leaf mass ratio (Witkowski and Lamont, 1991). Concerning leaf density, the sum of total non-structural carbohydrates (normally considered as the sum of soluble sugars and starch) and proteins may correspond from 30 to 60% of the leaf biomass (Poorter et al., 2009). The starch mass can represent ca 30 to 40% of total leaf dry mass across the wide range of species (Chatterton and Silvius, 1981; McDonald et al., 1986; Rufty et al., 1988). In our study, all primary metabolites analyzed represented from 12.5 to 24.1% of the leaf dry mass (Figure A.1), according to the increase in the N supply. The variation of amino acids, proteins, and starch content were highlighted. Amino acids (from 0.8 to 14.5% of the leaf dry mass) and protein (0.7 to 3.3% of the leaf dry mass) were positively correlated with N content, and negatively with SLA (Figure 4). On the other hand, starch (from 8.8 to 1.8% of the leaf dry mass) was negatively correlated with N content, and positively with SLA (Figure 4). Indeed, N deprivation leads to alterations in C metabolism, such as decreased levels of malate and other organic acids as well as increased levels of starch (Scheible et al., 1997; Stitt, 1999). Altogether, it seems reasonable to assume that the leaf N content through N metabolism-related metabolites synthesis is an important trait related to the increase of leaf density according to the increase of N supply.

Among the N metabolism-related metabolites, the possible role of the protein levels on SLA has been suggested (Poorter et al., 2009). In fact, leaf protein levels can sometimes vary quantitatively even more strongly than the variation in total non-structural carbohydrates (Poorter and Villar, 1997). However, our results suggested the variation in amino acids level is more determinant of leaf density than the protein variation as a consequence of the wide range of N supply (0 to 312.5 mg of N dm^{-3}) and variation of leaf N content (Figure A.1). Overall, as the changes in the relation between leaf area and leaf mass driven by nutrient stress are more related to alterations in leaf density than in leaf volume per area (Poorter et al., 2009; Xiong et al., 2016), we believe the general SLA response of both genotypes (Figure 1A) are explained, at least in part, by the role of N and C metabolism-related primary metabolites on leaf density.

As both genotypes displayed similar behavior of traits that influence the leaf density such as N and C metabolism-related metabolites (Figure A.1), we hypothesize the distinct SLA behavior between genotypes under moderate N deficiency to N sufficiency (Figure 1B and 2A) comes from their different leaf anatomical plasticity (Figure 2B, C and D). SLA in Biquinho decreased under N sufficiency due to the increment in leaf density and thicker palisade parenchyma (Figure 2C and 4). On the other hand, the leaf thickness variation (Jinwen et al., 2009) through changes in spongy parenchyma compensated the leaf density variation leading to no alteration of SLA in Habanero under the range of moderate N deficiency to N sufficiency (Figure 2B, D and 4). Furthermore, as the palisade parenchyma is denser than the spongy parenchyma (Bosabalidis and Kofidis, 2002), its alteration in Biquinho can explain the higher SLA sensitivity of this genotype under the range of moderate N deficiency to N sufficiency. Rice cultivars also showed distinct response in the leaf area to leaf mass ratio according to N supply, where LMA increased in the cultivar HHZ under N deficiency but decreased in the cultivar N22 and had no significant effect on other two rice cultivars (Xiong et al., 2016).

Hence, we suggest that the reductions in SLA, common for both genotypes under higher N supply, occurs because of the increment in the leaf density driven by shifts of N and C metabolism-related metabolites, mainly amino acids, protein and starch level (Figure A.1). However, the SLA behavior in the range of moderate N deficiency to N sufficiency is also influenced by genotypic anatomical plasticity. There is a convergent effect between the higher leaf density and thicker palisade parenchyma influencing the SLA of Biquinho while a divergent effect between the higher leaf density and thinner leaf thickness (as well as thinner spongy parenchyma) influencing the SLA of Habanero according to the increase in N supply. Although we did not find alterations in the proportion of intercellular spaces here it seems that further studies investigating the N supply effect on the number of leaf cells, cell wall thickness would lead to better understand the N influence on SLA related to organogenesis at the molecular level (Wany et al., 2018). Given that SLA is an important trait determining the fitness of different species in their own environment it will be interesting to further understand the molecular and physiological regulation of SLA in response to N supply and the suite of traits interconnected with it.

CONTRIBUTIONS

LAS, JAC-A and AN-N designed the research. LAS and JAC-A conducted the experiments and samplings. LAS, JAC-A, PMAC, EATP and NMS performed the analyses. LAS, EATP and AN-N wrote the manuscript. PMAC, AZ, WLA, RS, EATP and AN-N reviewed and corrected it. The authors declare that there is no conflict of interest.

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FIGURES

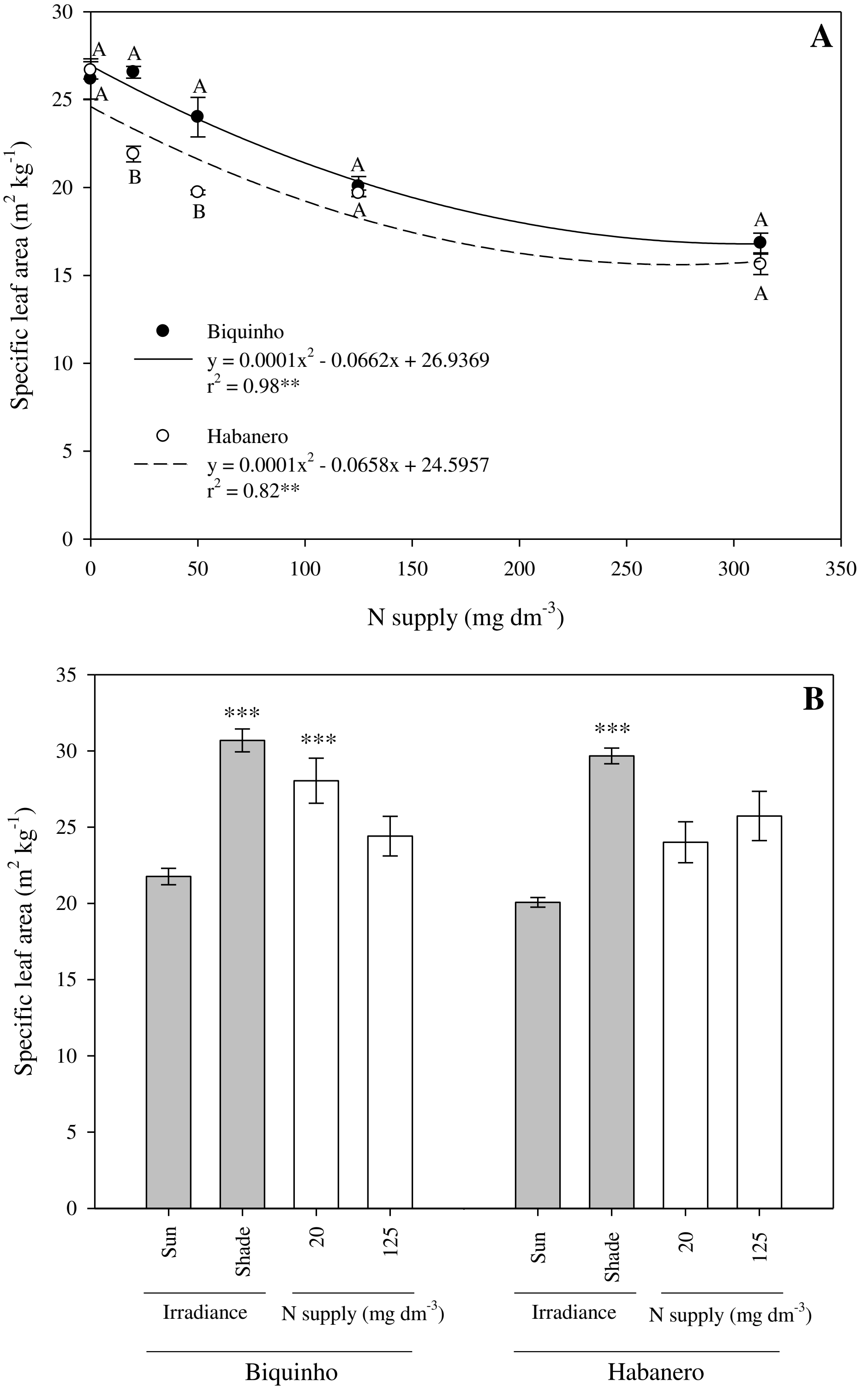

Figure 1. (A) Specific leaf area of two *C. chinense* genotypes, Biquinho (black) and Habanero (white), submitted to five doses of nitrogen under 500 µmol photons m^{-2} s^{-1}. Uppercase letters indicate significant differences between genotypes for each N dose at $P \leq 0.05$ according to Tukey's test. Regression models were adjusted to explain the SLA behavior according to the N supply for each genotype. The

regression models were tested by F-test and the coefficients by *t*-test at $P \leq 0.05$. Values are presented as means ± SE of five individual plants per treatment. (B) Results of the triple interaction between irradiance level (800 and 400 μmol photons m^{-2} s^{-1}), N supply (20 and 125 mg of N dm^{-3}) and genotypes (Biquinho and Habanero) to SLA. The difference in the mean values of SLA was evaluated among the different levels of shading or N supply according to the genotype. Asterisks indicate the statistical difference of the light condition or N supply at the level of each genotype at $P \leq 0.05$ according to Tukey's test.

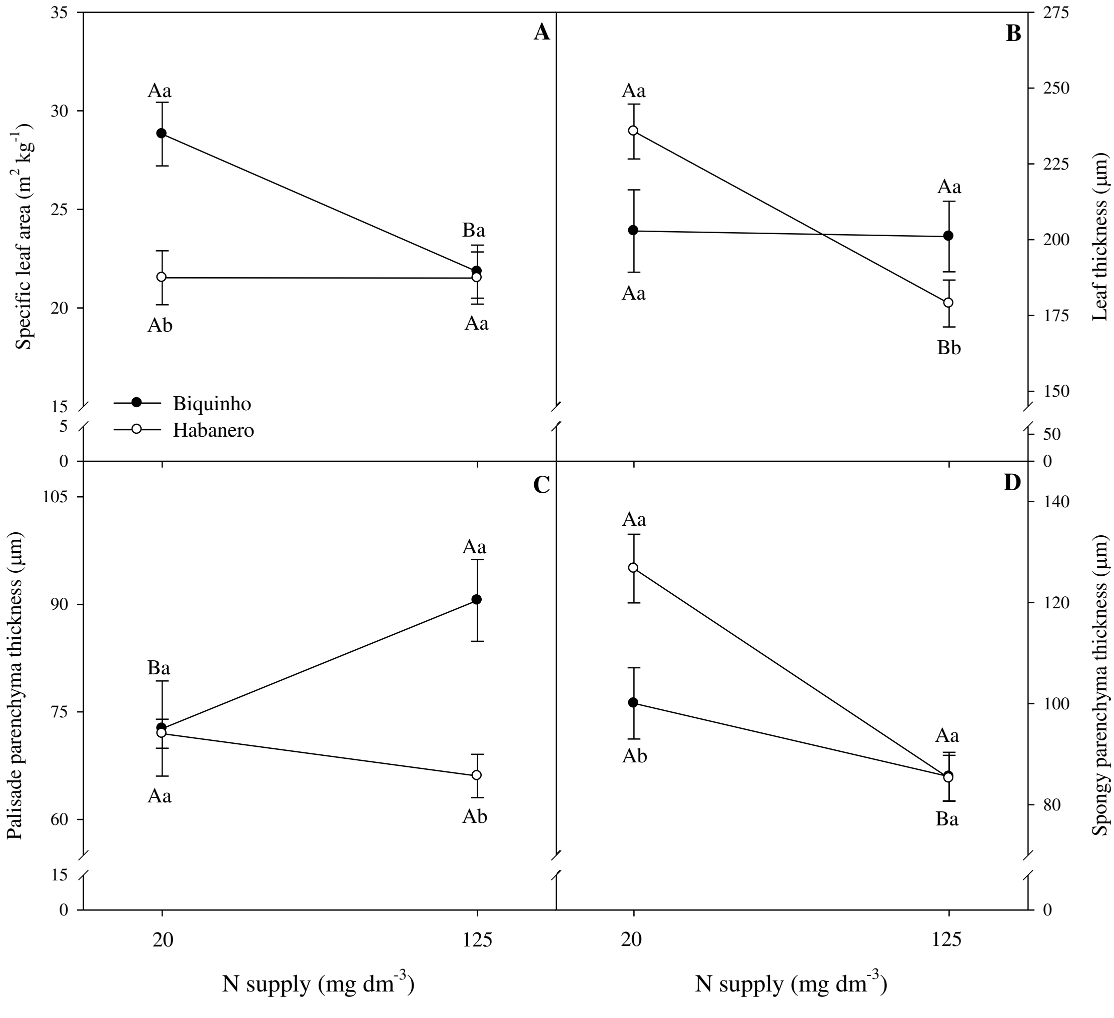

Figure 2. Interaction plots (mean ± SE) between the factors N supply (20 or 125 mg of N dm^{-3}) and genotype (Biquinho or Habanero) as indicated by ANOVA (Table A.1) for the (A) specific leaf area (total leaf area : dry weight of leaves ratio at 74 DAF) as well as (B) leaf thickness, (C) palisade and (D) spongy parenchyma. Uppercase letters compare the N dose for each genotype and lowercase letters compare the genotype for each N dose at $P \leq 0.05$ according to Tukey's test.

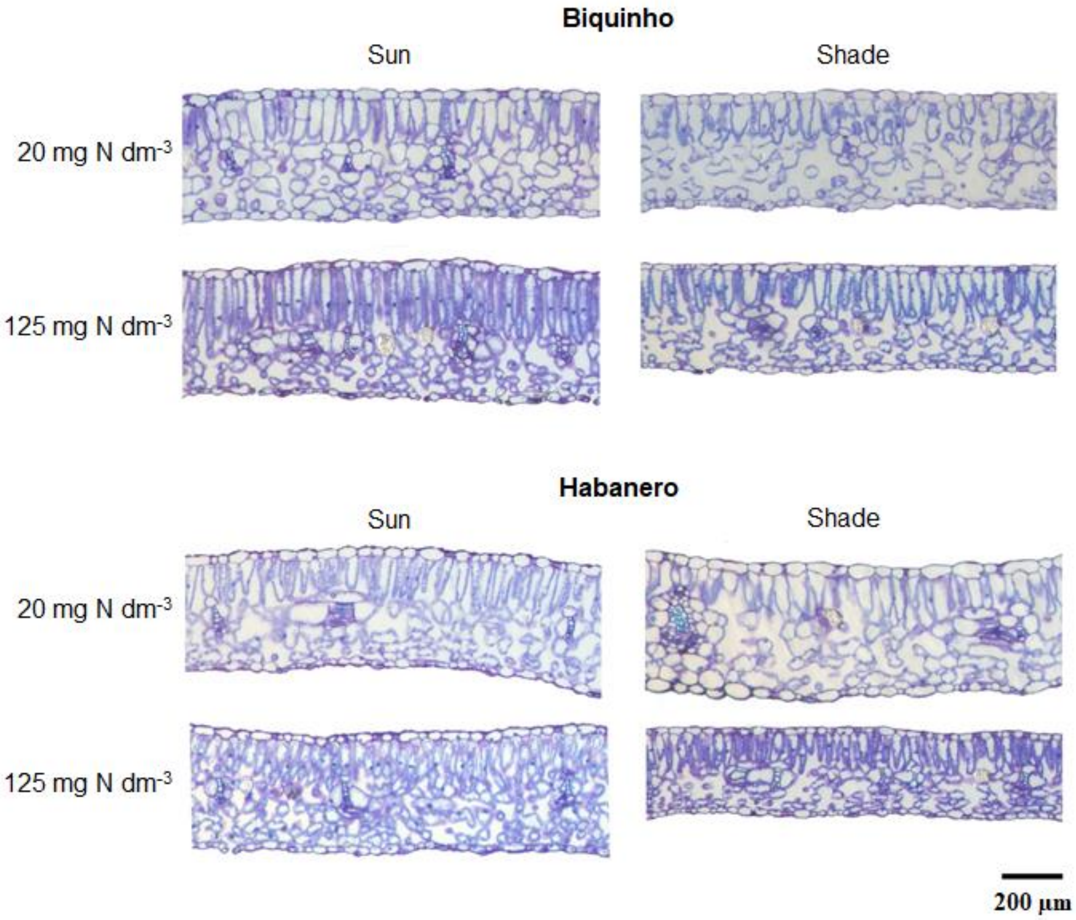

Figure 3. Representative pictures of mature leaves cross section in Biquinho and Habanero submitted to the moderate N deficiency (20 mg of N dm^{-3}) or N sufficiency (125 mg of N dm^{-3}) under sun or shade conditions. The scale of the pictures represents 200 µm.

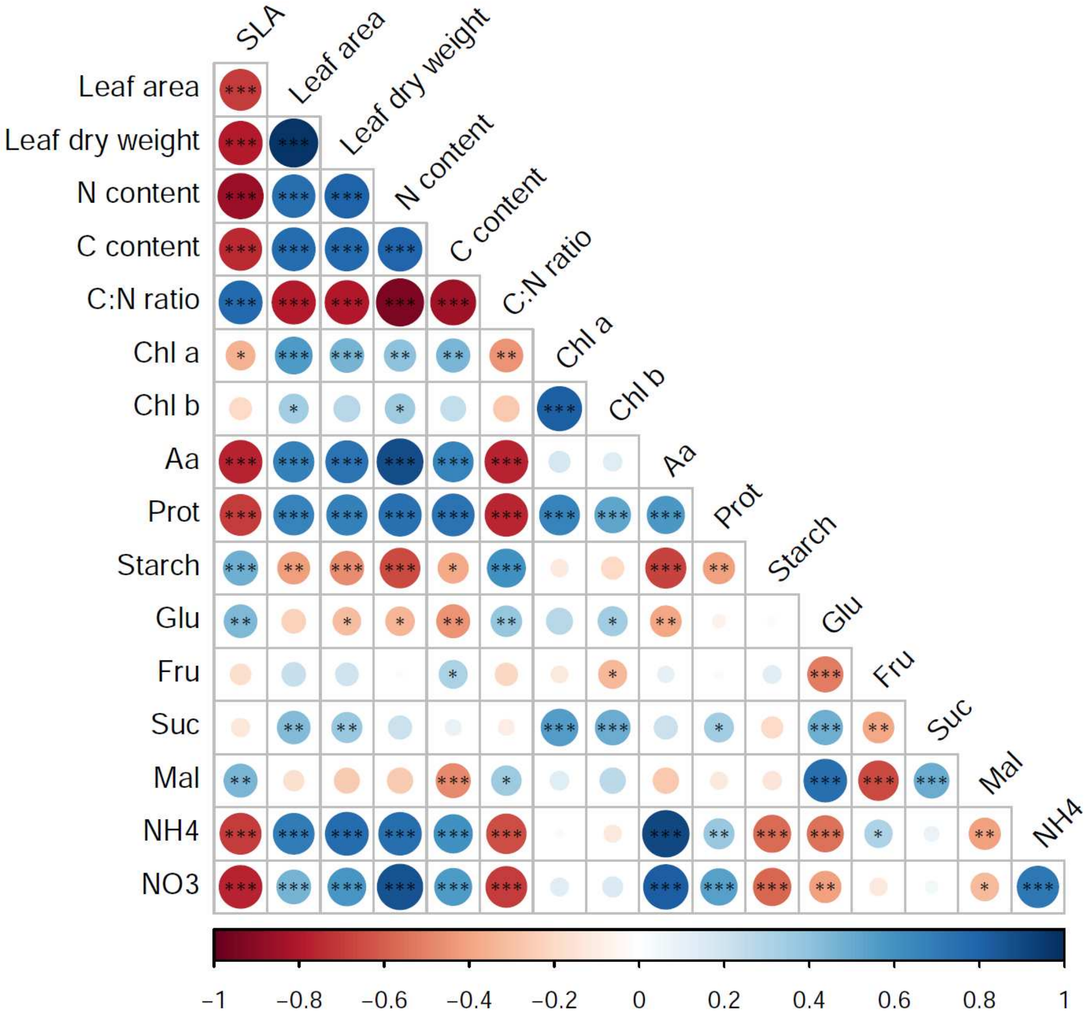

Figure 4. Pearson correlation between SLA, leaf traits, C content, N content, C and N metabolism-related metabolites obtained from two *C. chinense* genotypes, Biquinho and Habanero, submitted to five doses of nitrogen. Blue and red circles represent positive and negative correlations at $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Abbreviations: glucose (Glu), fructose (Fru), sucrose (Suc), malate (Mal), amino acids (Aa), proteins (Prot), NH_4^+ (NH4), NO_3^- (NO3), chlorophyll *a* (Chl a), and chlorophyll *b* (Chl b).

SUPPLEMENTAL MATERIAL

Table A.1. Three-way ANOVA performed to indicate the effect of shading intensity (sun or shade plants), N supply (20 or 125 mg of N dm^{-3}), genotype (Biquinho or Habanero) and their interactions on the specific as well as leaf area thickness of the mature leaves, palisade and spongy parenchyma (second experiment). The data are expressed as F-value and the statistical significance can be analyzed by the *P*-value.

	Specific leaf area and anatomical traits			
	Specific leaf area[a]		**Specific leaf area**[b]	
Source of Variation	F-value	*P*-value	F-value	*P*-value
Shading	718.563	**<0.001**	119.298	**<0.001**
Nitrogen	7.625	**0.009**	22.015	**<0.001**
Genotype	15.42	**<0.001**	26.139	**<0.001**
Shading x Nitrogen	0.16	0.691	0.0021	0.964
Shading x Genotype	0.959	0.333	0.095	0.759
Nitrogen x Genotype	60.103	**<0.001**	21.9	**<0.001**
Shading x Nitrogen x Genotype	4.443	**0.041**	0.266	0.609
	Leaf thickness		**Palisade parenchyma thickness**	
Source of Variation	F-value	*P*-value	F-value	*P*-value
Shading	7.900	**0.013**	6.854	**0.019**
Nitrogen	9.898	**0.006**	2.075	0.169
Genotype	0.332	0.573	9.172	**0.008**
Shading x Nitrogen	1.834	0.194	1.531	0.234
Shading x Genotype	0.731	0.405	1.222	0.285
Nitrogen x Genotype	8.700	**0.009**	8.156	**0.011**
Shading x Nitrogen x Genotype	0.163	0.692	0.349	0.563
	Spongy parenchyma thickness			
Source of Variation	F-value	*P*-value		
Shading	5.360	**0.034**		
Nitrogen	24.862	**<0.001**		
Genotype	5.503	**0.032**		
Shading x Nitrogen	0.720	0.409		
Shading x Genotype	0.023	0.882		
Nitrogen x Genotype	5.784	**0.029**		
Shading x Nitrogen x Genotype	0.022	0.883		

[a] SLA as described by Cavatte et al. (2012)

[b] SLA dividing the total leaf area by the total dry weight of leaves at 74 days after flowering

Table A.2. Specific leaf area, leaf thickness, thickness of palisade and spongy parenchyma considering the three factors: light intensity (sun or shade plants), N supply (20 or 125 mg N dm^{-3}) and cultivar (Biquinho or Habanero). The data are expressed as means ± standard error.

	Biquinho			
Parameters	**20 (sun)**	**125 (sun)**	**20 shade**	**125 shade**
Specific leaf area[a]	23.29 ± 0.31	20.24 ± 0.51	32.8 ± 0.68	28.58 ± 0.51
Specific leaf area[b]	24.43 ± 1.25	17.87 ± 0.83	33.21 ± 1.48	25.81 ± 0.99
Leaf thickess	211.74 ± 22.96	226.27 ± 2.95	193.98 ± 17.83	175.79 ± 5.59
Palisade parenchyma thickness	76.62 ± 12.02	102.11 ± 4.82	68.73 ± 7.76	79.00 ± 2.56
Spongy parenchyma thickness	105.01 ± 10.25	94.45 ± 5.64	95.10 ± 10.89	76.68 ± 2.15

	Habanero			
Parameters	**20 (sun)**	**125 (sun)**	**20 shade**	**125 shade**
Specific leaf area[a]	19.54 ± 0.33	20.50 ± 0.51	28.37 ± 0.48	30.37 ± 0.49
Specific leaf area[b]	17.75 ± 1.07	17.39 ± 0.36	25.30 ± 1.18	25.64 ± 0.88
Leaf thickess	240.36 ± 18.29	192.47 ± 9.07	231.00 ± 7.15	165.41 ± 5.77
Palisade parenchyma thickness	73.76 ± 3.58	70.56 ± 4.53	70.15 ± 2.09	61.57 ± 2.22
Spongy parenchyma thickness	130.00 ± 13.86	94.10 ± 4.55	123.44 ± 5.29	76.36 ± 1.80

[a] SLA as described by Cavatte et al. (2012)

[b] SLA dividing the total leaf area by the total dry weight of leaves at 74 days after flowering

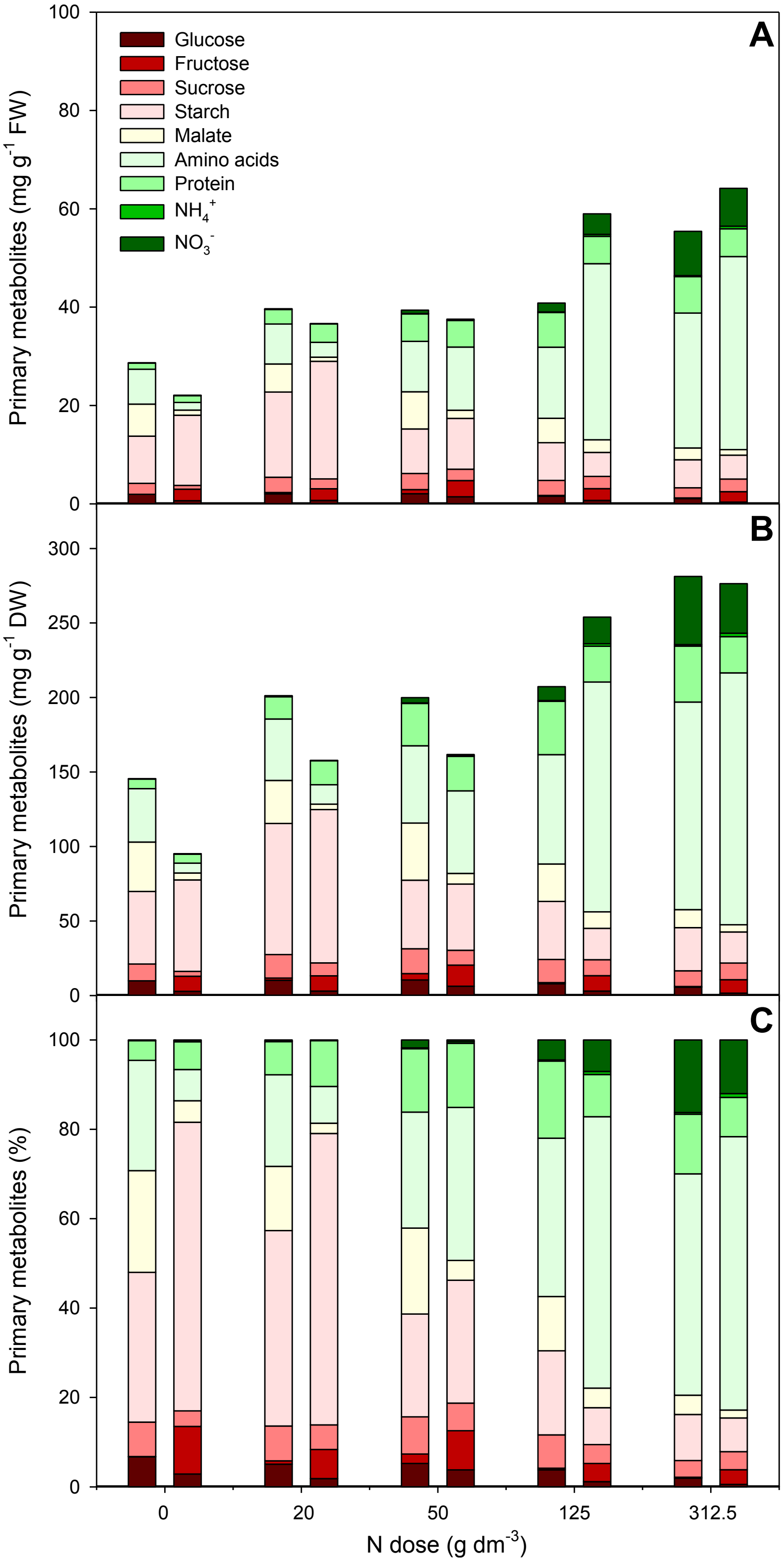

Figure A.1. Content of primary metabolites in leaves from two *C. chinense* genotypes, Biquinho (left) and Habanero (right), submitted to five doses of nitrogen. (A) Content of primary metabolites expressed by fresh weight and (B) dry weight of leaves. (C) Percentage composition of primary metabolites analyzed.

CHAPTER 4

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IDENTIFICATION OF METABOLITE TRAITS FROM THE CURRENT METABOLOMIC APPROACHES

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ABSTRACT

Metabolomics provide a qualitative and quantitative overview of the metabolites present in organisms, organs, tissues or specific cells. Different metabolomics approaches have been used for phenotyping populations and, together with mapping methods, allowed the identification of DNA sections associated with metabolite levels, defined as metabolic quantitative trait loci (mQTL). Many studies have provided a large number of mQTL and often candidate genes related to distinct metabolic traits. Since plant growth and development as well as responses to environmental changes lead to metabolic adjustments, the understanding of the genetic bases for metabolite changes is fundamental for plant physiology studies being also highly useful for plant breeding programs. Here, we revisited the progress on metabolite analysis currently used to characterize plant metabolite profile. We further describe few examples of the integration between metabolomic approaches and mapping methods allowing mQTL identification and its importance for plant physiology.

Keywords: plant metabolism, metabolomics, functional genomics, mQTL identification, candidate genes.

1. Introduction

The metabolome is the collection of all low molecular weight metabolites (usually <1000 Da) produced by cells that allows a functional readout of cellular activity and physiological status (Sun and Hu 2016). Plants contain the genetic background for several biosynthetic pathways, suggesting that plant metabolome is highly diverse and complex (Dixon and Strack 2003; Sumner et al. 2003; Wurtzel and Kutchan 2016). Altough an eukaryotic organism may contain between 4,000 and 20,000 metabolites, plant leaves are estimated to contain a number from 3,000 to 5,000 metabolites (Fernie et al. 2004). This diversity of plant metabolites include compounds of primary and secondary metabolism with wide variations in terms of chemical (molecular weight, polarity, solubility) and physical (volatility) properties (Eisenreich and Bacher 2007) as well as quantities, varying from traces (as hormones) to mM range such as sugars (Hounsome et al. 2008; Oklestkova et al. 2017).

Primary metabolites such as amino acids, organic acids and carbohydrates play essential roles in plants and are common to all species. On the other hand, secondary metabolites are often species-specific and are largely related to adaptation and protection during stresses conditions, accounting for the vast majority of the molecular diversity and plasticity in living organisms (Vogt 2010; Tissier et al. 2014; Yandeau-Nelson et al. 2015; Caretto et al. 2015). Due to the key role of metabolic changes on plant growth, stresses responses or food quality, combined technologies for metabolite analyses have been used to characterize the metabolome of a large number of species, growing under different conditions (Hounsome et al. 2008; Jorge et al. 2016; Ghatak et al. 2018). The list of species characterized include arabidopsis (Maruyama et al. 2014; Watanabe et al. 2014; Nagler et al. 2015; Wu et al. 2018), tomato (Roldan et al. 2014; Paupière et al. 2017; Zhu et al. 2018), rice (Gupta and De 2017), wheat (Qi et al. 2017), maize (Sun et al. 2016; Wen et al. 2018), soybean (Komatsu et al. 2014), cacao (Wang et al. 2016), grape berries (Wang et al. 2017), *Pinus* (Meijón et al. 2016), barley (Cao et al. 2017; Templer et al. 2017), poplar (Watanabe et al. 2018) and others (Ghatak et al. 2018). Moreover, intensive efforts have been made to reveal the genetic bases of the metabolic alterations in plants (Gilliland et al. 2006; Schauer et al. 2006; Lisec et al. 2008; Schauer et al. 2008; Riedelsheimer et al. 2012b; Ying et al. 2012; Li et al. 2013; Owens et al. 2014; Qin et al. 2014; Sauvage et al. 2014; Alseekh et al. 2015; Chen et al. 2016; Nimmakayala et al. 2016; Fusari et al. 2017; Han et al. 2018).

The advances in metabolite analyses and genotyping of large plant populations allowed us to obtain high-resolution maps of genomic regions associated to metabolite levels for distinct species (Scossa et al. 2016). Arabidopsis and tomato are classic examples of species in which mQTLs have been identified from the construction of genetic maps obtained by metabolite profiling characterization of segregating populations (Schauer et al. 2006, 2008; Calenge et al. 2006; Keurentjes et al. 2006; Rowe et al. 2008; Lisec et al. 2008, 2009; Fu et al. 2009; Toubiana et al. 2012a; Perez-Fons et al. 2014; Alseekh et al. 2015, 2017; Liu et al. 2016; Kazmi et al. 2017; Knoch et al. 2017). The distinct phenotypes observed in biparental segregating populations can be associated to genomic regions from the construction of linkage maps using molecular markers to precisely locate quantitative trait loci (QTL) (Salvi and Tuberosa 2005; Lippman et al. 2007). In addition, QTL are often identified by genome-wide association studies (GWAS) from the high-throughput genotyping of natural accessions and the analysis of statistical associations between the genotypes and the traits (phenotypes) of the individuals (Rafalski 2010; Burghardt et al. 2017). Thus, these mapping methods together with large-scale metabolite analyses enable the identification of genetic variants associated with metabolic traits (Yandeau-Nelson et al. 2015), frequently at the gene level. Indeed, many studies were able to identify candidate genes or even confirm their role by reverse genetic techniques after the mQTL identification (*e.g.* Lisec et al. 2008; Chan et al. 2010a; Chen et al. 2014; Alseekh et al. 2017; de Abreu e Lima et al. 2018; Wu et al. 2018).

The metabolic characterization and the understanding of its genetic base offers an adequate biological comprehension allowing the manipulation of metabolite levels during the plant growth and development as well as it provides insights into their interaction with the surrounding environment. Furthermore, this understanding provides valuable information that can be further used by plant breeders. In this review, we briefly describe and discuss the advances in analytical tools used for plant metabolomics, including the main features, advantages and disadvantages of each method. We further address the growing association of metabolomics and characterization of synthetic and natural populations for the identification of genomic regions and candidate genes associated with groups or individual metabolites.

2. Metabolomic approaches used for plant tissues

Fundamentally, metabolites can be analyzed by mass spectrometry (MS) and also molecular spectroscopy (Goodacre et al. 2004). The MS identifies and quantifies different classes of metabolites by the formation of gas-phase ions that are detected and characterized by their mass and charge (Murray et al. 2013). In the MS, complex samples can be used for direct injections and generation of the mass-to-charge (*m/z*) ratio used for putative metabolites identification (Lei et al. 2011). Plant metabolomics based on direct MS analyses is a rapid analysis that offers a feasible method of large-scale analyses such as metabolomics screening of large mutant population, functional genomics, and compound identification (Aharoni et al. 2002; Castrillo et al. 2003; Hirai et al. 2005; Oikawa et al. 2006; Allegrand et al. 2010; Giavalisco et al. 2011; Wang et al. 2015). However, it may lead to problems in the detection of many metabolites such as ion suppression effects (Lei et al. 2011). To avoid this problem and improve the metabolite coverage, tandem mass spectrometry

(MS/MS) has been developed and, in addition to the molecular ions *m/z* values, this approach allows the evaluation of fragments generated from precursor ions (Lopes et al. 2017). Furthermore, MS/MS allows two or more sequential stages of mass spectrometric analysis (tandem MS^n experiments, where *n* refers to the number of mass analysis steps) (Jorge et al. 2016), allowing highly accurate metabolite identification and even the empirical formula determination.

As the direct MS analyses alone cannot differentiate chemical isomers (*e.g.* hexoses) (Sumner et al. 2003), certain technologies such as chromatography are coupled to MS devise (MS-based approaches) increasing selectivity, sensitivity, and comprehensiveness of the obtained data (González-Domínguez et al. 2017). Consequently, MS coupled to chromatography leads to an increased time for analysis (Sumner et al. 2003; Goodacre et al. 2004; Lopes et al. 2017; Ghatak et al. 2018). The most used combined approaches for metabolite analyses in plants are gas chromatography (GC) and liquid chromatography (LC) coupled to MS, allowing metabolite determination with high resolution.

2.1. MS-based approaches

As mentioned above, coupled with chromatographic separation techniques, mass spectrometers are capable of detecting metabolites of low abundance (Tugizimana et al. 2013). By using GC or LC coupled to MS, metabolites of rather complex samples are first separated by one chromatography technique. Afterwards, sub fractions (chromatographic peaks) can be introduced directly (online) or indirectly (offline) into the MS. The online coupling allows the metabolite identification generating the retention time (RT) for each compound that is compared with authentic standards. Moreover, MS analysis provides structural information of the compound (Lopes et al. 2017) which is of crucial importance for flux analysis experiments (Heise et al. 2014). Thus, we will discuss the main advantages, disadvantages and applications of MS-based approaches such as GC-MS and LC-MS methods for determining the metabolite profile of plants.

2.1.1. Gas chromatography-mass spectrometry (GC-MS)

GC-MS is a relatively sensitive and a highly robust technology. In GC-MS-based methods, after extraction and sample drying, polar metabolites are derivatized to render them volatile and able to be separated by GC (Lisec et al. 2006). When combined with electron impact ionization, it results in highly reproducible fragmentation pattern that is essential for large-scale experiments (Obata and Fernie 2012; D’Amelia et al. 2018). Furthermore, GC-MS affords good coverage of primary metabolism, including sugars, organic and amino acids and their derivatives, by measuring hundreds of analytes (mass spectral features that are of either known or unknown chemical identity) in many samples (Alseekh and Fernie 2018; D’Amelia et al. 2018). The GC-MS analysis, in general, has a short running time and a relatively low running cost what explain the widespread use of this technology for metabolite profiling characterization. However, this technique is only capable to analyze volatile and thermally stable metabolites or metabolites that can be chemically modified to produce volatile derivatives (Jorge et al., 2016). Even so, GC-MS is currently the most accepted and applicable analytical technique in plant metabolomics studies.

2.1.2. Liquid chromatography-mass spectrometry (LC-MS)

LC-MS-based analysis provides the most comprehensive approach used in plant metabolomics. LC-MS does not require any prior sample treatment after extraction, has high sensitivity and separates the components in a liquid phase. Moreover, the metabolite separation occurs on the basis of differential chemical properties according to the choice of columns, including reversed phase, ion exchange, and hydrophobic interaction (Fernie and Tohge 2017; Alseekh and Fernie 2018). LC-MS is frequently used to profile secondary metabolites in plant metabolomics, but can also be used to primary metabolites, photosynthetic intermediates, lipids, fatty acids and phytohormones (Scherling et al. 2010; Meijón et al. 2016; Wang et al. 2017; Shimizu et al. 2018). Additionally, LC-MS has been used to resolve intracellular metabolic fluxes, especially regarding the central carbon metabolism (Szecowka et al. 2013; Heise et al. 2014).

The metabolome profiling by LC-MS is typically performed by targeted or untargeted methods (Gorrochategui et al. 2016). Non-target profiling have been used for monitoring several compounds belonging to different chemical classes, such as amino acids, lipids, organic acids, and flavonoids (Abrankó and Szilvássy 2015; Maldini et al. 2015; Ghosson et al. 2018). On the other hand, targeted approaches focus on identification and quantitation of a defined set of metabolites. Currently, targeted approaches use high sensitivity, simple and low price mass spectrometers, such as triple quadruple (QqQ). Consequently, these approaches allow versatile scan modes to profile phytohormones and stress responsible compounds (Forcat et al. 2008; Müller and Munné-Bosch 2011; Liu et al. 2012; Vital et al. 2017), providing complementary information to observed phenotypic responses.

Columns with different selectivity such as two-dimensional separation system (LCxLC and GCxGC) can be used to increase the peak heights, enhancing the metabolites detection sensitivity mainly for complex matrices (Guiochon et al. 2008; Gu et al. 2011; Almstetter et al. 2012; Tugizimana et al. 2013). Alternatively, the potential of LC-MS to analyze a wider variety of metabolites in plants has been improved after the development of ultra (high) resolution chromatography (UPLC). Compared with the conventional high-performance liquid chromatography (HPLC), UPLC allows higher peak resolution due to the use of capillary columns and nano flow for higher sensitivity (Obata and Fernie 2012; D'Amelia et al. 2018). Besides improving the metabolite coverage, UPLC has been used to solve the problems with ion suppression and pertinently requires lower sample volume for the metabolic phenotyping (Holmes et al. 2019). Currently, ~1000 metabolites can be detected in a single run using coupled UPLC high-resolution MS and MS/MS (Giavalisco et al. 2011). The robustness of UPLC-QqQ-MS system allows also analysis of hundreds of samples with minimal sample preparation and relatively low cost. For example, it was used for broad range profiling of complex secondary compounds such as flavonoid from contrasting genotypes in terms of herbivory tolerance (Gómez et al. 2018; Vital et al. 2018).

2.2.Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is also used in plant metabolomics. It is important to mention this technique is the only one that can track stable isotope labeling at specific atomic positions (isotopomer analysis) (Deborde et al. 2017). Although NMR is able to measure only highly abundant metabolites or at least highly concentrated samples, this technique is used either in isotope tracing experiments or in the elucidation of metabolite structure (Reuhs and Simsek 2017). NMR is an unbiased, rapid and non-invasive technique that requires little sample preparation, independent of the analyte polarity. Furthermore, this technique is not based on analyte separation as the chromatographic analysis and it does not require sample derivatization (Kim et al. 2010; Tugizimana et al. 2013). However, due to its lower sensitivity and dynamic range in comparison to MS, NMR-based plant metabolomics is most used as a metabolic fingerprinting technique that provides an overview of primary and secondary metabolites (Hall 2006; Kim et al. 2011). NMR has been used for many years and consequently was subjected to intensive validation. Moreover, specific computational analysis and chemometric software associated to the technique are available (Fernie et al. 2004).

3. Computational platforms for large-scale MS data processing, statistical validation, and metabolic interpretation

Physiological investigations usually involve complex experimental design, including treatments, biological and technical replicates to be analyzed in a single experiment. Typically, GC-MS and LC-MS analysis generate thousands of *m/z* features which might need computational tools for further data analysis. Metabolic profiling by the target and non-target metabolite analysis from LC-QqQ-MS spectra can be analyzed automatically using the Skyline platform (https://skyline.ms/project/home/begin.view?) configured for low mass analysis (Gómez et al. 2018; Vital et al. 2018). After manual inspections, the area of hundreds of XICs (*extracted ion chromatogram*) can be exported to perform the quantitative and integrative analysis. GC-MS raw data can be processed using computational packages such as the open-source R package TargetSearch (Cuadros-Inostroza et al. 2009) and TagFinder (Luedemann et al. 2008) enabling the identification of 150-180 compounds from leaf extracts with high quality scores (Llanes et al. 2016; Vital et al. 2017).

LC-MS non-target methods also generate a large amount of data, which requires specific features for evaluation and detection, normalization, label-free quantification, and identification. The XCMS platform (https://xcmsonline.scripps.edu/) has been extensively used for metabolite analyses (Forsberg et al. 2018) and allows the upload of the mass spectra for automatic remote processing optimized for several LC-MS systems. In addition, it is possible to perform multi-factor comparisons between different experimental contrasts, producing also, as outputs, tables containing precursor ion retention time (RT)/*m/z* values indicating changes in the relative abundances. The XCMS enable also the identification of putative compounds by MS and MS/MS search using the METLIN algorithm (Guijas et al. 2018) against spectral libraries. The XCM package can also be used in-house using R scripts to produce outputs containing feature information (RT and *m/z* values from precursor ions) that can be further used to perform statistical analysis and metabolite identification by formula predictor and MS/MS database search (Blaženović et al. 2018). Several algorithms are available, such as SIRIUS (Dührkop et al. 2013) and SigmaFit (Bruker), for obtaining the chemical formulas using both high resolution MS and MS/MS spectra. The putative formulas can be combined with fragmentation patterns, presents in the spectral libraries, such as METLIN and MASSBANK, to increase the confident identification ratios. The putative compounds can be subsequently confirmed using the RTs and MS/MS from authentic standards.

Statistical, functional and integrative analyses of metabolomic data can be performed from data generated by NMR and MS approaches, such as the output tables from TargetSearch and XCMS. Compound lists and *m/z* features can be automatically processed using the MetaboAnalyst platform (http://www.metaboanalyst.ca/). This platform is able to remove low quality data and also perform data normalization and transformation, clustering analysis, multivariate statistical tests and identification of the compounds most discriminants related to the treatments. Finally, the compounds and their concentrations or arbitrary abundances can be used for functional cluster analysis, indicating which pathways were significantly modified/perturbed in response, for instance, to environmental and/or genotypic constraints.

Bioinformatic tools such as MapMan and Vanted can be also used to visualize and statistically compare 'omics' data (Junker et al. 2006; Usadel et al. 2009). The MapMan was developed to analyze large dataset of transcripts from different plant species and it has been used to classify genes and metabolites as well as to provide a modular system to visualize the results in the context of different pathways and processes (Usadel et al. 2005, 2009). Although this software was initially developed for arabidopsis plants, it can be used for other plants through ontologies/mapping and comparisons with species already sequenced, and even among phylogenetically distant species (Usadel et al. 2009). Afterwards, with the emergence of Vanted, several biochemical data sets (*e.g.* transcript, protein, and metabolite) could be compared by statistical methods, allowing the production of clusters according to similar behavior and correlation networks (Junker et al. 2006). The Vanted offers visual exploration, statistical calculations such as the Student *t*-

test, outlier identification, correlation analysis, as well as biochemical data clustering with self-organizing maps (Junker et al. 2006). In this regard, it seems reasonable to anticipate that further development of bioinformatics tools are essential for eliciting biologically significant interpretations of a large amount of experimental data besides it may support growing comprehension of plant biological processes, for instance, under different growth conditions and time-points. Thus, bioinformatic tools such as MapMan and Vanted clearly provide significant help to scientists with the analysis and interpretation of large-scale biochemical datasets being useful tools for mQTL identification.

4. Metabolite profiling as a tool for understanding stress responses and functional genomics in plants

As aforementioned, metabolomics became an important tool not only in plant physiology and biochemistry but also in functional annotation of genes associated with metabolism. However, even with the significant progress of all techniques to characterize the metabolome, the amount of metabolites detected represents only approximately 7% of total metabolites of a typical plant cell (Alseekh and Fernie 2018). Therefore, improving the capacity to detect more metabolites remains a major technical challenge, particularly for plant metabolomics. Even so, studies aiming at the metabolite profile characterization still provide a considerable source of information that allows pertinent inferences about plant metabolism, including changes in metabolite levels caused by environmental factors. Just over two decades ago, the earliest metabolomics characterization largely used direct-injection in MS, GC-MS or LC-MS to profile transgenic and/or environmentally challenged plants (Fiehn et al. 2000; Roessner et al. 2001a, b; Aharoni et al. 2002; Bovy et al. 2002; Urbanczyk-Wochniak and Fernie 2005). In addition, metabolomics has been used as screening approaches (Catchpole et al. 2005; Meyer et al. 2007; Trenkamp et al. 2009). Currently, besides the use of LC-MS and GC-MS, other approaches are used to characterize plant metabolome and consequently to obtain new insights into stress tolerance in land plants.

Metabolite profiling determined by gas chromatography time of flight-mass spectrometry (GC-TOF-MS) of nodulated and non-nodulated *Casuarina glauca* provided evidence of the changes in the metabolic profile that led to salt stress tolerance (Jorge et al. 2017). Additionally, combined metabolic approaches were used to understand the primary and secondary metabolism throughout the development of grape berry fruits (4D GC×GC-TOF-MS and LC-ESI-MS/MS, respectively) as well as primary metabolism (GC-MS and LC-MS) and hormonome (HPLC-QqQ-MS) of barley roots under salt stress (Cao et al. 2017; Wang et al. 2017). ^{1}H-NMR was used for the metabolic profiling of *Miscanthus* genotypes under stress showing that 40 metabolites were different between sensitive and tolerant genotypes (Le Gall et al. 2016). In another study, metabolomic technologies involving chromatographic separation and MS techniques were used to characterize the seasonal changes in metabolite and lipid abundances in gray poplar (Watanabe et al. 2018). Many metabolites were measured in different poplar tissues including twig buds, leaves, bark, and wood. This allowed the identification of several metabolites, such as *i*) thiols and amino acids by a combination of monobromobimane or *O*-phthalaldehyde fluorescent labeling, respectively, followed by HPLC, *ii*) primary metabolites by GC-TOF-MS, *iii*) lipids by liquid chromatography electrospray ionization-mass spectrometry (LC-ESI-MS) and, *iv*) chlorophyll and protein content by spectrophotometric method to detect light absorbance in specific lengths. Remarkably, the usage of different technologies in this study allowed the identification of a wide range of metabolites at different time points as well as to C, N, and S storage and mobilization processes (Watanabe et al. 2018). Thus, it seems clear that the use of different metabolic profile approach allow a better understanding of plant metabolism, especially, but not only, regarding the metabolic responses displayed by environmental factors.

Metabolite profiling analyses are also essential for the functional annotation of genes associated with metabolism. For example, ultra-performance liquid chromatography coupled with Fourier transform mass spectrometry (UPLC-FT-MS) has been successfully used to characterize lipid metabolism in maize leaves and seedlings and allowed the identification of genes involved in lipid metabolism (de Abreu e Lima et al. 2018). UPLC-FT-MS also demonstrated the power of combining large-scale untargeted metabolomics with time-course-derived networks under different environments for identifying metabolite-gene associations in arabidopsis (Wu et al. 2018). Furthermore, the metabolomic profiling analyses allowed the detection of ~3000 metabolites which resulted in the identification of 123 highly resolved genomic regions associated with metabolites variation (mQTL) (Wu et al. 2018). In other study, candidate genes associated with amino acids levels were identified after metabolite characterization by LC-MS/MS (Angelovici et al. 2016). Moreover, as a result of primary metabolites quantified by GC-TOF-MS of different tissues, it was suggested 36 loci/genes to functional characterization studies or genetic improvement in maize (Wen et al. 2018). Additionally, other studies have suggested genomic regions or agronomic traits related to metabolites in distinct populations of tomatoes and maize through determination of the metabolic profile by MS and NMR approaches (Perez-Fons et al. 2014; López et al. 2015; Venkatesh et al 2016).

For a successful functional gene annotation, however, it is fundamental the integration of metabolite information with mapping methods. In this context, three approaches have been used to identify mQTL and candidate genes: forward genetics, biparental QTL mapping, and GWAS. The direct testing of candidate genes via the analysis of knock-out and knock-down mutants is an useful example of forward genetics. Although the recognized contribution of the forward genetics to identify genes of major effect, these technics are not efficient to identify genes with subtle effects (genes that do not provide statistically convincing evidence of a phenotypic effect). It also fails to provide direct information on whether allelic diversity at these loci is responsible for a phenotypic variation. Thus, as discussed within the next sections, several studies have used the biparental QTL mapping and GWAS to identify mQTL and candidate genes related to metabolite levels.

4.1. The potential use of metabolomic approaches combined with mapping methods to identify mQTL

The association between metabolism and economical relevant traits has encouraged many researchers to connect plant metabolites to genetics (Table 1). As a result, many genomic regions and/or candidate genes related to metabolite levels have been identified. As discussed in above sections, metabolic analysis techniques, genotyping technologies and bioinformatics have been improved along the years, making the knowledge on their applications or limitations decisive for future research.

A well-succeed mQTL identification is dependent on the outcome of five main tasks (Figure 1). The first is obtaining a large segregating population for an efficient fine-scale mQTL mapping consistent with the mapping method. Bi-parental populations such as recombinant inbred lines (RILs) (Rowe et al. 2008; Pollard 2012;

Wen et al. 2015; Kazmi et al. 2017; Knoch et al. 2017; de Abreu e Lima et al. 2018) and introgression lines (ILs) (Eshed and Zamir 1995; Schauer et al. 2006; Toubiana et al. 2012b; Perez-Fons et al. 2014; Alseekh et al. 2015) are generally used for classical mQTL mapping. On the other hand, landrace accessions are often used to mQTL mapping by GWAS (Chan et al. 2010a; Sauvage et al. 2014; Angelovici et al. 2016; Wu et al. 2016). Both biparental mapping and GWAS use nucleotide sequences called molecular markers to investigate the polymorphism present between the nucleotide sequences of different individuals (Nadeem et al. 2018). Nevertheless, GWAS only became available from the development of high-throughput genotyping technologies (Huang and Han 2014) such as genotype-by-sequencing (GBS) that uses restriction enzymes coupled with DNA barcoded adapters to reduce the genome complexity (Chung et al. 2017). After phenotyping and genotyping of the population, statistical methods performed by proper software allow the data integration according to the mapping method (Hackett 2002; Zou and Zeng 2008; Li et al. 2010; Korte et al. 2012; Xu et al. 2017; Wang et al. 2018). Finally, the performance of analysis to identify particular genetic variants that are likely to influence the examined trait, called fine mapping, facilitates the constraint of the locus related to the metabolic trait (Alseekh et al. 2017; Schaid et al. 2018). Afterwards, when overcome the lack of understanding of the pathways involved or the complex polygenic traits, candidate genes can be suggested and further validated by reverse genetic techniques (*e.g.* Lisec et al. 2008; Chan et al. 2010a; Kloosterman et al. 2010; Chen et al. 2014; Alseekh et al. 2017; de Abreu e Lima et al. 2018; Wu et al. 2018).

4.2. Development of mQTL studies

The potential for the combination of metabolic analyses and genetics is nicely exemplified by the identification and characterization of the QTL associated with total soluble solid content in tomato fruits, which contain the allele of the *Lycopersicum Invertase5* (*LIN5*) (Godt and Roitsch 1997). First, an ILs population of *Solanum lycopersicum* containing *S. pennellii* chromosome segments was obtained through repeated backcrossing and extensive genotyping (Eshed and Zamir 1995) and, together, the whole ILs population completely cover the whole *S. pennellii* genome in the commercial *S. lycopersicum* cultivar M82. By using this population, 23 QTL related to the increased Brix were identified (Eshed and Zamir 1995).

Subsequently, the QTL called *Brix-9-2-5* was mapped to a 9 centimorgan (cM) segment on chromosome 9 (Eshed and Zamir 1996). Further analysis, using the homozygous *S. lycopersicum* (cv. M82), the IL homozygous for the *S. pennellii* allele, their F_1 hybrids and recombinant families in *Brix-9-2-5* confirmed that wild-species allele lead to increased levels of glucose and fructose in fruits from cultivated tomato with various genetic backgrounds and under different environmental conditions (Fridman et al. 2000). Furthermore, mapping analysis delimited a single nucleotide polymorphism-defined recombination hotspot of 484 base pairs (bp) of a fruit-specific apoplastic invertase in the line 9-2-5 (Fridman et al. 2000). Indeed, apoplastic invertases catalyze the irreversible cleavage of sucrose to glucose and fructose, which are further transported into the cell. Afterwards, the same QTL was confirmed in both indeterminate and determinate growth habit tomato cultivars (Fridman et al. 2001). Additionally, the same authors revealed another QTL related to fruit soluble solids in determinate growth habit cultivars, called *PW-9-2-5* (Fridman et al. 2001). Furthermore, it was demonstrated that the higher Brix phenotype was associated with a single nucleotide substitution coding an amino acid residue close to the fructosyl-binding site of the apoplastic invertase, affecting the enzyme kinetics *in vitro* (Fridman et al. 2004). Last of all, RNA interference (RNAi)-based approach was used to evaluate the consequences of silencing *LIN5* on floral and fruit development (Zanor et al. 2009). By using metabolite profiling of ovaries, green and red fruits this study confirmed that the metabolic changes observed in the transformants were largely related to sugar metabolism, as previously suggested (Baxter et al. 2005). Altogether, these studies clearly improved our understanding on carbohydrate metabolism in tomato fruits and its impact on fruit yield and quality as well as its application in tomato breeding programs.

Glucosinolates mQTL is another example of a complex quantitative trait explained by the progress of results over time (Kliebenstein, 2009). Glucosinolates (S-glucopyranosyl thiohydroximates) are naturally occurring S-linked glucosides found mainly in Brassicaceae species which have metabolism modulated by environmental challenges (Martinez-Ballesta et al. 2013; Ishida et al. 2014). An initial study used 162 RILs that were genotyped by different molecular markers whereas metabolites levels were determined by HPLC allowed the identification of loci associated with total aliphatic glucosinolate accumulation, total indolic glucosinolate concentration and benzylic glucosinolate levels (Kliebenstein et al.

2001). Later on, four major genes related to the enzymes for the production of alkenyl glucosinolates (*AOP2*), 3C hydroxyalkyl glucosinolates (*AOP3*), methylthioalkylmalate (*MAM1*) and methylsulfinylalkyl ($FMO_{GS\text{-}OX1}$) were identified from results of previous QTL analysis (Kliebenstein et al. 2001; Kroymann et al. 2003; Hansen et al. 2007; Wentzell et al. 2007; Kliebenstein 2009). Furthermore, GWAS was used to map mQTL for glucosinolates (Chan et al. 2010b). In an experiment using 96 *A. thaliana* accessions, 43 glucosinolate phenotypes, and 229,940 single nucleotide polymorphisms (SNPs) was identified 172 genes with the potential to affect glucosinolates synthesis. Altogether, these studies revealed several mQTL and genes related to the glucosinolates biosynthetic pathway in arabidopsis (Sønderby et al. 2010), which is clearly applicable in breeding programs since glucosinolates are functional for supporting human health (Ishida et al. 2014).

To identify mQTL in tomato fruits another study used a population of 76 ILs and metabolite profiling by GC-MS (Schauer et al. 2006). In this work, 889 mQTLs related to 74 metabolites including amino acids and organic acids, sugars, sugar alcohols, fatty acids and vitamins C and E were identified. In a subsequent study, the mode of inheritance of mQTL and its consequences for the breeding of specific traits were investigated by studying the hereditability of the fruit metabolome though analysis of additional year's harvest and evaluation of the metabolite profiles of lines heterozygous for the introgression (ILHs) (Schauer et al. 2008). This study identified 332 putative QTL and revealed that most of QTL related to primary metabolites is dominantly inherited (Schauer et al. 2008). Recently, by using the same IL population, 679 mQTL related to secondary metabolites were identified for tomato fruits showing that some of them displayed a dominant-negative mode of inheritance (Alseekh et al. 2015). Afterwards, analyzing the levels of metabolites in tomato fruits from multiple harvests of the same ILs population, several mQTL that reduce variability for primary and secondary metabolites were identified, what suggests that the trait variation of some metabolites may be buffered by a regulatory locus (Alseekh et al. 2017). In addition, nine mQTL were further validated with higher mapping resolution using a population of backcross inbred lines (BILs) derived from the same parental genotypes. Altogether, these studies revealed several mQTL and also an important view of the mode of inheritance of metabolic traits in tomato fruits. Overall, many studies have identified genetic factors related to metabolites and are summarized in Table 1. Although these studies have allowed a substantially better characterization and comprehension of different pathways in plant metabolism, it seems reasonable to anticipate that they also provide the opportunity to greatly accelerate crop improvement, particularly those that have clearly lacked the attention they deserve. In addition, once the genomic basis for the metabolite levels is known, breeding and biotechnological programs can use this information to incorporate metabolic traits of interest and improve plant growth and/or fruit quality of crop plants grown under field conditions. It is highly likely that this knowledge will also enable us to pursue novel research avenues to increase our understanding of the complex networks governing metabolite function in different crop species.

4.3. Features of the mapping methods

Briefly, biparental mapping populations such as F_2, RIL, near-isogenic lines (NIL), backcrosses (BC), and doubled haploids (DH), come from the crossing of parental genotypes that clearly differ for traits of interest (Collard et al. 2005). Although the classical biparental mapping coupled with the metabolome characterization has successfully been used to identify mQTL, few recombination events occur during the generation of a biparental mapping population generally providing a limited amount of genetic variation (Ferreira et al. 2006; Kao and Zeng 2010; Xu et al. 2017). As a consequence, only QTL at which the two parents substantially differ can be detected (*e.g.* Yu et al. 1997; Xing et al. 2002; Collard et al. 2005; Bai et al. 2010; Liu et al. 2010). Moreover, to locate the QTL with high resolution requires an additional work on fine mapping once the linkage mapping allows the localization of QTL to 10-20 cM intervals (Mauricio 2001; Huang and Han 2014; Alseekh et al. 2017; Burghardt et al. 2017; Xu et al. 2017). Thus, following the rapid development of sequencing technologies and computational methods, many studies have identified mQTL and candidate genes related to metabolites by GWAS combined with the metabolome characterization for different species (Table 1).

Different from biparental mapping, GWAS include population scale samples to study the nonrandom association of alleles at different loci (called linkage disequilibrium) generated by ancestral recombination events. Consequently, GWAS normally allow finer resolution of QTL location due to the history of recombination events across a lineage (Nordborg and Weigel 2008; Rafalski 2010). Furthermore, GWAS offers the power to genetically map multiple traits simultaneously. However, a potential disadvantage of GWAS is the possibility of statistically significant results at loci unrelated to the trait, so called false positives (or spurious) associations (Burghardt et al. 2017). Sources of false positives associations result mainly from nonrandom associations due to the population structure, term used for the presence of subgroups in the population with ancestry differences (Aranzana et al. 2005; Zhao et al. 2007; Nordborg and Weigel 2008). Currently, the use of mixed-models, correction for multiple testing, inspection of Q-Q plots and Manhattan plots in addition to methods of correcting for population stratification ameliorate the occurrence of possible problems with false positives associations (Yu et al. 2005; Price et al. 2010; Korte et al. 2012; Korte and Farlow 2013). It is equally important to mention that possible false negatives results from the potential to miss signal due to the low power to detect relatively small genetic effects (Hong and Park 2012). False negative results occur mainly after the use of methods and software tools to correct the results for population structure or due to small sample size (Rafalski 2010; Hong and Park 2012). Therefore, GWAS is a useful and robust approach complementary to biparental mapping but, despite the strategies that can be used to avoid genetic associations with confounding factors in GWAS (Burghardt et al. 2017), the classical biparental mapping remains the method of choice for mapping the effects of alleles rare in germplasm collections (Rafalski 2010). In summary, wherever possible, the combination of different populations and mapping methods is a good strategy to identify genetic factors related to metabolites (Schauer et al. 2006; Lisec et al. 2008; Kliebenstein 2009; Chan et al. 2010a; Sauvage et al. 2014; Verslues et al. 2014; Han et al. 2018).

5. Concluding remarks

Although many studies have successfully used metabolic approaches in combination with mapping methods to identify genomic regions related to metabolites levels (Table 1), we posit that it is of pivotal importance that these emerging studies should be expanded. Remarkably, the vast majority of those studies discussed here have been obtained in model organisms and in specific some plants of agricultural relevance. Successfully transferring these achievements to major food crops, which are generally more recalcitrant to genetic manipulation, still remains a great challenge. Nevertheless, once identified, mQTL and candidate genes significantly improve our understanding of the plant metabolism and the complex network governing distinct important traits. Although important achievements have been made in metabolomics from the integration between metabolomic approaches and mapping methods over the last 20 years, there are still many opportunities to investigate the metabolic changes modulated by the environmental changes. Indeed, the combination of ‘omics’ and ecophysiology, recently defined as ‘ecophysiolomics’, is an up to date trend in plant physiology due to its potential applications (Flexas and Gago 2018). Moreover, a faster advancement of mQTL studies is expected for a wide range of species in the coming years. This is mainly due to the better cost-effective of both genotyping and metabolome characterization, as well as the development of sub-ILs, multiparent mapping populations, and the use of GWAS. From the metabolite characterization and identification of associated genomic regions and/or candidate genes in stress tolerant species, we will certainly get important insights into plant physiology and consequently advances in the crop breeding to achieve the challenge goal of increasing the food offer sustainably in the next years.

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FIGURES

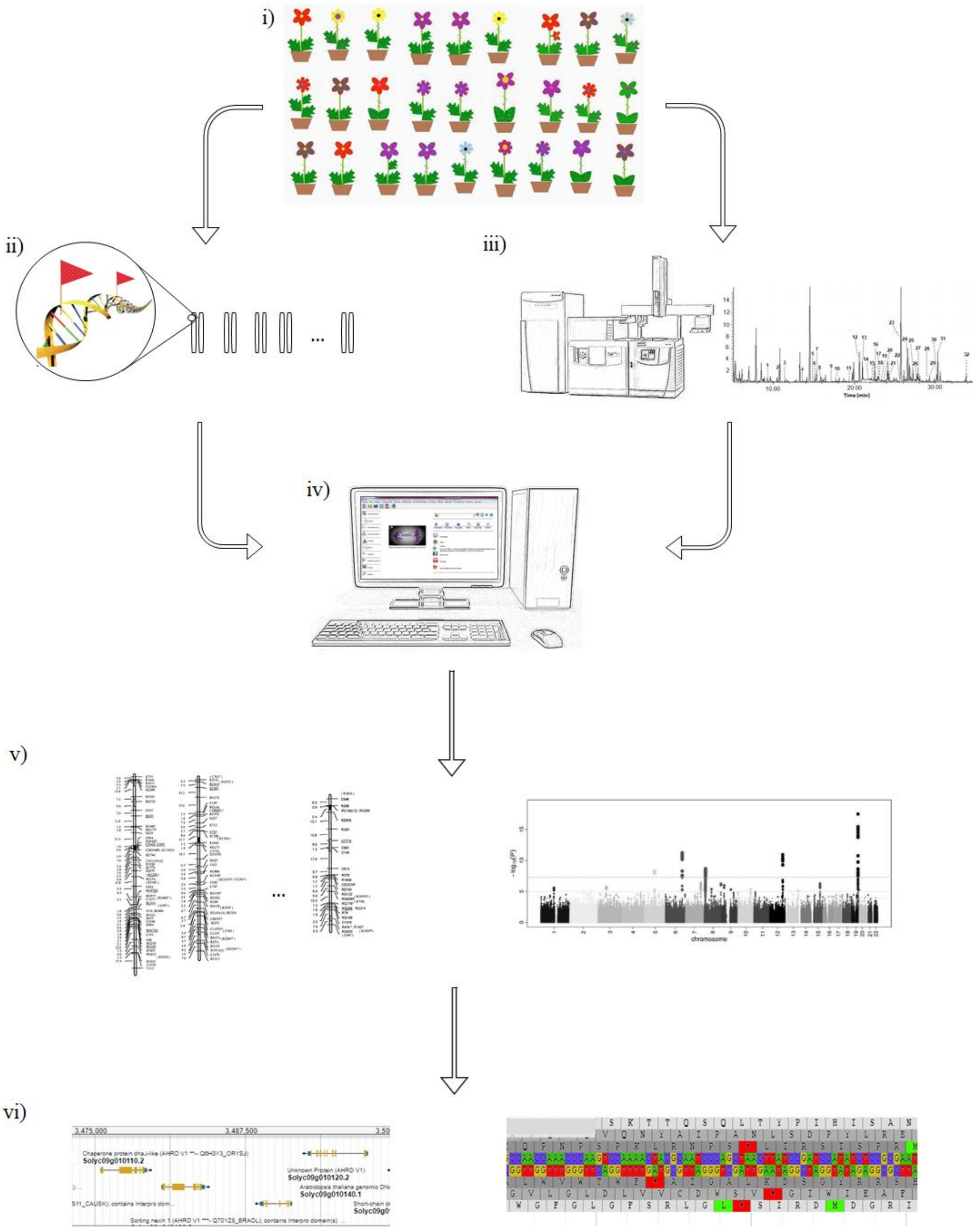

Figure 1. Main steps for a well-succeed mQTL identification. *i)* First, it is necessary to obtain a population genetically diverse and suitable according to the mapping method that will be used. *ii)* Afterwards, it is performed the genotyping by molecular markers that should be also available and *iii)* phenotyping through metabolome characterization. *iv)* Statistical methods allow the data integration while *v)* fine mapping facilitates the identification of mQTL. *vi)* Finally, candidate genes can be proposed and further confirmed by reverse genetic techniques.

Table 1. Examples of studies using metabolomics and mapping approaches to explore many distinct metabolites and identify mQTL in plants.

Species	Tissue	Mapping method	Population Type	Metabolic method	Reference
Apple	Fruit	LM[a]	F1	LC-QTOF-MS	(Khan et al. 2012)
Arabidopsis	Seedling	LM	LA[b], RILs	HPLC-QTOF-MS	(Keurentjes et al. 2006)
Arabidopsis	Leaf	LM	RILs, ILs	GC-MS	(Lisec et al. 2008)
Arabidopsis	Leaf	LM	RILs	GC-TOF-MS	(Rowe et al. 2008)
Arabidopsis	Seedling	LM	RILs, ILs	GC-TOF-MS	(Lisec et al. 2009)
Arabidopsis	Seedling	LM	RILs	HPLC-QTOF-MS	(Fu et al. 2009)
Arabidopsis	Leaf	GWAS	LA	GC-TOF-MS	(Chan et al. 2010a)
Arabidopsis	Leaf	GWAS	LA	GC-MS	(Wu et al. 2016)
Arabidopsis	Seed	GWAS	LA	LC-MS/MS	(Angelovici et al., 2016)
Arabidopsis	Seed	LM	RILs	GC-MS	(Knoch et al. 2017)
Arabidopsis	Leaf	GWAS	LA	LC-MS	(Wu et al. 2018)
Barley	Flag leaf	GWAS	LA, elite lines	IC-MS, HPLC	(Templer et al. 2017)
Maize	Leaf	GWAS	Inbred lines, testcrosses	GC-MS	(Riedelsheimer et al. 2012a)
Maize	Leaf	GWAS	Inbred lines	GC-MS	(Riedelsheimer et al. 2012b)
Maize	Kernel	GWAS	Inbred lines	UPLC-TOF-MS	(Shen et al. 2013)
Maize	Kernel	GWAS, LM	Inbred lines, RILs	LC-MS	(Wen et al. 2014)
Maize	Seedling, leaf, kernel	LM	RILs	GC-TOF-MS	(Wen et al. 2015)
Maize	Kernel	GWAS	RILs	LC-MS	(Wen et al. 2016)
Maize	Seedling, leaf, kernel	GWAS	Inbred lines, RILs	GC-TOF-MS	(Wen et al. 2018)
Maize	Leaf, seedlings	LM	RILs	UPLC-FT-MS	(de Abreu e Lima et al., 2018)
Potato	Tuber	LM	Diploid (C x E) mapping	GC-TOF-MS	(Carreno-Quintero et al. 2012)
Rice	Seed	LM	Back-crossed inbred lines	LC-Q-TOF-MS	(Matsuda et al. 2012)
Rice	Flag leaf, Germinating seed	LM	RILs	LC-EI-MS	(Gong et al. 2013)
Rice	Leaf	GWAS	LA	LC-MS	(Chen et al. 2014)
Rice	Leaf	GWAS	LA	LC-QTOF-MS	(Matsuda et al. 2015)
Rice	Grain	GWAS	LA	LC-ESI-MS	(Chen et al. 2016)
Rice	Leaf	GWAS	RILs	GC-TOF-MS	(Li et al. 2016)
Tomato	Fruit	LM	ILs	GC-MS	(Schauer et al. 2006)
Tomato	Fruit	LM	ILs	GC-MS	(Schauer et al. 2008)
Tomato	Fruit	LM	ILs	GC-MS	(Toubiana et al. 2012)
Tomato	Fruit	LM	ILs	GC-MS, LC-MS, NMR, HPLC-PDA	(Perez-Fons et al. 2014)
Tomato	Fruit	GWAS	LA	GC-MS	(Sauvage et al. 2014)
Tomato	Fruit	LM	ILs	UPLC-MS	(Alseekh et al. 2015)
Tomato	Fruit	LM	Sub-ILs	GC-MS, LC-MS, UPLC-FT-MS	(Liu et al. 2016)
Tomato	Germinating seed	LM	RILs	GC-TOF-MS	(Kazmi et al. 2017)
Tomato	Fruit	LM	ILs	UPLC-MS	(Alseekh et al. 2017)
Wheat	Flag leaf	LM	Doubled haploid lines	GC-MS	(Hill et al. 2013)

Wheat	Flag leaf	LM	Doubled haploid lines	LC-ESI-MS	(Hill et al. 2015)
Wheat	Leaf	GWAS	Elite lines	GC-MS	(Matros et al. 2017)

[a] Linkage mapping, [b] Landrace accessions

CONCLUDING REMARKS

In this study I have demonstrated that Biquinho and Habanero are distinct cultivars in terms of yield-related parameters, leaf morphology, metabolic and physiological traits. Firstly, I took advantage of this phenotypic variation to understand the behavior of *C. chinense* yield-related parameters under crescent N supply. The first experiment allowed the definition of the N supply needed to obtain plants with moderate N-deficiency and N-sufficiency. The cultivars displayed modest differences in their vegetative biomass according to the N supply. While N-deficiency is marked by decreased in biomass production and starch accumulation in leaves, under N-excess condition increased NH_4^+ levels and the relative biomass allocation in leaves were observed mainly in Habanero, which has lower fruit set and higher fruit size. Thus, I propose that the young reproductive sink strength is an important factor for plant tolerance to toxic NH_4^+ levels. Furthermore, I observed that N-deficiency in Capsicum plants reduces the fruit yield through the lower development of new reproductive and vegetative tissues. Plants under N-deficiency have a lower number of flowers but heavier fruits. In contrast, plants under higher N supply have lower CO_2 assimilation and favor the vegetative growth instead reproductive one. Probably this behavior is due to unbalanced sucrose supply between these organs. Under N excess, the lower CO_2 fixation and the internal competition for assimilates between the vegetative and reproductive organs can be responsible for the decrease in the weight of individual fruits and the decrease in the number of flowers, fruit set and yield.

In the second chapter, I focused in the search for metabolic traits related to fruit set. For this, I used the difference between the metabolite content at dusk and dawn (or the balance of metabolites) (Aloni *et al.* 1996) in Biquinho and Habanero under moderate N-deficiency or N-sufficiency combined with unshaded or shaded conditions. Previous studies highlighted the partition of C metabolism-related metabolites in sink organs, mainly flowers, to explain the fruit set (Turner & Wien 1994; Aloni *et al.* 1996). Indeed, the results obtained in the present study indicate the balance of sugars, amino acids and malate in flowers influence the fruit set of *C. chinense*. Moreover, the obtained results suggest starch turnover as well as export of sucrose and amino acids from source leaves as central players on the metabolic mechanism to explain how source strength can indirectly influence the fruit set.

In the third chapter, I explored the effect of N supply on SLA, an important trait related to photosynthetic capacity and plant growth (Potter & Jones 1977; Mooney *et al.* 1978; Poorter & Remkes 1990; Poorter *et al.* 1990, 2009; Witkowski & Lamont 1991; Reich *et al.* 1994). The results suggest that the SLA decrease, observed in both genotypes under higher N supply, occurs because of the increment in the leaf density driven by the variation in N and C metabolism-related metabolites, mainly the amino acids, protein and starch level. However, the SLA behavior in the range of moderate N deficiency to N sufficiency is influenced by genotypic anatomical variations. There is a convergent effect between the higher leaf density and thicker palisade parenchyma influencing the SLA of Biquinho according to the increase in N supply. On the other hand, there is a divergent effect between the higher leaf density and thinner leaf thickness (as well as thinner spongy parenchyma) influencing the SLA of Habanero according to the increase in N supply.

In the fourth chapter, I revisited the metabolite analysis currently used to characterize plant metabolite profile. Furthermore, examples describing mQTL identification via the integration between metabolomic approaches and mapping methods were shown. As a faster advancement of QTL studies is expected for a wide range of species in the coming years, I believe these set of studies revealed important physiological and metabolic traits associated with Capsicum yield-related parameters worthy to be further investigated. Indeed, further studies can take advantage of the great genetic diversity of *C. chinense* populations (Jarret & Berke 2008; Finger *et al.* 2010; Rosado-Souza *et al.* 2015; Baba *et al.* 2016; Moreira *et al.* 2018) to identify and introduce many economical relevant traits in Capsicum breeding programs.

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