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Photosynthesis and photoprotection in coffee leaves is affected by nitrogen and light availabilities in winter conditions

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ABSTRACT

Coffee is native to shady environments but often grows better and produces higher yields without shade, though at the expense of high fertilization inputs, particularly nitrogen (N). Potted plants were grown under full sunlight and shade (50%) conditions and were fertilized with nutrient solutions containing either 0 or 23 mM N. Measurements were made in southeastern Brazil during winter conditions, when relatively low night temperatures and high diurnal insolation are common. Overall, the net carbon assimilation rate was quite low, which was associated with diffusive, rather than biochemical, constraints. N deficiency led to decreases in the concentrations of chlorophylls (Chl) and total carotenoids as well as in the Chl/N ratio. These conditions also led to qualitative changes in the carotenoid composition, e.g., increased antheraxanthin (*A*) and zeaxanthin (*Z*) pools on a Chl basis, particularly at high light, which was linked to increased thermal dissipation of absorbed light. The variable-to-maximum fluorescence ratio at predawn decreased with increasing *A* + *Z* pools and decreased linearly with decreasing N. We showed that this ratio was inadequate for assessing photoinhibition under N limitation. Expressed per unit mass, the activities of superoxide dismutase and glutathione reductase were not altered with the treatments. In contrast, ascorbate peroxidase activity was lower in low N plants, particularly under shade, whereas catalase activity was lower in shaded plants than in sun-grown plants, regardless of the N level. Glutamine synthetase activity was greater in sun-grown plants than in shaded individuals at a given N level and decreased with decreasing N application. Our results suggest that the photoprotective and antioxidant capacity per amount of photons absorbed was up-regulated by a low N supply; nevertheless, this capacity, regardless of the light conditions, was not enough to prevent oxidative damage, as judged from the increases in the H_2O_2 and malondialdehyde concentrations and electrolyte leakage. We demonstrated that N fertilization could adequately protect the coffee plants against photodamage independently of the anticipated positive effects of N on the photosynthetic capacity.

Introduction

Leaves cannot utilize all of the photosynthetically active radiation (PAR) absorbed during exposure to full sunlight for photosynthesis, resulting in what is often described as excess excitation energy (Müller et al., 2001). To protect against this excess energy, plants can avoid light absorption, e.g., through low chlorophyll (Chl) contents and steep inclinations to and large reflectance of incident radiation (Adams et al., 2004). Nonetheless, if excess excitation energy arises, it can potentially lead to the production of reactive oxygen species (ROS) that can oxidize pigments and proteins of the photosynthetic machinery, particularly the D1 protein subunit of the photosystem (PS) II reaction center (Asada, 1999). To avoid photooxidative damage, plants possess complex photoprotective mechanisms. One key mechanism involves the dissipation of excess absorbed light as heat in the antenna pigment complexes of PSII. This process is related to the xanthophyll cycle, in which violaxanthin (V) is de-epoxidated to antheraxanthin (*A*) and zeaxanthin (*Z*). The de-epoxidation state (DEPS) of this cycle safely dissipates

Abbreviations: A, antheraxanthin; APX, ascorbate peroxidase; Car, carotenoid; CAT, catalase; Chl, chlorophyll; C_i/C_a, internal-to-ambient CO_2 concentration ratio; *D*, fraction of the absorbed PAR dissipated as heat; DEPS, de-epoxidation state of the xanthophyll cycle; DW, dry weight; F_0, initial Chl fluorescence; F_m, maximum Chl fluorescence; F_v/F_0, variable-to-initial Chl fluorescence; F_v/F_m, variable-to-maximum Chl fluorescence; FW, fresh weight; GR, glutathione reductase; g_s, stomatal conductance; GS, glutamine synthetase; HL, high light; HN, high nitrogen; LL, low light; LN, low nitrogen; MDA, malondialdehyde; N, nitrogen; *P*, fraction of the absorbed PAR used in photochemistry; PAR, photosynthetically active radiation; P_E, fraction of the absorbed PAR neither used in photochemistry nor dissipated thermally; P_N, net carbon assimilation rate; PS, photosystem; ROS, reactive oxygen species; SOD, superoxide dismutase; V, violaxanthin; Z, zeaxanthin; γ-GH, γ-glutamyl-hydroxamate.

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excess excitation energy before it reaches the PSII reaction centers (Müller et al., 2001; Demmig-Adams and Adams, 2006). Other photoprotective mechanisms include (i) antioxidant enzymes such as superoxide dismutase (SOD), which catalyzes the reaction of superoxide to form H_2O_2, and catalase (CAT) and ascorbate peroxidase (APX), which detoxify the H_2O_2 produced (Asada, 1999; Logan et al., 2006) as well as (ii) low molecular weight antioxidants, both lipophilic (α-tocopherol and β-carotene) and hydrophilic (ascorbate and glutathione), which are efficient quenchers of some ROS, including singlet oxygen and superoxide anions (Asada, 1999; Logan et al., 2006).

Under conditions of environmental stresses, such as nitrogen (N) deficiency, the amount of excess energy can even increase due to stress-induced decreases in CO_2 fixation capacity (e.g., DaMatta et al., 2002; Cruz et al., 2003). This lowered capacity means that a given light intensity is potentially in greater excess under N deficiency (Verhoeven et al., 1997). As a consequence, the capacity for photoprotection is expected to be up-regulated to protect low N leaves from photooxidative damage under high light, as shown by several investigators (e.g., Khamis et al., 1990; Verhoeven et al., 1997; Chen and Cheng, 2003). Nonetheless, contrasting results have also been observed. For example, both xanthophyll cycle-dependent thermal energy dissipation and the ability to scavenge ROS are up-regulated by a high N supply in *Coffea arabica* (Ramalho et al., 1998, 2000), whereas N supply was observed to have no effect on the major protective carotenoids and antioxidants in *Pinus radiata* (Posch et al., 2008).

Despite evolving in African forest understories and thus being considered as a shade-requiring species, the coffee tree appears to have enough plasticity to be cultivated in both exposed and shaded environments (DaMatta, 2004; Araújo et al., 2008; Chaves et al., 2008; Matos et al., 2009). However, in the main Brazilian coffee-producing regions, the species is often subjected to a combination of chilling night temperatures (minimum temperatures typically ranging from 5 to 12 °C) and high diurnal solar radiation during winter months. These stressful conditions can lead to marked decreases in both vegetative growth (Barros et al., 1997; DaMatta et al., 1999) and photosynthetic rates (Silva et al., 2004; Chaves et al., 2008; Partelli et al., 2009), thereby contributing to the creation of excess excitation energy in the photochemical apparatus (DaMatta et al., 1997; Ramalho et al., 2003; Chaves et al., 2008) which, ultimately, may lead to severe oxidative stress (Fortunato et al., 2010). Furthermore, since up to 95% of total plant N can be taken up by fruits in heavily bearing coffee trees (Cannell, 1975), remarkable decreases in the leaf N concentration are common following harvests, which take place in mid-late autumn and early winter in most Brazilian coffee-producing regions. In fact, the N concentration has been shown to be minimal in coffee leaves during winter months, even in trees that receive extra N (DaMatta et al., 1999). Taken together, these conditions would exacerbate the occurrence of photoinhibition and photodamage to the photosynthetic apparatus, unless robust photoprotective mechanisms can be developed.

Previous studies examining the effects of N supply on coffee photoprotection have been restricted to short-term (from hours to days) experiments conducted in Portugal by Ramalho et al. (1997, 1998, 2000). Therefore, the interactive effects of light and N supply have received little attention in this species. It has been hypothesized that both accumulation of protective carotenoids and the antioxidant system are directly up-regulated by an increased N supply regardless of whether the plants are grown under low or high light availability. Measurements were made during the winter season when photosynthetic rates are minimal in coffee (Silva et al., 2004); therefore, the anticipated positive effects of N on photoprotection should be largely manifested independently of N-induced changes in photosynthetic capacity. To test this hypothesis, changes in gas exchange, thermal dissipation, xanthophyll pool size and composition, antioxidants and cellular damage were analyzed in a factorial experiment using coffee plants grown under two N applications and two light regimes.

Materials and methods

Plant material and experimental design

The experiment was conducted in Viçosa (20°45′S, 42°54′W; 650 m a.s.l.), southeastern Brazil. Uniform seedlings of coffee (*Coffea arabica* L. cv 'Red Catuaí IAC 44′), obtained from seeds, with 3 leaf pairs, were transplanted (December 2006) into 12-L pots containing a mixture of soil and sand (2:1, v/v). After transplantation, the seedlings were randomly submitted to two light treatments, i.e., plants receiving either 100% or 50% solar radiation [hereafter referred to as high light (HL) and low light (LL) plants, respectively]. For the LL plants, one shade enclosure was constructed using neutral density black nylon netting. Maximum solar radiation reached more than 2000 μmol m^{-2} s^{-1} of PAR. During the first 45 days after transplantation, the plants were fertilized fortnightly with 250 mL of complete Hoagland's nutrient solution, after which they were randomly assigned to two N treatments (January 2007): irrigation once a week with 250 mL of Hoagland's solution containing either 0 or 23 mM N [hereafter referred to as low N (LN) and high N (HN) plants, respectively]. Nutrient solutions were supplied until the LN plants had visual symptoms of N deficiency, older leaves in particular; the youngest, fully expanded leaves, corresponding to the third or fourth pair from the apex of plagiotropic branches, were then sampled and measurements were performed (July 2007). Throughout the experiment, the plants were grown under naturally fluctuating conditions of temperature and air relative humidity, and were irrigated as necessary. The pots were randomized periodically to minimize any variation within each light environment.

The experiment was a completely randomized design, with 4 treatment combinations, forming a 2 × 2 factorial (2 light and 2 N levels) with 10 plants in individual pots per treatment combination as replication. The experimental plot was one plant per container. Unless otherwise indicated, samplings and measurements were conducted using 6 replicates per treatment combination. The experiments were repeated in time giving similar results.

Photosynthetic measurements

The net carbon assimilation rate (P_N), stomatal conductance to water vapor (g_s) and the internal-to-ambient CO_2 concentration ratio (C_i/C_a) were measured with a portable open-flow gas exchange system (LICOR 6400, Li-COR, Lincoln, NE, USA). Measurements were made at midday at ambient temperature and CO_2 conditions, under artificial light, i.e., 1000 and 500 μmol photons m^{-2} s^{-1} at the leaf level, which corresponded approximately to the ambient irradiance intercepted by the sampled leaves (in their natural angles) from HL and LL plants, respectively. During the measurements, the leaf-to-air vapor pressure deficit was about 3.0 kPa in both light environments. The Chl *a* fluorescence was determined using a portable pulse amplitude modulation fluorometer (FMS2, Hansatech, Norfolk, UK). Following dark adaptation for 30 min, the leaf tissue was illuminated with a weak modulated measuring beam to obtain the initial fluorescence (F_0). A saturating white light pulse of 6000 μmol m^{-2} s^{-1} was applied for 1 s to ensure the maximum fluorescence emission (F_m), from which the $F_v/F_m = [(F_m - F_0)/F_m)]$ and the F_v/F_0 ratios were calculated. The leaf tissue was exposed to actinic PAR (1000 and 500 μmol m^{-2} s^{-1} for sun and shade leaves, respectively) for 300 s to obtain the steady-state fluorescence yield (F_s). Subsequently, a saturating white light pulse (6000 μmol m^{-2} s^{-1}; 1 s) was applied

to achieve the light-adapted maximum fluorescence (F_m'). The actinic light was then turned off and a far-red illumination was applied to measure the light-adapted initial fluorescence (F_0'). Following Demmig-Adams et al. (1996), the fractions of the absorbed PAR used in photochemistry [$P=(F_m'-F_s)/F_m'$], dissipated as heat [$D=1-(F_v'/F_m')$] and the fraction neither used in photochemistry nor dissipated thermally [$P_E=(F_v'/F_m')-P$] were calculated. Measurements were made immediately after the gas exchange was gauged and also at predawn (F_0 and F_m only).

The rate of $^{14}CO_2$ uptake in leaf discs (14 mm diameter) isolated from the experimentally treated plants was assessed in a leaf-disc oxygen electrode (LD2/2, Hansatech, Kings Lynn, Norfolk, UK) under saturation with CO_2 (~5 kPa) at a PAR of 900 μmol photons m^{-2} s^{-1} at 35 °C for 30 min. Previous assays showed that incorporated radioactivity was maximal at that temperature. The CO_2 was supplied from 400 μL of 1 M NaH $^{14}CO_3$ (specific activity of 1.96 GBq $mmol^{-1}$), pH 9.3, placed on a felt mat at the base of the leaf chamber. Leaf discs were then flash frozen and stored at −80 °C until required. The incorporated radioactivity into the leaf discs was assessed using a liquid scintillation analyzer (Beckman LS 6500, Beckman Instruments, Fullerton, USA). The rate of $^{14}CO_2$ uptake was expressed as Bq m^{-2} s^{-1}.

Biochemical assays

Leaf discs were collected in cloudless days at about midday for biochemical determinations and cellular damage measurements. Leaf discs were also collected before dawn (at 0500 h) for pigment analyses. All leaf tissues were flash frozen in liquid nitrogen and stored at −80 °C until analysis. Each sample was replicated twice.

Free amino acids were assayed as described elsewhere (Praxedes et al., 2006) using the ninhydrin method. Amino acid concentration was estimated using a standard curve with an equimolecular mixture of glycine, glutamic acid, phenylalanine and arginine in 70% (v/v) aqueous ethanol. Soluble protein concentration was determined by the method of Bradford (1976) using BSA as a standard. Total N was estimated as the sum of amino acids-N, ammonium-N and nitrate-N pools, as described in DaMatta et al. (1999). Chlorophylls and total carotenoids were extracted using 80% (v/v) aqueous acetone and quantified spectrophotometrically according to Lichtenthaler (1987). Carotenes and xanthophylls were extracted with ice-cold 90% (v/v) acetone and assayed by HPLC as reported in Matos et al. (2009). Detection of pigments was carried out at 440 nm using a UV/VIS detector. For identification and quantification of peaks, pure commercial standards (DHI, Denmark) were used. DEPS was calculated as $(Z+0.5A)/(V+A+Z)$.

Glutamine synthetase (GS; EC 6.3.1.2) was extracted and assayed as previously documented (Cruz et al., 2004). Activity of GS was measured by the production of γ-glutamyl-hydroxamate (γ-GH) at 530 nm. Key antioxidant enzymes, including SOD (EC 1.15.1.1), APX (EC 1.11.1.11), catalase (CAT; EC 1.11.1.6) and glutathione reductase (GR; EC 1.6.4.2), were extracted using a cold mortar and pestle with polyvinylpolypyrrolidone and appropriate extraction buffers as described exactly in Pinheiro et al. (2004). Total SOD activity was determined by measuring its ability to inhibit the photochemical reduction of *p*-nitro-blue-tetrazolium chloride at 560 nm. The activity of CAT was estimated by measuring the rate of decomposition of H_2O_2 at 240 nm; total APX activity was estimated by monitoring the decline in absorbance at 290 nm, whereas GR activity was assessed by measuring the rate of NADPH oxidation at 340 nm. Further details have been documented previously (Pinheiro et al., 2004).

Cellular damage was analyzed through (i) electrolyte leakage, assayed immediately after leaf sampling using a conductivity meter; (ii) malondialdehyde (MDA) accumulation, estimated as the content of total 2-thiobarbituric acid-reactive substances; and (iii) H_2O_2 accumulation, estimated via the ferrous oxidation-xylenol orange method. Further details have been reported elsewhere (Lima et al., 2002).

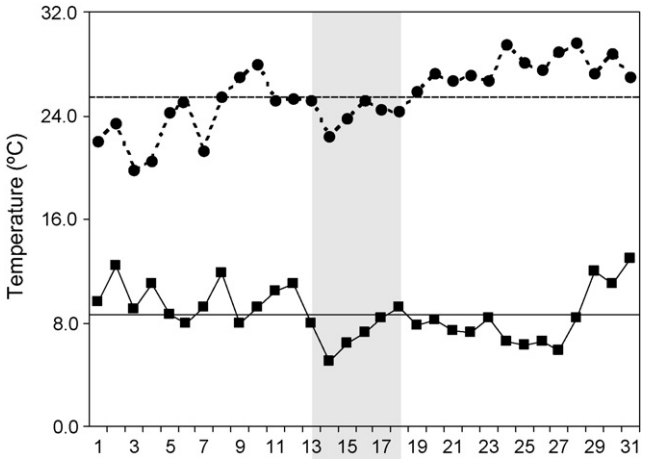

Fig. 1. Time course of maximum (circles) and minimum (squares) air temperature throughout July 2007 at Viçosa, southeastern Brazil. The horizontal lines represent the monthly mean maximum and minimum temperatures. The gray-marked part denotes the days when samplings and measurements were performed.

Statistical analyses

Data were statistically examined using a fixed-model ANOVA (with light levels and N supply levels considered fixed, and replicates and observations random) following a completely randomized design, and significant differences between treatments were analyzed using the Newman–Keuls test at $P \leq 0.05$. Assumptions of normality were determined using the Kolmogorov–Smirnov test; when necessary, data were transformed to attain a normal distribution. Mean comparisons were performed using Statgraphics Plus Version 5.1 (StatPoint, Inc., Herndon, VA, USA). Correlation coefficients were calculated using Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

In July 2007, the minimum and maximum average temperatures were 8.7 °C and 25.5 °C, respectively, but temperatures below 6 °C and above 26 °C were common (Fig. 1). Overall, relatively cool nights were followed by warm, sunny days, with the maximum PAR reaching more than 2000 μmol photons m^{-2} s^{-1} (data not shown).

Regardless of the light conditions, the leaf N concentration was, on average, 58% higher in HN plants (>30 g kg^{-1} DW) than in LN individuals (Table 1). The increased N concentration was accompanied by an increase in the concentrations of free amino acids (30%) and soluble proteins (146%) (Table 1).

Irrespective of the treatments, both P_N and g_s were quite low, ranging from 1.5 to 2.1 μmol CO_2 m^{-2} s^{-1} and from 11 to 19 mmol H_2O m^{-2} s^{-1}, respectively. The C_i/C_a was also low, ranging from 0.41 to 0.55 (Table 2). Similar results were obtained in the early morning (data not shown). In contrast, the rate of $^{14}CO_2$ uptake (assessed under saturating CO_2 and therefore in the absence of diffusion-mediated limitations of photosynthesis—thus reflecting the mesophyll capacity for carbon fixation) was lower (6–10%) in LL plants than in HL plants. Moreover, the potential capacity to fix $^{14}CO_2$ was about 33% lower in LN plants than in HN plants in either light treatment (Table 2).

In HL plants, both of the F_v/F_m and F_v/F_0 ratios at predawn were relatively low and similar irrespective of the N level; however, these ratios increased in HN plants relative to LN plants at midday (Fig. 2).

Table 1
The effects of N supply (0 or 23 mM, respectively, for low and high N applications) on the concentrations of total N, amino acids and proteins in coffee plants grown under either low light or full sunlight conditions. Different capital letters denote significant differences between means for each parameter within each N application, and different small letters denote significant differences for each parameter within each light condition ($P \leq 0.05$, Newman–Keuls' test). Data are expressed as means ± SE, $n = 6$.

Characteristics	Low nitrogen		High nitrogen	
	Low light	High light	Low light	High light
Total N (g kg^{-1} DW)	20.6 ± 0.9 Ab	20.0 ± 0.8 Ab	30.4 ± 1.2 Aa	33.6 ± 1.1 Aa
Amino acids (mmol kg^{-1} FW)	9.6 ± 1.1 Ab	11.0 ± 1.8 Aa	14.0 ± 1.1 Aa	12.8 ± 1.7 Aa
Total protein (g kg^{-1} FW)	51.4 ± 2.7 Bb	66.9 ± 3.5 Ab	131 ± 5 Ba	160 ± 5 Aa

Table 2
The effects of N supply (0 or 23 mM, respectively, for low and high N applications) on the rate of net carbon assimilation (P_N), stomatal conductance (g_s), internal-to-ambient CO_2 concentration ratio (C_i/C_a) and the rate of $^{14}CO_2$ uptake in coffee plants grown under either low light or full sunlight conditions. Different capital letters denote significant differences between means for each parameter within each N application, and different small letters denote significant differences for each parameter between means within each light condition ($P \leq 0.05$, Newman–Keuls' test). Data are expressed as means ± SE, $n = 10$ (except for the rate of $^{14}CO_2$ uptake, in which $n = 6$).

Characteristics	Low nitrogen		High nitrogen	
	Low light	High light	Low light	High light
P_N (μmol CO_2 m^{-2} s^{-1})	2.1 ± 0.2 Aa	1.5 ± 0.1 Ba	2.1 ± 0.3 Aa	1.6 ± 0.1 Aa
g_s (mmol m^{-2} s^{-1})	13 ± 0.7 Ba	19 ± 0.7 Aa	11 ± 1.0 Aa	12 ± 0.1 Ab
C_i/C_a	0.46 ± 0.04 Aa	0.55 ± 0.04 Aa	0.41 ± 0.04 Aa	0.46 ± 0.05 Aa
$^{14}CO_2$ uptake (Bq m^{-2} s^{-1})	765 ± 13 Bb	822 ± 12 Ab	1131 ± 12 Ba	1204 ± 14 Aa

For example, the F_v/F_m ratio was about 0.74 in HN plants compared with 0.64 in LN individuals. On the other hand, F_v/F_m was similar (above 0.80 at predawn and slightly below 0.80 at midday) in LN plants and HN plants under shade. In contrast, F_v/F_0 was significantly lower in LN plants than in HN individuals at both predawn and midday (Fig. 2).

The fraction of absorbed PAR used in photochemistry (P) was lower and the fraction dissipated as heat (D) was higher in HL plants compared with LL plants and was independent of the N level, although P was higher and D was lower under HN conditions within a given light level (Fig. 3). The fraction of absorbed PAR neither used in photochemistry nor dissipated thermally (P_E) was significantly higher in HL plants than in LL plants regardless of the N supply (Fig. 3).

In dark-adapted leaves, F_v/F_m at predawn decreased with increasing $A+Z$ pools on a Chl basis (but not with DEPS; data not shown) and decreased linearly with decreasing N concentrations (Fig. 4).

As expected, the concentrations of Chl ($a+b$) and total carotenoids decreased remarkably with a diminishing N supply.

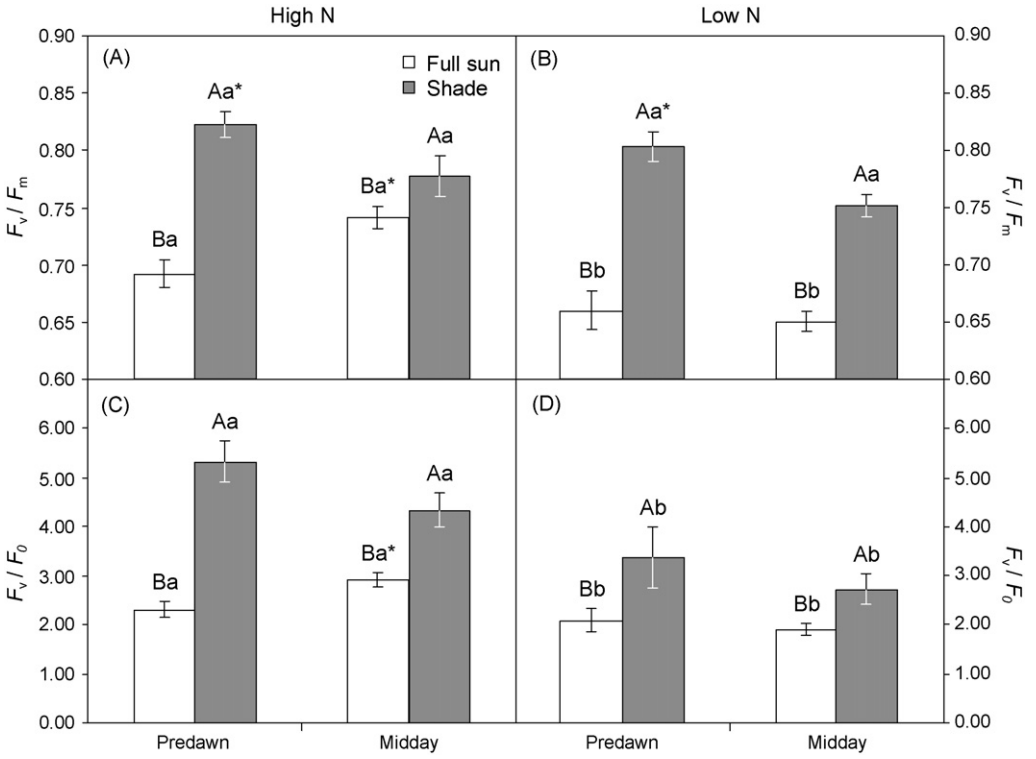

Fig. 2. The effects of N supply (0 or 23 mM, respectively, for low and high N applications) on the ratios of variable-to-maximum (F_v/F_m; A, B) and variable-to-initial (F_v/F_0; C, D) chlorophyll fluorescence in coffee plants grown in full sunlight and shade conditions. Measurements were made at predawn and midday. Different capital letters denote significant differences between the means for each parameter within each N application, and different small letters denote significant differences for each parameter within each light condition at either predawn or midday. Asterisks represent significant differences for a given parameter within the sampling schedule ($P \leq 0.05$, Newman–Keuls' test). Data are expressed as means ± SE, $n = 6$.

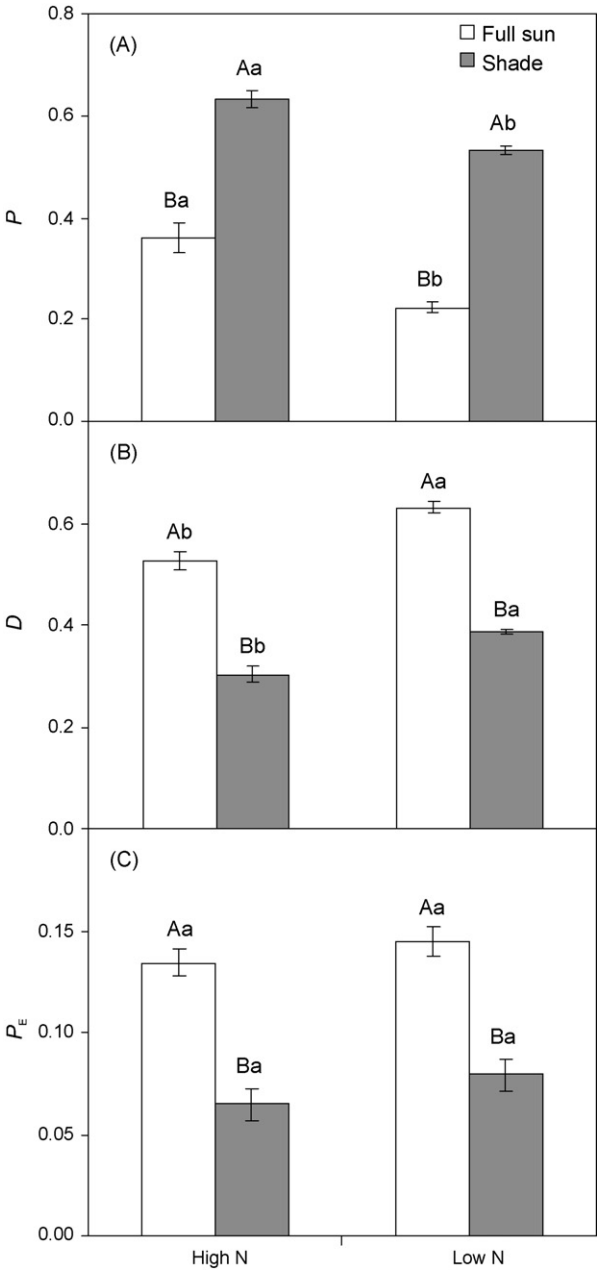

Fig. 3. The effects of N supply (0 or 23 mM, respectively, for low and high N application) on the fractions of absorbed photosynthetic active radiation used in photochemistry (*P*; A) or dissipated as heat (*D*; B) and the fraction neither used in photochemistry nor dissipated thermally (P_E; C) in coffee plants grown in full sunlight and shade conditions. Measurements were made at midday. Different capital letters denote significant differences between means for each parameter within each N application, and different small letters denote significant differences for each parameter within each light condition ($P \leq 0.05$, Newman–Keuls' test). Data are expressed as means ± SE, $n = 6$.

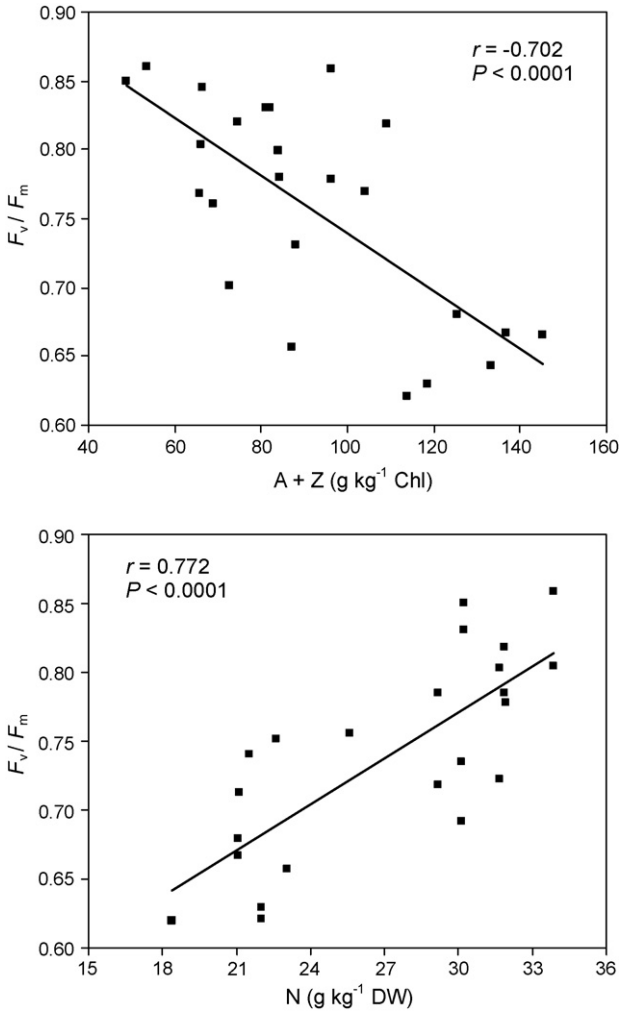

Fig. 4. The variable-to-maximum chlorophyll (Chl) fluorescence ratio (F_v/F_m) in relation to the concentration of antheraxanthin (*A*) + zeaxanthin (*Z*) on a Chl basis at predawn (upper) and in relation to the concentration of nitrogen (N) (lower) in coffee plants.

These concentrations also decreased, though to a lesser extent, with increasing light availability (Table 3). However, the ratios of Chl *a*/*b* and Chl/Car remained unchanged regardless of the treatments. A decrease in the Chl concentration was associated with a decrease in the Chl/N ratio, particularly under HL conditions (Table 3).

Independently of the light environments, HN plants, compared with their LN counterparts, exhibited much larger concentrations per unit mass of neoxanthin (150–197%), violaxanthin (244–683%), lutein (~120%), α-carotene (214–229%) and β-carotene (50–115%). Concentrations per unit mass of *A* and *Z* also tended to be higher (22–46%) in HN than in LN plants, although the differences were only statistically significant for *A* under shade conditions (Table 4). Within each N condition, the concentrations of all carotenoids on a mass basis were higher in LL plants than in HL individuals, with the exception of *A* and *Z*, which were similar between the light treatments. A quite different picture emerged when the concentrations of the above carotenoids were expressed on a Chl basis (Table 4). Under HL conditions, LN plants showed significant increases in *A* (148%), *Z* (117%) and β-carotene (67%) compared with HN plants. Under shade, LN plants exhibited decreases in *V* (70%) and α-carotene (28%) and an increase in DEPS (35%) relative to HN individuals. By comparing the light treatments, increases in *Z* (72–125%) and *A* + *Z* (41–115%) were found in HL plants compared with LL plants, whereas the concentration of α-carotene was 48% higher in HN plants under shade as compared with their counterparts under full sunlight. Unless otherwise stated above, no further significant differences in the carotenoid concentrations on a Chl basis were detected in response to the treatments.

Expressed per unit mass, the activities of SOD and GR were not significantly affected by the treatments. In contrast, APX activity was lower in LN plants, particularly under shade conditions, whereas CAT activity was lower in LL plants compared with HL plants, regardless of the N level (Table 5). GS activity was significantly higher in HL plants than in LL plants at a given N level and was 115% higher in HN plants than in LN plants within each light treat-

Table 3
The effects of N supply (0 or 23 mM, respectively, for low and high N applications) on the concentrations of total chlorophylls (Chl) and total carotenoids (Car) as well as on the ratios of Chl *a*/*b*, Chl/Car and Chl/N in coffee plants grown under either low light or full sunlight conditions. Samples were collected at midday. Different capital letters denote significant differences between means for each parameter within each N application, and different small letters denote significant differences for each parameter between means within each light condition ($P \leq 0.05$, Newman–Keuls' test). Data are expressed as means ± SE, $n = 6$.

Characteristics	Low nitrogen		High nitrogen	
	Low light	High light	Low light	High light
Chl (*a* + *b*) (g kg^{-1} FW)	1.62 ± 0.10 Ab	1.03 ± 0.17 Bb	3.82 ± 0.38 Aa	2.56 ± 0.14 Ba
Car (g kg^{-1} FW)	0.35 ± 0.04 Ab	0.29 ± 0.03 Ab	0.79 ± 0.07 Aa	0.53 ± 0.05 Ba
Chl *a*/*b* (kg kg^{-1} FW)	2.88 ± 0.25 Aa	2.83 ± 0.18 Aa	2.63 ± 0.03 Aa	2.55 ± 0.05 Aa
Chl/Car (kg kg^{-1} FW)	2.97 ± 0.23 Aa	2.23 ± 0.17 Aa	3.21 ± 0.08 Aa	2.95 ± 0.32 Aa
Chl/N (mg kg^{-1} DW)	81.1 ± 9.1 Ab	50.4 ± 8.5 Bb	99.2 ± 4.9 Aa	75.9 ± 6.1 Ba

Table 4
The effect of N supply (0 or 23 mM, respectively, for low and high N applications) on the carotenoid composition expressed in both mg kg^{-1} fresh weight (FW) and g kg^{-1} Chl in coffee plants grown under either low light or full sunlight conditions. Samplings were made at midday. Different capital letters denote significant differences between means for each parameter within each N application, and different small letters denote significant differences for each parameter between means within each light condition ($P \leq 0.05$, Newman–Keuls' test). Data are expressed as means ± SE, $n = 6$.

Characteristics	Low nitrogen		High nitrogen	
	Low light	High light	Low light	High light
mg kg^{-1} FW				
Neoxanthin	51.6 ± 6.4 Ab	29.6 ± 5.2 Bb	129 ± 8 Aa	87.9 ± 7.6 Ba
Lutein	136 ± 17 Ab	105 ± 12.1 Ab	298 ± 23 Aa	236 ± 28 Ba
α-Carotene	14.5 ± 1.3 Ab	6.5 ± 1.4 Bb	45.6 ± 2.2 Aa	21.4 ± 3.6 Ba
β-Carotene	37.2 ± 3.9 Ab	29.7 ± 4.0 Ab	80.1 ± 6.2 Aa	44.5 ± 4.9 Ba
Violaxanthin	8.0 ± 1.5 Ab	5.4 ± 1.0 Ab	62.7 ± 8.0 Aa	18.6 ± 4.0 Ba
Antheraxanthin (*A*)	20.6 ± 2.9 Ab	19.8 ± 1.7 Aa	53.2 ± 6.1 Aa	24.2 ± 2.3 Ba
Zeaxanthin (*Z*)	83.0 ± 8.7 Aa	96.8 ± 8.0 Aa	121 ± 18 Aa	137 ± 26.1 Aa
A + *Z*	103 ± 11 Ab	116 ± 9 Ab	174 ± 24 Aa	162 ± 240 Aa
g kg^{-1} Chl				
Neoxanthin	32.4 ± 4.2 Aa	28.4 ± 3.0 Aa	34.7 ± 0.8 Aa	34.5 ± 2.3 Aa
Lutein	85.6 ± 11.4 Aa	114 ± 13 Aa	79.3 ± 2.4 Aa	92.5 ± 9.6 Aa
α-Carotene	9.3 ± 1.3 Aa	6.2 ± 0.9 Aa	12.9 ± 1.4 Aa	8.7 ± 1.5 Ba
β-Carotene	23.8 ± 3.1 Aa	30.1 ± 2.6 Aa	21.7 ± 1.5 Aa	18.0 ± 2.1 Ab
Violaxanthin	5.0 ± 0.9 Ab	5.4 ± 0.7 Aa	16.7 ± 1.8 Aa	7.3 ± 1.5 Ba
Antheraxanthin	13.0 ± 1.9 Ba	23.6 ± 3.8 Aa	13.8 ± 1.0 Aa	9.5 ± 0.7 Ab
Zeaxanthin	52.2 ± 5.9 Ba	117 ± 20 Aa	31.4 ± 3.3 Aa	54.1 ± 9.9 Ab
A + *Z*	65.1 ± 7.2 Ba	140 ± 24 Aa	45.1 ± 3.5 Ba	63.6 ± 10.0 Ab
DEPS	0.84 ± 0.02 Aa	0.87 ± 0.01 Aa	0.62 ± 0.03 Bb	0.78 ± 0.05 Aa

ment (Table 5). Nevertheless, different results were found when the enzyme activities were expressed on a protein basis. The activities of SOD, APX and GR were remarkably higher in LN plants and independent of the light conditions, whereas the activity of CAT was greater in LN plants at full sunlight and were similar among the other treatments. GS activity was lower in LL plants under HN supply and remained unchanged in the other treatments (Table 5).

The H_2O_2 concentration was considerably higher in LN plants (144–473%) than in HN individuals irrespective of the light conditions (Table 6). Moreover, HL plants showed higher H_2O_2 concentrations (66–290%) than LL plants within each N level tested. In contrast, the light treatments affected neither MDA concentration nor electrolyte leakage, which were significantly greater in LN plants (92–150%) than in HN plants (Table 6).

Discussion

The LN plants were N deficient, since N concentrations below 23 g kg^{-1} have been shown to induce visual symptoms of N deficiency in coffee (Moraes, 1981). Other N deficiency symptoms, such

Table 5
The effects of N supply (0 or 23 mM, respectively, for low and high N applications) on the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and glutamine synthetase (GS), expressed both per fresh weight and protein mass, in coffee plants grown under either low light or full sunlight conditions. One unit of SOD was defined as the amount of enzyme that inhibited the reduction of *p*-nitro-blue tetrazolium by 50%. Samples were collected at midday. Different capital letters denote significant differences between means for each parameter within each N application, and different small letters denote significant differences for each parameter between means within each light condition ($P \leq 0.05$, Newman–Keuls' test). Data are expressed as means ± SE, $n = 6$.

Characteristics	Low nitrogen		High nitrogen	
	Low light	High light	Low light	High light
SOD (U kg^{-1} FW)	0.86 ± 0.19 Aa	1.01 ± 0.19 Aa	0.77 ± 0.17 Aa	0.96 ± 0.04 Aa
APX (mmol ascorbate min^{-1} kg^{-1} FW)	45.3 ± 2.9 Bb	57.5 ± 2.3 Ab	73.6 ± 4.1 Aa	72.0 ± 4.6 Aa
CAT (mmol H_2O_2 min^{-1} kg^{-1} FW)	2.2 ± 0.6 Ba	3.2 ± 0.5 Aa	2.7 ± 0.5 Ba	3.9 ± 0.4 Aa
GR (mmol NADPH min^{-1} kg^{-1} FW)	1.8 ± 0.1 Aa	1.8 ± 0.2 Aa	2.0 ± 0.2 Aa	2.1 ± 0.2 Aa
GS (mmol γGH h^{-1} kg^{-1} FW)	3.7 ± 0.3 Bb	4.8 ± 0.3 Ab	8.0 ± 0.6 Ba	10.3 ± 0.7 Aa
SOD (U kg^{-1} protein)	32.5 ± 5.7 Aa	33.5 ± 2.2 Aa	8.1 ± 1.5 Ab	12.7 ± 1.0 Ab
APX (mmol ascorbate min^{-1} kg^{-1} protein)	1096 ± 124 Ba	1479 ± 238 Aa	627 ± 53 Ab	572 ± 56 Ab
CAT (mmol H_2O_2 min^{-1} kg^{-1} protein)	16.0 ± 3.2 Ba	57.3 ± 5.3 Aa	16.6 ± 2.1 Aa	15.6 ± 2.1 Ab
GR (mmol NADPH min^{-1} kg^{-1} protein)	14.6 ± 0.8 Aa	15.3 ± 1.6 Aa	6.9 ± 0.6 Ab	7.2 ± 0.5 Ab
GS (mol γGH h^{-1} kg^{-1} protein)	76.1 ± 3.1 Aa	86.0 ± 6.7 Aa	50.0 ± 2.5 Bb	79.9 ± 7.1 Aa

Table 6
The effects of N supply (0 or 23 mM, respectively, for low and high N applications) on hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and electrolyte leakage in coffee plants grown under either low light or full sunlight conditions. Samples were collected at midday. Different capital letters denote significant differences between means for each parameter within each N application, and different small letters denote significant differences for each parameter between means within each light condition ($P \leq 0.05$, Newman–Keuls' test). Data are expressed as means ± SE, $n = 6$.

Characteristics	Low nitrogen		High nitrogen	
	Low light	High light	Low light	High light
H_2O_2 (mmol kg^{-1} DW)	3.8 ± 0.1 Ba	6.4 ± 0.3 Aa	0.7 ± 0.1 Bb	2.6 ± 0.1 Ab
MDA (μmol kg^{-1} DW)	190 ± 19 Aa	186 ± 8 Aa	79 ± 4 Ab	97 ± 6 Ab
Electrolyte leakage (%)	6.1 ± 0.4 Aa	6.1 ± 0.6 Aa	2.8 ± 0.2 Ab	2.4 ± 0.2 Ab

as a decreased total leaf area and lower specific leaf area, were also found in LN plants (data not shown).

Neither light nor N supply substantially affected the P_N rates. These rates were quite low, which had already been demonstrated by other investigators (DaMatta et al., 1997; Silva et al., 2004; Chaves et al., 2008) studying coffee plants during winter conditions in southeastern Brazil. It has been suggested that low night temperatures may largely depress g_s, even when the daytime temperature is adequate for gas exchange in coffee (DaMatta et al., 1997). The low g_s and the corresponding low C_i/C_a ratio suggest that diffusive limitations, rather than biochemical constraints, greatly accounted for the low P_N. In contrast, the data on $^{14}CO_2$ assimilation suggested that the mesophyll photosynthetic capacity for CO_2 fixation was improved by HL availability and particularly by the HN supply. In fact, HN plants in both light environments displayed similar $^{14}CO_2$ assimilations in comparison to that of unstressed coffee trees analyzed during the growing season (see DaMatta et al., 2008). Taken together, these results indicate that the anticipated positive effects of N nutrition on coffee photosynthesis (Ramalho et al., 2000; DaMatta et al., 2002) are apparently dampened or even suppressed by the high diffusive resistance to CO_2 influx into leaves, which is presumably imposed by low night temperatures, especially in HL plants.

Considering that carbon fixation, the usual main sink for the absorbed PAR in chloroplasts, was quite depressed regardless of the N and irradiance treatments, adjustments in light capture, use and dissipation are required to provide photoprotection to the photosynthetic apparatus, particularly under full sunlight. Here, we showed that decreased light capture via strong reductions in the concentrations of both Chl *a* and Chl *b* were evident in response to both high light and N deficiency. These reductions were linked to a decrease in the Chl/N ratio, which results from a decrease in biosynthesis and/or an increase in Chl degradation.

As expected, the higher *P* of HN plants was accompanied by a lower *D*, which differed from that found in LN plants in both light treatments. The higher *D* of LN plants is likely associated with the higher $A + Z$ pool on a Chl basis (as well as higher DEPS in LN compared with HN plants under shade). However, the decreased *P* was not entirely offset by the increase in *D* under full sun, which instead led to a higher P_E. The increase in P_E suggests that down-regulation of PSII in order to prevent over-reduction of Q_A was insufficient to compensate for the decreased demand for electrons through $NADP^+$ consumption. This in turn may potentially result in singlet oxygen formation and damage to membrane components (Müller et al., 2001).

The F_v/F_m ratio is often used as an indicator of the degree of photoinhibition in PSII (Maxwell and Johnson, 2000; Demmig-Adams and Adams, 2006). We believe that the lower predawn F_v/F_m, particularly in LN plants in high light, may be partially associated with sustained xanthophyll cycle-dependent thermal energy dissipation due to nocturnal retention of zeaxanthin under cold conditions, as previously suggested to occur in coffee by Ramalho et al. (2003) and recently confirmed by Partelli et al. (2009) in several coffee genotypes when exposed to night temperatures below 13 °C. Lower predawn F_v/F_m under N limitation has also been reported for other woody species, e.g., apple (Cheng, 2003) and grapevine (Chen and Cheng, 2003). Nonetheless, the treatment effects on the F_v/F_m must be cautiously considered. While the decrease in F_v/F_m in LN plants was probably accompanied by photodamage, no evidence of oxidative damage could be observed in HN plants grown in the open despite their higher P_E compared with individuals under shade. In addition, HN plants displayed the highest $^{14}CO_2$ fixation rates; therefore, we propose that the sustained decreases in F_v/F_m under full sun exposure are likely associated with photoprotection. One must also be cautious in interpreting the similar effects of shading on both P_E and F_v/F_m: because the F_v/F_m ratio was above 0.80 at predawn, the slight decreases in that ratio at midday should be considered, at a first glance, as an indication of dynamic, rather than chronic, photoinhibition (Demmig-Adams and Adams, 2006). Nevertheless, more sensitive indices for photoinhibition, such as the F_v/F_0 ratio, which shows significantly higher amplitudes than F_v/F_m (Lima et al., 1999; Maxwell and Johnson, 2000), could also be used to detect photoinhibition in response to N deficiency under shade. In fact, shaded LN plants showed clear signs of photooxidative damage (as judged from the increases in the H_2O_2 and MDA concentrations and electrolyte leakage) and, above all, displayed the lowest $^{14}CO_2$ fixation rates in this study.

Several investigators have demonstrated that N deficiency does not affect F_v/F_m (e.g., Khamis et al., 1990; Lu and Zhang, 2000; Chen and Cheng, 2003; Posch et al., 2008; Kumagai et al., 2009), which has been interpreted as protection against photoinhibitory damage in N-deficient plants. Proper adjustments in light capture and the dissipation of excess energy have usually been invoked to explain the lack of effect of N deficiency on PSII photochemical efficiency. Nonetheless, in the above quoted studies, cellular damage markers were not assessed, and consequently we cannot draw a firm conclusion regarding the association of N deficiency with photodamage. In a few studies (e.g., Huang et al., 2004) in which cellular damage has been examined, lipid peroxidation (analyzed through MDA accumulation) increased in LN plants with no commensurate change in F_v/F_m. In fact, F_v/F_m is relatively stable and does not respond readily to small changes in F_v or F_0, since F_m is the sum of F_v and F_0 (Babani and Lichtenthaler, 1996). According to these authors, F_m does not change at all, e.g., when F_v slightly decreases and F_0 slightly increases. Hence, changes in PSII photochemical efficiency could be masked by calculation of the F_v/F_m ratio. We therefore propose that the stability of F_v/F_m cannot always be considered as evidence of proper photoacclimation during N deficiency. It is noteworthy that F_v/F_m has also been shown to be quite stable under other stressful conditions, such as drought, despite the occurrence of remarkable photodamage, as previously demonstrated in coffee (Lima et al., 2002; Pinheiro et al., 2004).

Although the activity of CAT, an important enzyme in the photorespiratory pathway, is much more responsive to light availability than to N supply, the activity of GS, an enzyme that is thought to catalyze the rate-limiting step of photorespiration (Hoshida et al., 2000), was remarkably lower in LN plants than in HN plants within each light environment. This could be considered as circumstantial evidence that photorespiration has limited importance as an additional sink for the excitation energy in the photosyn-

thetic apparatus of LN plants. In addition, the rate of superoxide formation may increase considerably in N-deficient coffee plants under HL conditions (Ramalho et al., 1998), but no corresponding increase in the activity of SOD per unit mass could be found in the present study, what agrees with the findings of Fortunato et al. (2010) for cv. 'Catuaí' under low temperature exposure. Most importantly, a significant reduction in APX activity per unit mass was detected, which may explain the remarkable accumulation of H_2O_2 in the LN plants, a fact further supported by the negative correlation ($r = -0.845$, $P < 0.001$) between APX activity and H_2O_2 concentration (data not shown). Furthermore, the degree of unsaturation of chloroplast membranes has been shown to be greater in LN coffee plants than in their HN counterparts (Ramalho et al., 1998). These changes may decrease the stability of the chloroplast membranes in addition to making them more susceptible to peroxidative attack (Müller et al., 2001), which also contribute to explain the increased lipid peroxidation and electrolyte leakage found in LN individuals.

In conclusion, even though the absolute amounts of protective carotenoids increased with increasing N supply, the concentrations of these carotenoids on a Chl basis were similar (e.g., neoxanthin and lutein) or higher (e.g., A and Z) in LN plants when compared with HN individuals. Similarly, the activities on a protein basis (as well as on a Chl basis; data not shown) of most of the antioxidant enzymes analyzed were also remarkably higher in LN plants, particularly at full sunlight. This demonstrates that the antioxidant enzyme activities are subject to individual regulation and not merely responding to a general inhibition of leaf protein synthesis under N deficiency (Logan et al., 1999). Taken together, our results suggest stoichiometric increases in the photoprotective and antioxidant capacity of leaves per amount of photons absorbed, i.e., such a capacity was up-regulated by the LN supply. This conclusion is in contrast with our working hypothesis, as well as with Ramalho et al. (1997, 1998, 2000), who proposed that additional N could up-regulate protective carotenoids and detoxifying enzymes in coffee plants in high light. However, in their studies, Ramalho and colleagues expressed the results on the basis of mass or area; different conclusions might have been reached if the results were expressed on a Chl or protein basis. Nevertheless, the up-regulation of the photoprotective capacity by LN was insufficient to prevent photoinhibitory damage, probably because the light-capture and light-utilization processes were imbalanced. In any case, a genotype-dependent ability to trigger acclimation processes should not be excluded when comparing our results with those of Ramalho and coworkers, as coffee genotypes/species may display quite different responses to low temperature conditions (Ramalho et al., 2003; Fortunato et al., 2010). Finally, we demonstrated that N fertilization could adequately protect the coffee plants even in stressful conditions (e.g., low night temperature and full sunlight) against photooxidative damages through mechanisms that are apparently independent of the anticipated positive effects of HN supply on the carboxylation processes.

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