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Antioxidant metabolism in coffee (*Coffea arabica* L.) plants in response to nitrogen supply

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Abstract Nitrogen (N) is the main element required for plant development. N fertilization interferes directly in N content in tissues, antioxidant systems, chlorophyll content and photosynthesis. We investigated the action of three levels of N (0, 150 and 300 kg N ha^{-1}) in coffee (*Coffea arabica* L.) cv. Catuaí Vermelho IAC 44 leaves of plants during distinct fruit development stages (from pinhead drop to dried fruit). Leaf N content, net photosynthesis, chlorophyll content, total soluble protein and the activities of catalase, superoxide dismutase, guaiacol peroxidase and ascorbate peroxidase were analyzed. Leaf N content, net photosynthesis, chlorophyll and protein content increased significantly with N supply (N deficient plants exhibited visual symptoms of chlorosis). Antioxidant enzymes showed increased specific activities during fruit development, and decreases with N fertilization, being higher in absence of N. We identified two bands of Mn–SOD with increased activities and one of Fe–SOD, but they did not exhibit high SOD activity remaining essentially constant. Curiously, Cu/Zn–SOD isoenzymes were not detected, despite the fact that they are frequently abundant in plants. A relationship between N fertilization and antioxidant enzyme activities were founded in coffee leaves during fruit development indicating high activity of enzymatic antioxidant system during the coffee fruit ripening stage.

Keywords Antioxidants · Oxidative stress · Plant nutrition · *Coffea arabica* · Nitrogen nutrition

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1 Introduction

Despite being a species originating in shaded environments, coffee typically has high yields in sunny environments, but high management fertilization is required, especially in N (Pompelli et al. 2010). Nitrogen (N) is the main essential nutrient for the growth and development of plant species (Lea and Azevedo 2006, 2007; Andrews et al. 2011, 2013; Neto et al. 2015). Grain yield increases linearly with the

levels of N fertilizers applied to plants, which is a primordial nutrient responsible for crop productivity (Reis et al. 2011). Therefore, a better understanding of how plants respond to N supply is required for several reasons (Carswell et al. 2003), and many traits have been studied to improve this understanding, for example chlorophyll concentration, plant height, timing of leaf senescence, activities of enzymes controlling the assimilation of N and quantitative trait loci (Majerowicz et al. 2002; Ray et al. 2003; Andrews et al. 2007). In N fertilization experiments, the reduction in plant height, leaf area index and biomass production have been reported in N-deficient coffee plants (Da Matta et al. 2002; Netto et al. 2005; Fenilli et al. 2007; Reis et al. 2009a, b, 2011; Neto et al. 2011). This is because insufficient N input leads to the reduction in plant growth that should be attributed primarily to reduce photosynthesis (Chen et al. 2013). Leaves with high N content have a higher maximum net photosynthesis rate and higher carboxylation efficiency in saturation light with limiting carbon dioxide supply than those in N-deficient plants (Andrews et al. 2009). However, these photosynthetic characteristics increase curvilinearly with increasing levels of protein and consequently the rate of carbon dioxide assimilation per unit of N is lower in leaves with high N content than with low N content (Da Matta et al. 1999; Lawlor et al. 2001; Da Matta et al. 2002; Reis et al. 2006).

Plants under N deficiency lead to reprogramming their primary and secondary metabolism (Kováčik and Bačkor 2007). Senescence and nutrient remobilization from leaves to grains during ripening stage are common phenomena in coffee plants, independent of N levels (Barros et al. 1999; Reis et al. 2006, 2009a, b) and oxidative stress share common symptoms, like loss of chloroplastic pigments and proteins, lipid peroxidation and membrane alterations, leading to a progressive decrease in photosynthetic capacity (Cassano et al. 1994; Carvalho et al. 2011). However, in N deficiency situations, these processes can occur ealier (Camargo et al. 2011). In addition, Logan et al. (1999) reported an increase in activities of antioxidant enzymes in N-deficient spinach plants.

An adequate N nutritional status might induce the antioxidant systems to prevent damage on photosynthesis (Medici et al. 2004). To protect the photosynthetic apparatus from oxidative stress, plants must dissipate the excessive light energy (Carvalho et al. 2011), which can be achieved by the down-regulation of photochemical efficiency via xanthophyll cycle (Demming-Adams et al. 1996) or by maintenance of electron flux involving alternative pathways such as photorespiration and the Mehler peroxidase reaction (Asada 1999). However, both pathways lead to an increase in reactive oxygen species (ROS) generation.

ROS are highly reactive and cytotoxic to all organisms that their production must be minimized (Gratão et al. 2005; Ghelfi et al. 2011; Gill et al. 2013; Su et al. 2014). The accumulation of ROS in cells is usually low but under adverse environmental factors the production of ROS may be enhanced (Fidalgo et al. 2004; Monteiro et al. 2011; Cia et al. 2012). Therefore, in order to cope with ROS production within the cell, plants have a complex enzymatic antioxidant system that includes key enzymes in cellular detoxification, such as SOD (EC 1.15.1.1), which dismutates O^{-2} to H_2O_2 which may be detoxified to H_2O by ascorbate peroxidase (APX) (EC 1.11.1.11), catalase (CAT) (EC 1.11.1.6) and guaiacol peroxidase (GPOX) (EC 1.11.1.7) (Resende et al. 2012; Dourado et al. 2013; Gratão et al. 2015). SOD isoenzymes are usually present in distinct cell compartments; for instance, chloroplasts contain basically Cu/Zn–SOD and Fe–SOD isoenzymes, whereas in mitochondria only the Mn–SOD isoenzyme has been clearly localized (Alscher et al. 2002; Gratão et al. 2005). In peroxisomes, both Cu/Zn–SOD and Mn–SOD have been found either as soluble proteins in the organelle matrix or bound to membranes in the case of Mn–SOD (del Río et al. 2003).

There is a lack of information regarding the role of N on antioxidant stress responses in coffee plants (Gomes-Junior et al. 2007; Rendón et al. 2013). To our knowledge, no research has been carried out so far to specifically examine the antioxidant metabolism and the photosynthetic induction property of coffee leaves in relation to N fertilizer management under field conditions. In this study, changes due to N fertilizer were described in relation to antioxidant enzymes such as SOD, CAT, APX and GPOX, nutrient concentration and photosynthesis in coffee leaves during fruit development stages (from pinhead drop to dried fruit).

The aim of this study was to evaluate the response of net photosynthesis, chlorophyll content, protein and antioxidant enzymes in coffee leaves under different N supply and at different fruit developmental stages, establishing a possible relationship between different

levels of N on the activation key of the cellular antioxidant enzymes system.

2 Materials and methods

2.1 Site description and experimental design

The experiment was carried out in the experimental farm of the University of São Paulo (USP/ESALQ), at Piracicaba, São Paulo State, Brazil (22°42′S, 47°38′W and 580 m of altitude). Coffee plants (*Coffea arabica* L.*)* cv. Catuaí Vermelho IAC-44 were used, from a 6-year-old coffee orchard, spaced 1.75 m between rows and 0.75 m between plants (density of 7.633 plants per hectare). The region is characterized as a Cwa type climate, or tropical of altitude, with annual average temperatures of 21.1 °C, and mean rainfall of 1.257 mm. The raining period occurs between October and March and the dry period between June and September. The climate data was daily monitored by the computerized agrometeorological station of USP/ESALQ setting at the experiment location.

The soil in the experimental area is classified as a neutroferric Red Nitosol, moderate A horizon and clayey texture, which chemical attributes were determined in samples from 0 to 200 mm depth. The soil analysis results were: pH ($CaCl_2$): 5.3; OM: 31 g dm^{-3}; P (resin): 8 mg dm^{-3}; K: 4.3 $mmol_c$ dm^{-3}; Ca: 29 $mmol_c$ dm^{-3}; Mg: 20 $mmol_c$ dm^{-3}; H + Al: 30 $mmol_c$ dm^{-3}; CEC: 83.1 $mmol_c$ dm^{-3}; and base saturation: 64 %.

The experimental design was in randomized complete blocks arranged in a 3 × 6 factorial with five replications. The treatments consisted of three levels of N applied as ammonium sulfate: T1: absence of N fertilization, T2: 150 kg N ha^{-1} and T3: 300 kg N ha^{-1}. Six periods of evaluation during fruit developmental stages were considered: pinhead drop, rapid expansion, ripening, green, cherry and dried fruit. The highest N level (T3 = 300 kg N ha^{-1}) was estimated based on the yield expectancy (Reis et al. 2006) and an intermediate level of N fertilization was considered as half the maximum dosage (T2: 150 kg N ha^{-1}). In addition, 300 kg ha^{-1} of potassium (applied as KCl) was applied split in two applications, 150 kg ha^{-1} in November, 2005 and in January, 2006. The levels of fertilizer were based on the expectation of yield and official recommendation for coffee plants cultivated in São Paulo State (Brazil).

2.2 Nitrogen content determination

For N analysis, coffee leaves samples were obtained for each plot, consisting of 50 leaves removed from the third leaf pairs, starting at the end of the branches in the upper third of the plant (Reis et al. 2011). Leaves were dried in a forced-air oven at 65 °C during 48 h and ground. Thereafter, the samples were digested and chemically analyzed. Total-N was determined by the Kjeldahl method, which consist in the sulfuric digestion of the plant material to decompose the organic-N into ammonium, which is then converted to ammonia, distilled, and the amount of ammonia was determined by back-titration (Reis et al. 2009a).

2.3 Measurement of photosynthesis

Photosynthetic parameters were estimated from three plants per plot in fully expanded leaves on the third or fourth leaf pairs from the apex on lateral branches in the upper third of the plant, as described by Reis et al. (2009b). During the measurements, air relative humidity was about 80 % and leaf temperature ranged from 22 to 24 °C. Carbon dioxide assimilation was measured in the morning under artificial, saturating photosynthetic photon flux (PPF: 0.85–0.90 mmol m^{-2} s^{-1}) supplied by two 1000 W halogen tubes at an ambient CO_2 concentration of about 355 µmol L^{-1}, with a portable photosynthesis meter (PPS—model LI-6400, Li-Cor, Inc., Lincoln, NE, USA). In general, maximum rates occurred near 08:00 am (solar time) throughout the warm, rainy season, and around mid-morning during the cool, dry season. Measurements were taken throughout a cloudless day during fruit developmental stages using the same PPF described above.

2.4 Chlorophyll extraction

For the analysis of chlorophyll content, about 15 leaves were removed from the third leaf pairs, starting at the end of the branches in the upper third of the plant. The leaf disc was cut into fine strips and placed in a test tube containing 5 mL acetone 80 % (v/v: acetone/water). Extracted in the dark, a 3 mL aliquot was analyzed spectrophotometrically at 645 and 663 µm. The total chlorophyll levels were determined according to the equation proposed by Arnon (1949).

2.5 Enzyme extraction and assays

For enzyme extraction, the coffee leaves were collected directly into liquid N and stored at −80 °C for further analysis. Compound samples were obtained for each plot, consisting of 10 leaves removed from the third leaf pairs, starting at the end of the branches in the upper third of the plant. The following steps were carried out at 4 °C unless stated otherwise. The coffee leaves were homogenized (2:1 - buffer volume:fresh weight) in a mortar with a pestle with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenedinitrilotetraacetic acid (EDTA), 3 mM β-mercaptoethanol and 5 % (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 10,000×*g* for 30 min and the supernatant was kept stored in separate aliquots at −80 °C, prior to CAT, APX, GPOX and SOD analyses (Azevedo et al. 1998).

The total protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard (Bio-Rad Protein Dye Reagent).

2.6 Polyacrylamide gel electrophoresis (PAGE) and SOD activity staining

Electrophoretic analysis was carried out under non-denaturing condition in 9 % polyacrylamide gels, followed by SOD activity staining as described by Garcia et al. (2006), with equal amounts of protein (60 μg) being loaded on to each lane. SOD activity was determined as described by Azevedo et al. (1998). The gel was rinsed in distilled-deionized water and incubated in the dark for 30 min at room temperature in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitrobluetetrazolium, and 0.3 % N,N,N′,N′ tetramethyl ethylenediamine (TEMED). At the end of this period, the reaction mixture was poured off, the gel rinsed with distilled-deionized water and then illuminated in water until the development of colourless bands of SOD activity on the purple-stained gel. A standard sample of bovine SOD (Sigma Chemical Co.) was applied to all gels to be used as a positive control for SOD activity.

2.7 SOD densitometry analyses

SOD isoenzymes bands were observed following the staining of PAGE 9 % for enzyme activity and the intensity of the band was recorded according to a densitometry analysis through the use of Kodak Digital Science—1D (Image Analysis Software version 3.0.1).

2.8 SOD isoenzymes

An identical assay was carried out in parallel to classify the SOD isoenzymes by testing using potassium cyanide (KCN) and hydrogen peroxide (H_2O_2) as inhibitors of specific activities (Vitória et al. 2001). SOD isoenzymes were classified as described previously by Azevedo et al. (1998) according to their sensitivity to KCN (Cu/Zn–SOD inhibitor) and/or H_2O_2 (Cu/Zn–SOD and Fe–SOD inhibitor).

2.9 CAT assay

CAT activity was determined as described by Monteiro et al. (2011), with some minor modifications. CAT activity was assayed spectrophotometrically at 25 °C in a reaction mixture containing 1 mL 100 mM potassium phosphate buffer (pH 7.5), containing 2.5 μL H_2O_2 (30 % solution) prepared immediately before use. The reaction was initiated by the addition of 15 μL of plant extract and activity determined by monitoring the degradation of H_2O_2 at 240 nm over 1 min against a plant extract-free blank. CAT activity was expressed as μmol min^{-1} mg^{-1} protein.

2.10 APX assay

APX activity was determined by the method of Cia et al. (2012), by monitoring the rate of ascorbate (AsA) oxidation at 290 nm at 30 °C. The reaction medium contained 50 mM phosphate buffer, pH 7.0, 0.5 mM AsA, 0.1 mM EDTA, 0.1 mM H_2O_2 and 100 μL of enzyme extract, in a 1 mL of total volume. The reaction was started by the addition of AsA. The decrease in absorbance was followed from 10 to 60 s and APX activity was expressed as μmol AsA min^{-1} mg^{-1} protein.

2.11 GPOX assay

GPOX activity was determined as described by Gratão et al. (2012). The reaction medium contained 250 μL phosphate–citrate buffer (sodium phosphate dibasic 0.2 M: citric acid 0.1 M) pH 5.0, 150 μL enzyme

extract and 25 µL 0.5 % guaiacol, which was vortex shaken and incubated at 30 °C for 15 min. The reaction was stopped by quickly cooling in an ice-water bath, followed by the addition of 25 µL of sodium metabisulphide 2 % solution. The reaction mixture was held for 10 min and the GPOX activity was evaluated by monitoring the absorbance at 450 nm. One enzyme activity unit (u) corresponds to an increase of 0.001 in absorbance per min.

2.12 Statistical analysis

The data was submitted to analysis of variance using the SAS program of statistics, *System for Windows* 6.11 (SAS Inst. 1996). According to the F test significance level for the treatments (N levels and periods of evaluation), the data was submitted to linear and quadratic regression analysis using the GLM (general linear model) and the Tukey test (0.05) for mean comparisons. The correlations among dependent variables (leaf nitrogen, net photosynthesis, SOD, CAT, APX, and GPOX activity) were obtained by means of CORR procedure (Pearson correlation coefficient), using the SAS—*System for Windows* 6.11 (SAS Inst. 1996).

3 Results

3.1 SOD activity

SOD activity staining following non-denaturing PAGE revealed the existence of three bands in coffee leaves, including two Mn–SOD and one Fe–SOD (Fig. 1). The SOD bands were classified according to their metal cofactor based on their inhibitory pattern to hydrogen peroxide and/or potassium cyanide. A Cu/Zn–SOD inhibited by both compounds was not detected. SOD activity staining revealed two major Mn–SOD isoenzymes, I and II (resistant to both inhibitors), and a SOD isoenzyme III, classified as Fe–SOD (inhibited by hydrogen peroxide) as shown in Fig. 1.

SOD activity was enhanced significantly in coffee plants under N deficiency during fruit development (Fig. 2a). Moreover, bands I and II (Mn–SOD)

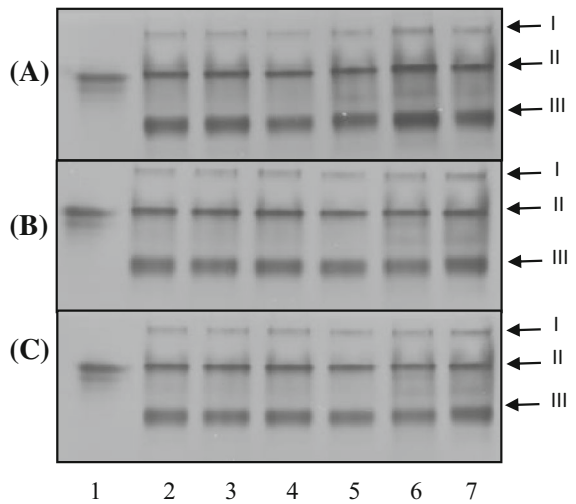

Fig. 2 Activity staining for SOD determined in coffee leaves extracts at different stages of fruits development, under non-denaturating conditions (PAGE 9 %). **a** Low N = absence of N (0 kg N ha^{-1}); **b** medium N = 150 kg N ha^{-1} and **c** high N = 300 kg N ha^{-1}. The *lines* listed are: (1) bovine SOD standard, (2) pinhead-drop, (3) rapid expansion, (4) ripening, (5) green, (6) cherry and (7) dried fruits. The SOD isoforms are *I* Mn–SOD, *II* Mn–SOD; *III* Fe–SOD

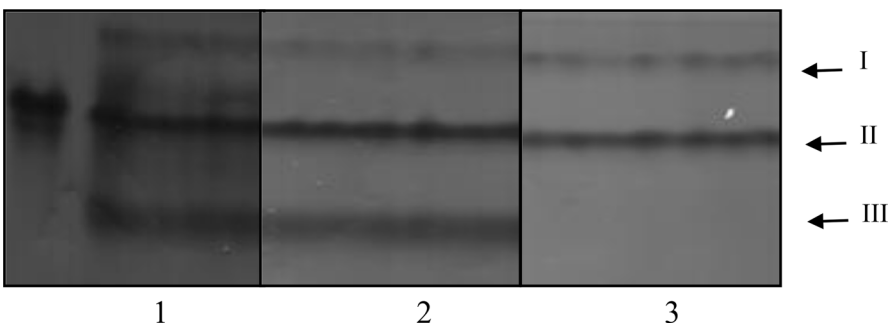

Fig. 1 Isoforms of SOD determined under non-denaturating conditions (PAGE 9 %) in coffee leaves extracts. *Lane 1* pre incubating gel in revelation buffer without any inhibitor; *lane 2* revelation buffer plus 2 mM potassium cyanide (Cu/Zn–SOD inhibitor) and *lane 3* revelation buffer plus 5 mM hydrogen peroxide (Cu/Zn–SOD and Fe–SOD inhibitor). The SOD isoforms are *I* Mn–SOD, *II* Mn–SOD; *III* Fe–SOD

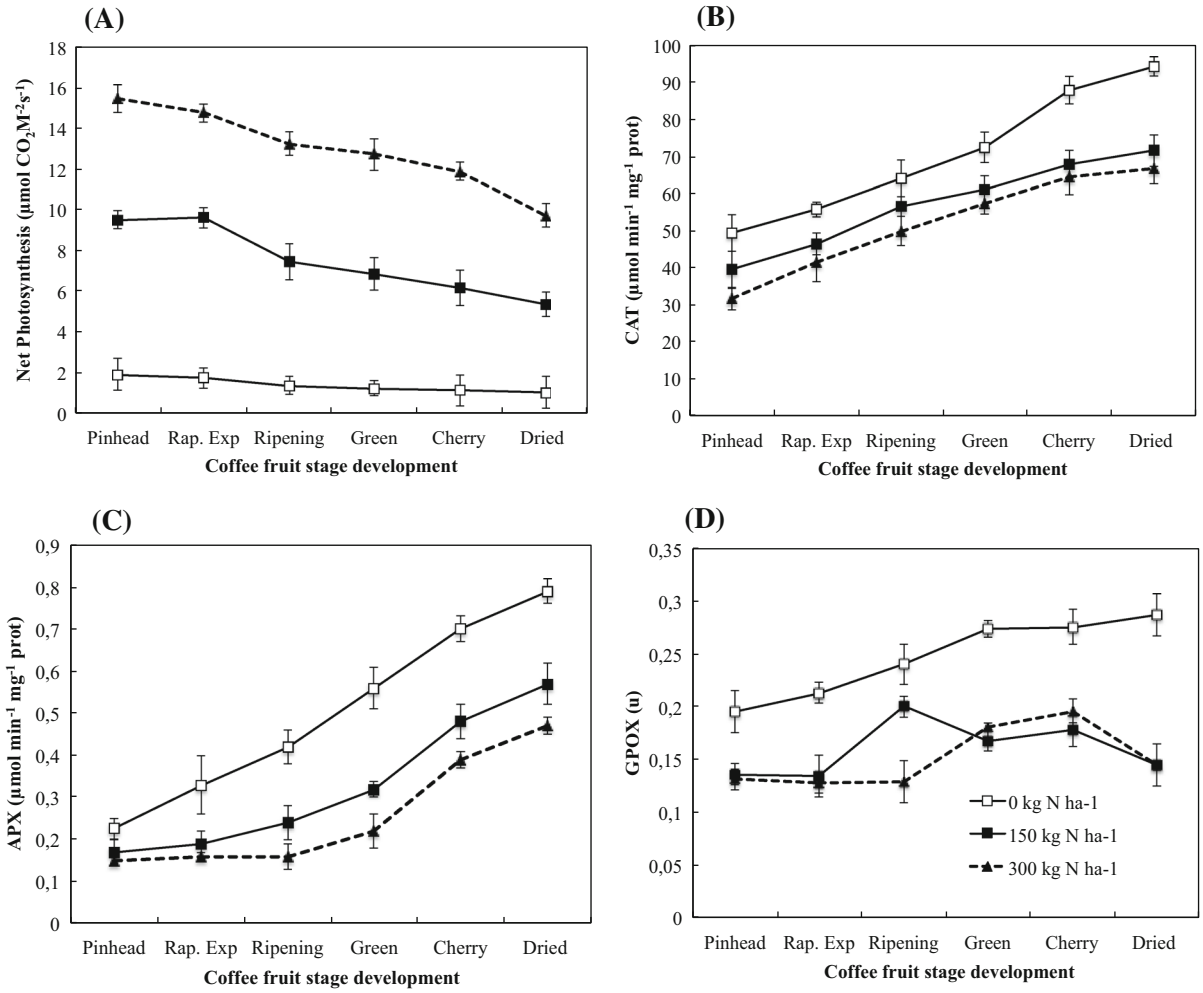

Fig. 3 Photosynthesis (**a**); CAT specific activity (µmol min^{-1} mg^{-1} prot) (**b**); APX specific activity (µmol min^{-1} mg^{-1} prot) (**c**) and GPOX (u) (**d**) in coffee leaves during fruits development in response to N supply. Low N = 0 kg ha^{-1} (*open square*); medium N = 150 kg ha^{-1} (*filled square*) and high N = 300 kg ha^{-1} (*filled triangle*). Values are the means of five replicates ± SEM. *Rap. Exp* Rapid Expansion

increased the intensity, as well as the thickness of bands of SOD on the native gels of coffee leaves under N deficiency (Fig. 2a, Lane 1–6). In addition, the band III (Fe–SOD) also increased, mainly in line 6–7 in all N treatment but specially in N-deprived plants, and its activity is higher than the others two (Fig. 2). These results showed that bands of Mn–SOD and Fe–SOD, indicating that possibly more active in ROS production in N-deficient coffee plants than chloroplasts in every stage of fruit development.

3.2 Photosynthesis and antioxidant enzyme activities

Photosynthetic rates in coffee leaves increased significantly with leaf N concentration until critical N values have been reached, after which photosynthetic rates remained constant despite further increases in N (Fig. 3a). Photosynthetic rates exhibited decreases in all treatments during fruit development until the end of the experiment (Fig. 3a). Nitrogen deficiency increased the activities of CAT, APX and GPOX

Table 1 Relationship among levels of N (kg ha^{-1}), CFDE (coffee fruit developmental stage), TSP (total soluble protein, mg mL^{-1}), GPOX (u), CAT (μmol min^{-1} mg^{-1}prot), APX (μmol min^{-1} mg^{-1}prot), P_n (net photosynthesis, μmol CO_2 m^{-2} s^{-1}), Chl (total chlorophyll, mg g-FW), and leaf N (g kg^{-1}) in response to N supply

	Levels	CFDE	TSP	GPOX	CAT	APX	Photo	Chl	N
Levels	1	ns	0.75*	−0.72*	−0.48*	−0.53*	0.96*	0.90*	0.90*
CFDE		1	−0.47*	0.32**	0.84*	0.80*	ns	ns	ns
TSP			1	−0.76*	−0.77*	−0.76*	0.87*	0.93*	0.87*
GPOX				1	0.72*	0.67*	−0.75*	−0.81*	−0.80*
CAT					1	0.95*	−0.65*	−0.68*	−0.67*
APX						1	−0.68*	−0.69*	−0.70*
Photo							1	0.95*	0.94*
Chl								1	0.96*
N									1

ns Not significant

*,** Significant at 1 and 5 %, ($p < F$ 0.0001)

determined by the spectrophotometer assays (Fig. 3b–d). These results indicated a clear inverse relationship between antioxidant enzymes activities and levels of N applied in coffee plants during fruit developmental stages (Table 1). The levels of N induced a different behavior in antioxidant enzyme activities, which was more active in N-deficient plants, and then continuously to increase during coffee fruit maturation.

3.3 Nitrogen, chlorophyll and protein content

From pinhead drop up to the dried fruit stage of development, fluctuation of total N concentration, total soluble protein and chlorophyll concentration were measured in coffee leaves (Fig. 4). A eightfold amplitude between the extreme values was observed during fruit development and a break down of leaf pigments was observed (Fig. 4c). In addition, total leaf N, chlorophyll content and total soluble protein decreased in all treatments as shown in Fig. 4.

4 Discussion

4.1 Photosynthesis and antioxidant responses to N supply

N supply usually affects the plant biomass, metabolism, regulation, structure and crop production (Lawlor et al. 2001; Reis et al. 2009a; Ker et al. 2014). Understanding the mechanisms by which crops respond to N is the key to maintaining and improving crop growth and yield, and the efficiency with which N is used and other resources as well (Da Matta et al. 1999, 2008; Reis et al. 2009b).

It has been suggested that in N-deficient plants the primary targets of N limitations are the meristems, leading to the well characterized down regulation of photosynthetic activity in source leaves (Logan et al. 1999). The surplus electron flow could enhance oxygen photoreduction in the chloroplast via the Mehler reaction (Asada 1999; Roberty et al. 2014) leading to the production of elevated levels of reduced oxygen species. In this case, one might also expect chloroplasts of N-deficient plants exhibit elevated activities of antioxidant enzymes such as SOD and APX, since these constituents are involved in the detoxification of reduced ROS (Polessakaya et al. 2006). As shown in the results, the coffee leaves submitted to N deficiency showed optimum activity of all enzymes antioxidants analyzed, however, in the case of SOD, this increase is due to the Mn–SOD and Fe–SOD isoforms especially in lines 6–7 in N-deprived plants. ROS are continuously produced even under optimum growth conditions being important in leaf expansion and root growth of maize, making it possible the increase of some antioxidant enzyme activities that degrade H_2O_2 (Rodrigues et al. 2002; Polessakaya et al. 2004). Under conditions of prolonged stress, enhanced generation of ROS disturbs

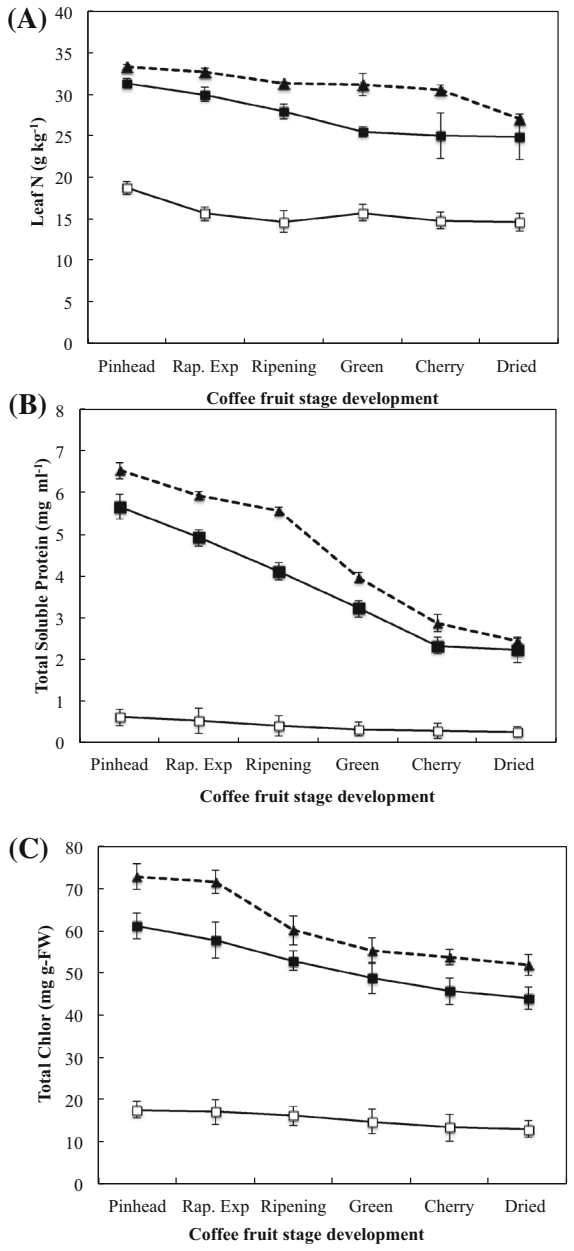

Fig. 4 Leaf N content (**a**); total soluble protein (**b**); and total chlorophyll content (**c**) in coffee leaves during fruits development in response to N supply. Low N = 0 kg ha^{-1} (*open square*); medium N = 150 kg ha^{-1} (*filled square*) and High N = 300 kg ha^{-1} (*filled triangle*). Values are the means of five replicates ± SEM

the normal redox environment of cells (Foyer and Noctor 2012; Keunen et al. 2013).

In coffee leaves and during leaf expansion fruit developmental stages, antioxidant enzymes such as CAT, APX and GPOX were shown to increase in activity, mainly in N-deficient plants, as shown in Fig. 3b–d, corroborating the previous report by Logan et al. (1999) and Huang et al. (2004) who observed increases in the activities of antioxidant enzymes in N-deficient spinach and rice plants, respectively. Increases in the activities of CAT, APX and GPOX relate well with the increased H_2O_2 concentration in N-deficient plants (Foyer and Noctor 2013). Since over 90 % of total leaf APX activity and approximately 80 % of total GR activity are localized in the chloroplast in plants grown under favorable conditions (Alscher et al. 1997), these trends for foliar APX and GR levels presumably represent a predominant chloroplastidic response (Logan et al. 1999).

The enzyme SOD is unique in that its activity can affect the O^{-2} radical, cell singlet oxygen and H_2O_2 concentrations (Foyer and Noctor 2013). The number of isoenzymes of each type of SOD varies greatly among plant species (Gratão et al. 2005) and we have been able to identify and classify up to three distinct SOD isoenzymes in coffee leaves. SOD activity, observed in coffee N-deficient plants, increased during fruit development as shown by the increased in SOD bands intensity on native gels (Fig. 2—lane 5). ROS generation may be further accentuated by limiting CO_2 uptake efficiency and accumulating reducing power in N-deficient plants can be due to accumulation of H_2O_2, which is known to decrease stomatal opening (Neill et al. 2002).

The high SOD activity may suggest that enhanced O^{-2} radical generation during photosynthesis in leaves of the N-deficient plants occurred and the identification of the distinct classes of SOD isoenzymes indicates their likely cellular organelle location (Azevedo et al. 1998; Lee and Lee 2000; Vitória et al. 2001; Gratão et al. 2005; Gomes-Junior et al. 2007; Tezotto et al. 2012). Two bands of Mn–SOD exhibited increases in activity in coffee leaves during fruit development in response to N supply (Fig. 1). One band of Fe–SOD isoenzyme activity exhibiting very low activity has also been observed following electrophoresis, but they remained essentially constant without any major changes during fruit ripening stage. Fe–SOD isoenzymes have been detected in plants, but not to the same extent as Mn–SODs, and have been shown to be associated with chloroplasts (Vitória et al. 2001; Gomes-Junior et al. 2007). The chloroplasts of the coffee plants, in this study, apparently were not majorly exposed to ROS, since that other SOD

isozyme (Mn–SOD), resident in the mitochondria, proved to be more active (Figs. 1, 2). The decrease on net photosynthesis can be partially attributed to other factors rather than the direct overproduction of ROS in the chloroplasts in response to N starvation, such as low chlorophylls content (Fig. 4).

In conclusion, alteration in activated oxygen metabolism was detected by increases of CAT, APX, GPOX and SOD activities during leaf nutrient remobilization to coffee fruits particularly in N-deficient coffee plants. Cu/Zn–SOD isoenzymes were not detected in coffee leaves, whereas two bands of Mn–SOD, sited in mitochondria, exhibited increases in activity in coffee leaves during fruit development in response to N supply. Net photosynthesis, leaf N concentration and total protein content increased in response to N supply. Our results suggest that the relationship between the increased leaf antioxidant enzyme activities and N nutritional status during fruit development indicated that the antioxidant system is very active during the coffee fruit ripening stage.

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