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Nitrogen metabolism in coffee plants in response to nitrogen supply by fertigation

Ana Paula Neto · José Laércio Favarin · André Rodrigues dos Reis · Tiago Tezotto · Rodrigo Estevam Munhoz de Almeida · José Lavres Junior · Luiz Antonio Gallo

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Abstract Coffee plants require high application rates of nitrogen (N) to produce coffee beans. However, information regarding plant N uptake and assimilation under intensive high-technology cultivation systems is scarce, a situation which restrains the maximizing productivity with minimal N rates. The aim of this study was to evaluate N assimilation enzymes such as nitrate reductase (EC 1.6.6.1), glutamine synthetase (EC 6.3.1.2), urease (EC 3.5.1.5), and N compounds of coffee plants during a phenological cycle in response to N applied by fertigation. Our study was carried out with 7-year-old trees of *Coffea arabica* L., under a center pivot sprinkler irrigation and fertigation system, with five N rates (0, 200, 400, 600, and 800 kg ha^{-1}) applied to the coffee field. The results of the present study suggest that both N metabolism enzymes, and the content of nitrate and ammonium in coffee were directly related to the phenological cycle. The N fertigation rates are correlated with the N and amino acid contente. Nitrate reductase and urease showed higher activities before anthesis and during grain filling. Glutamine synthetase showed highest activities during rapid grain expansion. The data also indicate that there is merit in gaining greater understanding of N metabolism in coffee plants grown under high-intensive systems. There is therefore merit in conducting further research on how monitoring the N assimilation enzymes might be used to improve fertilizer management of coffee in commercial orchards.

Keywords *Coffea arabica* · Glutamine synthetase · Nitrate reductase · Urease

A. P. Neto · J. L. Favarin · T. Tezotto
Laboratório Multiusuário em Produção Vegetal, Departamento de Produção Vegetal, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo, Piracicaba, SP CEP 13418-900, Brazil

A. R. dos Reis
Engenharia de Biossistemas, Universidade Estadual Paulista 'Julio de Mesquita Filho', Tupã, SP CEP 17602-496, Brazil

T. Tezotto (✉)
Crop Science Department, Luiz de Queiroz College of Agriculture, University of São Paulo, Av. Páduas Dias, 11, CP 9, Piracicaba, SP CEP: 13418-900, Brazil
e-mail: tiagotezotto@gmail.com; tiago.tezotto@usp.br

R. E. M. de Almeida
Embrapa Pesca e Aquicultura, Palmas, TO CEP 77020-020, Brazil

J. Lavres Junior
Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, SP CEP 13418-900, Brazil

L. A. Gallo
Departamento de Ciências Biológicas, Escola Superior de Agricultura 'Luiz de Queiroz', Universidade de São Paulo, Piracicaba, SP CEP 13418-900, Brazil

1 Introduction

In the last decade coffee (*Coffea arabica* L.) cultivation has expanded to marginal areas such as Western Bahia State, Brazil, with an irregular rainfall distribution throughout the year. This region presents a smooth topography, which facilitates the adoption of high technology such as center pivot sprinkler irrigation (Neto et al. 2011). Coffee plants of this region have high rates of vegetative growth and productivity index averaged 2,400–3,600 kg ha^{-1} per year (CONAB 2010; Neto et al. 2011) and 600–800 kg ha^{-1} N as urea applied to maintain this productivity. The high vegetative growth and yield occur primarily because of the greater number of sunlight hours due to less cloudiness, and average temperatures around 20 °C in the months of autumn and winter. This temperature above that of traditional cultivation regions (Neto et al. 2011), might provide better physiological conditions for growth.

N is the nutrient of greatest demand by coffee plants. N assimilation is a vital process for plant growth, being directly responsible for crop biomass production and grain yield (Fenilli et al. 2007; Reis et al. 2011). Regarding the total N absorbed by coffee plants, nitrate is the predominant form transported in xylem sap, representing more than 50 % of the total N (Mazzafera and Gonçalves 1999) that is reduced to nitrite by nitrate reductase (Wray and Fido 1990).

However, the quantitative contribution of nitrate sources as fertilizer is low and urea is the most widely used nitrogen fertilizer in agriculture (http://faostat.fao.org). Although urea is converted in part to ammonium and nitrate in the soil, little is known about urea uptake and metabolism in plants (Witte 2011). In addition, significant amounts of plant N flow through urea, which is derived from arginine degradation by arginase (Polacco et al. 2013). The N present in urea is unavailable to the plants unless hydrolyzed by the tissue-ubiquitous urease (Polacco et al. 1985). The N product of urease activity—ammonia—is incorporated into organic compounds mainly by glutamine synthetase (Sirko and Brodzik 2000). Therefore, nitrate reductase and glutamine synthetase activities might represent the rate-limiting step in N assimilation, as they can be used as a marker for the capacity of roots and shoots to assimilate external N in coffee plants (Reis et al. 2009). Due to the importance of N as the main nutrient for plants and its regulatory role in plant metabolism, it has been suggested that nitrate reductase activity is related to plant productivity and/or to a significant response to N fertilization (Malavolta et al. 2004; Reis et al. 2007, 2009).

Official bulletins of fertilization recommend about 400 kg ha^{-1} of N per year (Raij et al. 1996) split into twice or four applications. Coffee growers in the western part of Bahia State, Brazil, generally apply high N at rates between 600 and 800 kg ha^{-1} as urea, distributed as much as 26 times during coffee cycle, without a clear knowledge on the impact on coffee plant physiology. In fact, because of rapid vegetative growth and high productivity, several coffee farmers in Minas Gerais and Bahia State, Brazil, have been applying excessive N-urea fertilizer in an attempt to prevent N deficiency, thus further reducing crop efficiency. Although there is considerable knowledge available on N fertilization in coffee, the effects of such high rates on coffee physiology are not known. Moreover, the application of high N rates can increase losses by lixiviation, volatilization, and denitrification or erosion (Peoples et al. 1995). These losses can be minimized by synchronizing the fertilizer application with the plant's physiological demand (Matson et al. 1998; Panek et al. 2000; Matějková et al. 2010).

The aim of our work was to evaluate the changes in the N metabolic (assimilatory) enzymes as well as the nutritional status of N in coffee plants due to high N-urea supplied by fertigation during phenological phases.

2 Materials and methods

A field experiment was conducted between August 2008 and July 2009 on the Morena Farm located in Luiz Eduardo Magalhães, Bahia State, Brazil, 11°46′00″S and 45°43′32W, at an altitude of 740 m, with an average annual temperature of 23 °C and rainfall of 1,500 mm. The time course of monthly mean precipitation and air temperature from August 2008 to July 2009 is illustrated in Fig. 1. Plants of *Coffea arabica* L. var. Catuaí. Vermelho H-2077-144 with seven years-old and grown with spacing of 3.8 × 0.5 m (5,263 plants per hectare) were used in the study. Coffee trees were arranged circularly, with a central pivot for directed irrigation and fertirrigation. The region's climate is classified as Aw or

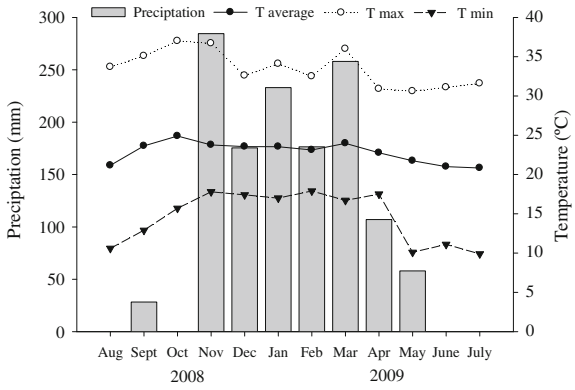

Fig. 1 Time course of monthly mean precipitation (mm) and air temperature (°C) from August 2008 to July 2009

savanna on the Köppen scale, with hot and wet summers and cool and generally dry winters. The soil is classified as Oxisol (Red-Yellow Latosol), with medium texture. Soil sample were collected at depth of 0–200 mm and the following parameters were determined: pH 4.7 (1:2.5 soil:0.01 mol $CaCl_2$); organic matter (OM): 25 g dm^{-3} (colorimetric method); sulfur (S): 10 mg dm^{-3} (turbidimetric method); phosphorus (P): 114 g dm^{-3}, potassium (K^+): 2 $mmol_c$ dm^{-3}, calcium (Ca^{2+}): 23 $mmol_c$ dm^{-3} and magnesium (Mg^{2+}): 9 $mmol_c$ dm^{-3} (ion exchange resin); aluminum (Al): 3 $mmol_c$ dm^{-3} (titrimetric method); potential acidity (H + Al): 31 $mmol_c$ dm^{-3} (pH SMP method); cation exchange capacity (CEC): 65 $mmol_c$ dm^{-3}; base saturation (V), 52 %; boron (B): 0.74 mg dm^{-3} (hot water); copper (Cu^{2+}): 9.6 mg dm^{-3}, iron (Fe^{2+}): 82 mg dm^{-3}, manganese (Mn^{2+}): 3.1 mg dm^{-3}, and zinc (Zn^{2+}): 3.6 mg dm^{-3} (2:1 soil:DTPA–TEA solution; 0.005 mol diethylenetriamine acid + 0.1 mol triethanolamine + 0.01 mol $CaCl_2$).

Fertigation is performed on the farm site using low-energy precision application (LEPA), in which water plus fertilizers are sprinkled locally on the rows of plants. Coffee trees were irrigated throughout the year, except during harvest, with application of an average of 3–4 mm per day on alternate days. The LEPA sprinkler was closed during fertigation with N-urea in the experimental plots. The application of N-urea in each experimental plots was performed manually, simulating the LEPA sprinkler to distribute the solution over the coffee canopy. N-urea was applied every 15 days, starting on August 1st 2008 and ending on July 17th 2009.

Experimental plots were composed of three central plants with four bordering plants on each side. The experimental setup was completely randomized with five treatments and four replications. The treatments corresponded to the following rates of N: T0, no N application; T1–200 kg ha^{-1} N (38 g $plant^{-1}$ N; 84 g $plant^{-1}$ urea); T2–400 kg ha^{-1}N (76 g $plant^{-1}$ N; 164 g $plant^{-1}$ urea); T3–600 kg ha^{-1} (114 g $plant^{-1}$ N; 253 g $plant^{-1}$ urea); and T4–800 kg ha^{-1} (152 g $plant^{-1}$ N; 338 g $plant^{-1}$ urea). The experimental field received 380 kg ha^{-1} potassium (K), 2.4 kg ha^{-1} zinc (Zn), 6.7 kg ha^{-1} boron (B), 9.8 kg ha^{-1} manganese (Mn), and 0.6 kg ha^{-1} copper (Cu), supplied from September 2008 to June 2009 by fertigation.

Samples to evaluate the N assimilation enzymes and N nutritional status were obtained during the phenological phases, with a total of six samplings at: vegetative phase (VG), 56 days before anthesis (−56); anthesis (A) (0); pinhead drop (PD) (42 days after anthesis, DAA); rapid expansion (RE) and grain filling (GF) (126–168 DAA); and maturation (MA) (266 DAA). Leaves of the third pair collected at the midpoint of the canopy (collected for nutrient status diagnosis) were stored on ice and immediately transported to the laboratory for enzymatic assays and analyses of amino acids, N, nitrate and ammonium.

2.1 Nitrate reductase activity

The in vivo nitrate reductase activity was determined according to the procedure of Radin (1974). Leaf samples were collected at 12:00 pm. Freshly collected leaf tissue was stored in plastic bags, transported to the laboratory on ice, and rinsed with deionized water. Afterwards, 100 mg of fresh tissue cut in discs was transferred to assay tubes containing 5 mL of phosphate buffer solution pH 7.4 (50 mM Na-phosphate buffer + 200 mM KNO_3). Thereafter, the assay tubes (wrapped in aluminum foil to protect from the light) were incubated in a 37 °C water bath for 30 min. The reaction was stopped by adding 1 mL of 1 % sulfanilamide in 2 M HCl followed by 1 mL of 0.05 % naphtylenediamine solution. The nitrite (NO_2^-) produced was measured in a spectrophotometer at 540 nm, using a nitrite standard calibration curve.

The enzyme activity was directly related to the amount of NO_2^- and the results were expressed in μmol NO_2^- g^{-1} h^{-1} FW (fresh weight).

2.2 Glutamine synthetase activity

Leaves were initially frozen with liquid N_2. Before tissue maceration, a 2.0 mL aliquot of the extract buffer (200 mM Tris–HCl, pH 7.5) was added and the material was homogenized and further centrifuged at $3.248 \times g$ for 5 min at 4 °C. The supernatant was reserved for the enzyme activity.

Glutamine synthetase activity was measured by the method proposed by Elliott (1953), which is based on the enzyme biosynthetic activity in the formation of λ-glutamyl hydroxamate. This reaction was carried out in assay tubes under continuous shaking in a water bath at 30 °C, with the addition of: 0.25 mL extraction buffer solution; 0.1 mL 50 mM ATP, pH 7.0; 0.25 mL 500 mM sodium glutamate; 0.05 mL 1 M $MgSO_4$; 0.05 mL 100 mM cysteine; 0.15 mL 100 mM hydroxylamine, pH 7.0; and 0.15 mL of the crude extract supernatant. After intervals of incubation, the reaction was stopped by the addition of 1 mL of a 1:1:1 mixture of 10 % $FeCl_3$: 24 % (w/v) TCA: 6 M HCL, leading to the formation of a brown-yellowish precipitate. Thereafter, the mixture was centrifuged at $3.248 \times g$ and the supernatant color was analyzed in a spectrophotometer at 540 nm to determine the formation of λ-glutamyl hydroxamate (λ-GH), using a standard calibration curve. GS specific activity was expressed as μmol λ-GH h^{-1} mg^{-1} protein.

The total soluble protein concentration was determined according to the Bradford (1976) procedure, using tissue extracts prepared with Tris-buffer (200 mM Tris–HCl, pH 7.5). To a 100 μL aliquot extract sample 5 mL Bradford reagent was added and the absorbance was read at 595 nm. Total soluble protein concentrations was calculated using by referring to a bovine serum albumin (BSA) standard calibration curve. The results were expressed in mg g^{-1} fresh tissue.

2.3 Urease activity

Urease activity was measured according to the whole tissue method described by Hogan et al. (1983), with ammonium determination as suggested by McCullough (1967). One hundred mg fresh tissue cut in discs was transferred to assay tubes containing 8 mL 50 mM phosphate buffer (pH 7.4) 0.2 M urea and 0.6 M n-propanol for a period of 3 h. After incubation, an aliquot of 0.5 mL supernatant was added to 2.5 mL Reagent I (0.1 M phenol and 170 μM sodium nitroprusside). Afterwards, 2.5 mL Reagent II (0.125 M NaOH + 0.15 M $Na_2HPO_4.12H_2O$ + NaOCl (3 % Cl_2) was added for determination of ammonium. This reaction was carried out in capped assay tubes under continuous shaking in a water bath at 37 °C for 35 min. Ammonium was measured in a spectrophotometer at 625 nm, using a NH_4Cl standard calibration curve and urease activity was expressed as μmol N–NH_4^+ h^{-1} g^{-1} FW.

2.4 Amino acids

Amino acids were extracted from lyophilizated leaves (~0.2 g) with methanol:chloroform:water (12:5:3, v/v/v) (Bielesky and Turner 1966; Andrade et al. 2010). Amino acid concentrations were quantitatively estimated using a ninhydrin reagent (Cocking and Yemm 1954) using a leucine standard calibration curve.

2.5 Total nitrogen, nitrate and ammonium determination

Coffee leaf samples were dried in a forced-air oven at 65 °C, 72 h and ground with a mortar and pestle. Total-N was determined by the Kjeldahl method (Bremer 1965). The concentrations of nitrate and ammonium in plant tissues were determined using a 1 M KCl solution as described by Tedesco and Gianello (1979).

2.6 Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS) software for Windows 6.11 (SAS 1996). Variance analysis (F-test) was employed to evaluate the significance of the treatments. Pearson correlation among N-rate, leaf N, NH_4^+, NO_3^-, glutamine synthetase, nitrate reductase, urease and amino acids were obtained and tested by the CORR procedure of SAS. Graphs were made with the SigmaPlot® software.

3 Results

Maximum, minimum and average air temperature and precipitation from August 2008 to July 2009 are shown in Fig. 1. Minimum air temperature start to increase by early August and precipitation by November. This period showed the lowest thermal amplitudes and the highest minimum temperatures.

During the annual reproductive cycle, coffee plants usually exhibit changes in nitrate uptake and assimilation capacity, which have been associated with the various physiological developmental stages of flowers and fruits. Figure 2a–d show the changes of N nutritional status (nitrogen, amino acids, nitrate, and ammonium content) of coffee plants during phenological development stages. Leaf-N and amino acids concentration increased in coffee phenological phase during anthesis stage but slowed down through pinhead stage. However, nitrate content increased on anthesis until maturation stage (Fig. 2c) and ammonium increased on pinhead and grain filling stage (Fig. 2d).

Figure 3a–c show the activities of N assimilation enzymes during the flower and grain development stages in response to N-urea supply by fertigation. High nitrate reductase enzyme activities were observed during pre-anthesis (VG) probably due to the beginning of rainfall season and concomitant temperature increase, which leads to the increase of NO_3^- uptake. GS highest activity was achieved during rapid expansion stage and urease activity late on grain fillign phase.

Correlation among N rates and all variables analyzed during phenological phase (vegetative,

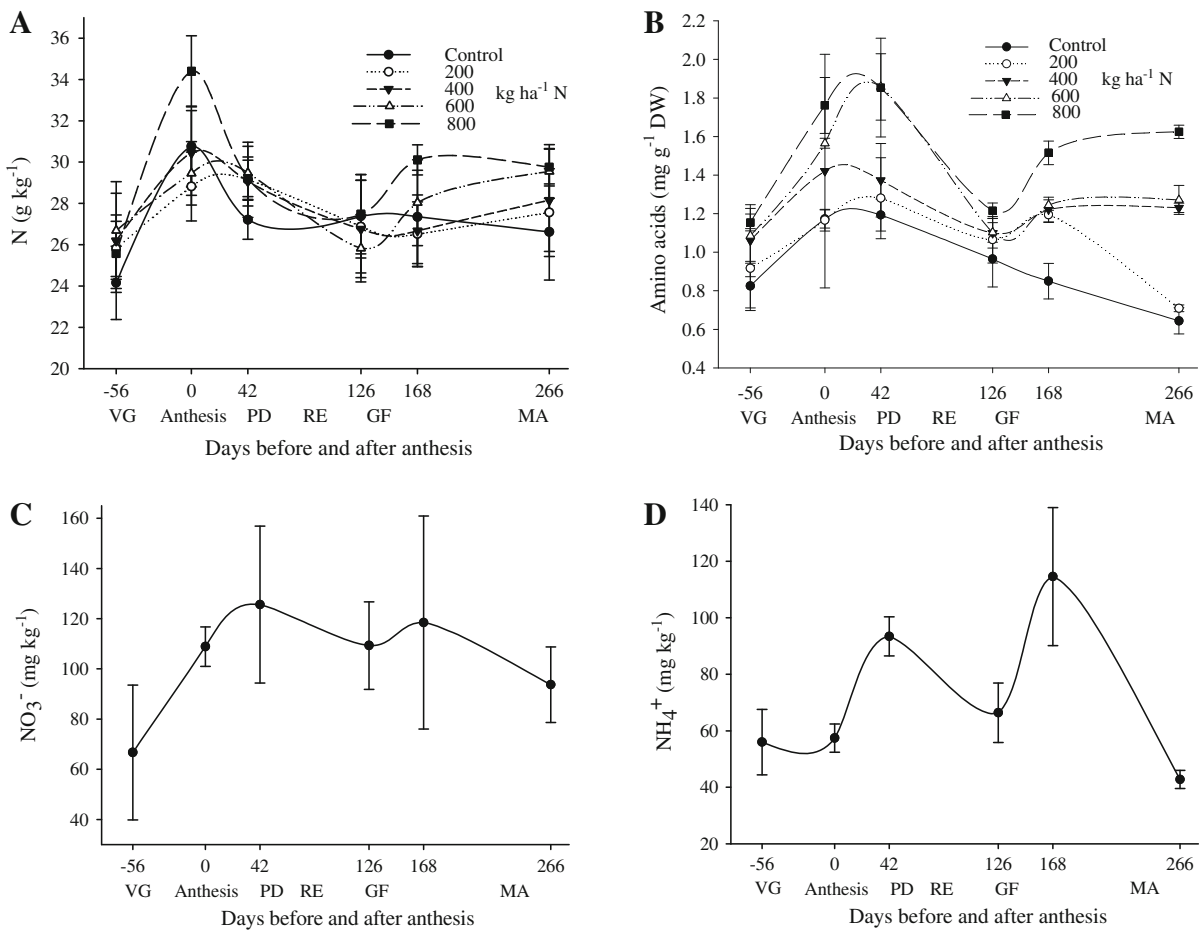

Fig. 2 Nitrogen (**a**), amino acids (**b**), nitrate (**c**), and ammonium content (**d**) in *Coffea arabica* leaves during grain development (vegetative phase (VG), anthesis, pinhead drop (PD), rapid grain expansion (RE), grain filling (GF), maturation (MA)) due to N fertilization by fertigation. *Error bars* represent standard deviation (SD)

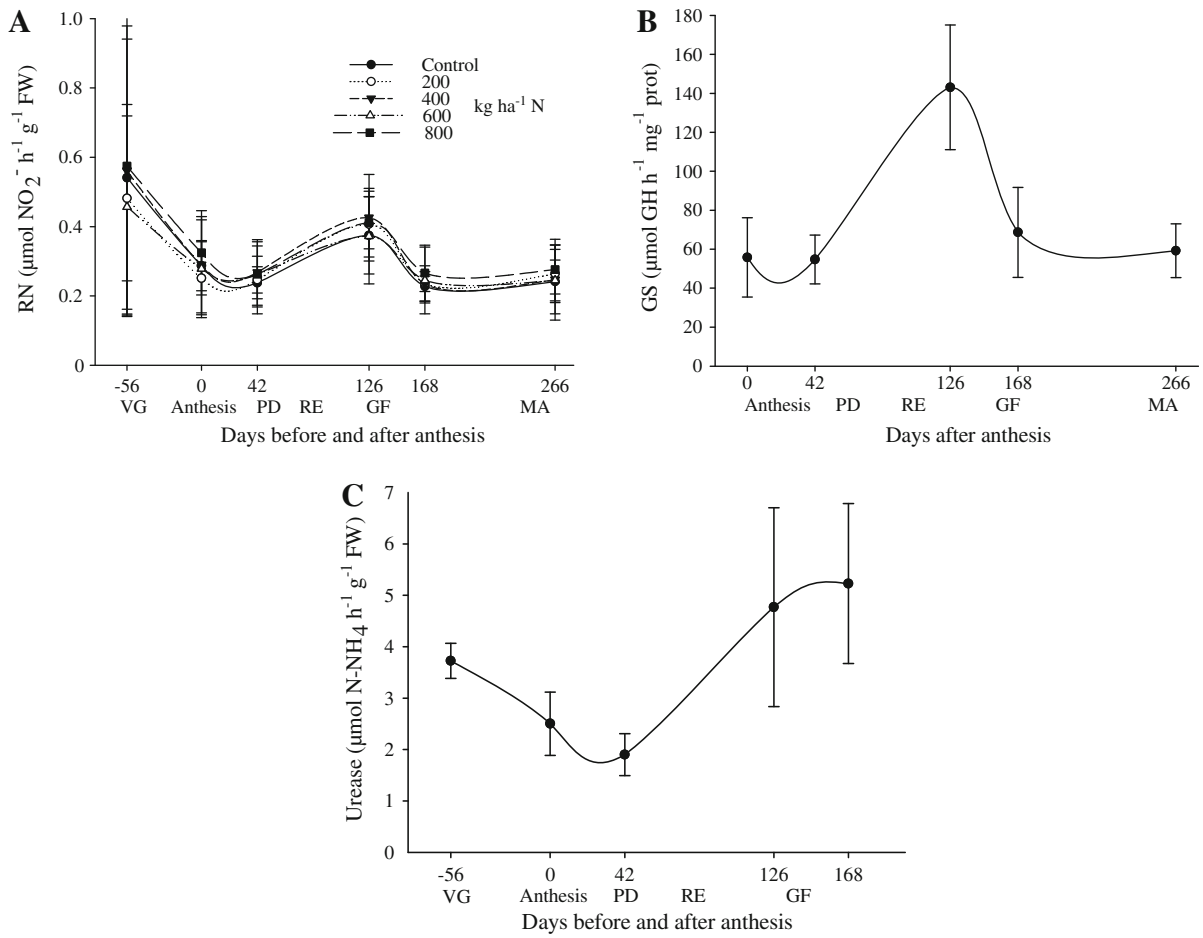

Fig. 3 Nitrate reductase (**a**), glutamine synthetase (**b**), and urease activity (**c**) in *Coffea arabica* leaves during grain development (vegetative phase (VG), anthesis, pinhead drop (PD), rapid grain expansion (RE), grain filling (GF), maturation (MA)) due to N fertilization by fertigation. *Error bars* represent standard deviation (SD)

anthesis, pinhead drop, rapid expansion, grain filling and maturation stages) are presented in Table 1. Correlation was found between N rates and amino acids at all phenological phase evaluated ($P < 0.01$).

There was a significant interaction between N rates and phenological phase of coffee plants for leaf-N, amino acids, NO_3^- and GS activity. NH_4^+ and RN did not vary with N rate, although they changed during phenological phase (Table 2).

4 Discussion

The first application of N in the fertigation system of coffee trees was during vegetative stage (56 days before anthesis) when leaves showed low levels of N (25.7 g kg^{-1} DW) and amino acids (1.01 mg g^{-1} DW) (Fig. 2a, b) due to strong remobilization of nitrogen compounds to the fruit during the previous reproductive phase. From the vegetative stage to anthesis N and amino acid contents in the leaves increased in all treatments, including the control plants. This increase in N accumulation by plants coincided with the start of the rainy season and its increased temperature. These are factors which favor greater release of N in the soil, and the re-use of nitrogen accumulated over the winter in the reserve organs (DaMatta et al. 1999; Amaral et al. 2001).

The increase in the N concentration during anthesis was partly due to the effect of N fertilization, started 70 days before the evaluation. In addition, the greatest temperature and water availability occurred in this

Table 1 Pearson correlation for N-rate, leaf N, NH_4^+, NO_3^-, glutamine synthetase (GS), nitrate reductase (NR), urease and amino acids (AA) during phenological phase: vegetative phase (VG), anthesis, pinhead drop (PD), rapid grain expansion (RE), grain filling (GF), maturation (MA)

	N	NH_4^+	NO_3^-	GS	NR	Urease	AA
Vegetative phase (56 days before anthesis)							
Rate	ns	ns	ns	ns	ns	ns	0.65**
N		ns	ns	ns	ns	ns	ns
NH_4^+			ns	ns	ns	ns	ns
NO_3^-				ns	ns	ns	ns
GS					ns	ns	ns
NR						ns	ns
Urease							ns
Anthesis (0 day)							
Rate	0.56**	ns	ns	ns	ns	ns	0.67**
N		ns	ns	ns	ns	ns	0.48*
NH_4^+			ns	ns	ns	ns	ns
NO_3^-				ns	ns	ns	ns
GS					−0.43*	ns	ns
NR						ns	ns
Urease							ns
Pinhead drop (42 days after anthesis)							
Rate	0.45*	ns	ns	−0.53**	ns	ns	0.81**
N		ns	ns	ns	ns	ns	0.44*
NH_4^+			ns	ns	ns	ns	ns
NO_3^-				ns	ns	ns	ns
GS					ns	ns	−0.55**
NR						ns	ns
Urease							ns
Rapid expansion (126 days after anthesis)							
Rate	ns	0.42*	ns	ns	ns	ns	0.67**
N		ns	ns	ns	ns	ns	ns
NH_4^+			ns	ns	ns	ns	ns
NO_3^-				ns	ns	ns	ns
GS					0.40*	ns	ns
RN						0.49*	ns
Urease							ns
Grain filling (168 days after anthesis)							
Rate	0.5*	0.38*	ns	ns	ns	ns	0.89**
N		ns	ns	0.56**	0.54**	ns	0.48**
NH_4^+			ns	ns	ns	ns	ns
NO_3^-				ns	ns	ns	ns
GS					ns	ns	ns
NR						ns	ns
Urease							ns
Maturation (266 days after anthesis)							
Rate	0.57**	ns	ns	−0.76**	ns	ns	0.96**
N		ns	ns	0.54**	ns	ns	0.52**

Table 1 continued

	N	NH_4^+	NO_3^-	GS	NR	Urease	AA
NH_4^+			ns	ns	−0.40*	ns	ns
NO_3^-				ns	ns	ns	ns
GS					ns	ns	−0.67**
NR						ns	ns
Urease							ns

ns Non-significant

** and * Significant at 0.01 and 0.05 levels respectively

Table 2 Summary of statistical analyses for rate, phenology, and interaction between N rate and phenology

Variable	N rate	Phenology	N rate vs phenology
Leaf-N	0.0250*	<0.0001*	0.0446*
Amino acids	<0.0001*	<0.0001*	<0.0001*
NO_3^-	0.4904	<0.0001*	<0.0001*
NH_4^+	0.1414	<0.0001*	0.2248
RN	0.0110	<0.0001*	0.2320
GS	0.1007	<0.0001*	0.0017*
Urease	0.7524	<0.0001*	<0.0001*

* Significant at 5 % level by F-test ($p < 0.05$)

phase (Fig. 1). Part of the N also may have come from the mineralization of the N present in the soil, suggested by the increased N concentration even before this nutrient was applied (Neto et al. 2011). Another explanation for the increased N concentration during anthesis can be redistribution of nitrogenated compounds from other organs, such as roots, stem, and branches, which store compounds in the winter that are redistributed at the start of the rainy season (Reis et al. 2009, 2011).

At anthesis, 34.4 g kg^{-1} (DW) leaf N was observed with application of 800 kg ha^{-1} N-urea, while amino acids increased gradually as the N-rate increased, from the anthesis to the pinhead drop phase (PD). Then, after anthesis, N and amino acid contents in the leaves decreased progressively until grain filling phase (GF) when an increase was observed in the 400, 600 and 800 kg ha^{-1} treatments. This drop in the GF phase is probably due to N remobilization from the leaves to the developing beans (Rena et al. 2001; Reis et al. 2011). Nitrate content in the leaves showed a twofold increase at the beginning of the fertigation and remained constant during fruit development (Fig. 2c). However, ammonia content showed a bimodal curve with two peaks, the first at PD phase and the second at GF phase. Taken together, N, amino acid, nitrate and ammonium contents showed similar variation curves, with a decrease in the GF phase, that indicates the high demand by nitrogen compounds for the formation of the coffee fruit (Rena et al. 2001): N is the second nutrient most demanded by grains and even applications of the order of 800 kg N ha^{-1} does not prevent this reduction of nitrogen leaf (Fig. 2a).

Regarding the uptake of nitrogen forms by coffee plants, Mazzafera and Gonçalves (1999) stated that 52 % of the N present in the xylem of coffee was NO_3^-, which is rapidly incorporated by the nitrate reductase. Similarly, Carelli et al. (1989) found that leaf nitrate reductase activity in coffee plants growing under natural light-temperature regimes, showed higher values prior to anthesis and at the end of fruit development. Such results indicate that the differential nitrate uptake, associated with the phenological phases, might be an important factor in the control of leaf nitrate reductase during the annual cycle of the mature coffee plant. This is independent of the variations promoted by seasonal environmental conditions (Carelli et al. 2006; Reis et al. 2007).

In Costa Rica, Carvajal et al. (1969) and in Brazil, Reis et al. (2009) observed that nitrate uptake rates of mature coffee plants were higher before anthesis and at the beginning of fruit maturation. Glutamine synthetase and urease activity did not change as a function of N-urea application, but only with phenological phases. On the other hand, NH_4^+ was not detected in xylem even when NH_4^+ was present in solution. Although urea transporters are identified in plants (Witte 2011), there is still no information on the conditions under which plants can take up urea from the soil. In this work the urease activity varied only in phenological phase and not with the N-urea applied.

The highest glutamine synthetase activity in the leaves was observed during the rapid expansion (RE) of grains (Fig. 2c). In all other stages the activity remained at similar levels and three times lower than the peak observed at RE stage. Glutamine synthetase is responsible for re-assimilation of NH_4^+ from photorespiration, proteolysis and amino acid catabolism during mobilization of N for translocation and/or storage in senescent tissues (Lea et al. 1990; Kamachi et al. 1991), which may explain the increased levels of amino acids and nitrogen in plants after the period of highest enzyme activity. A previous report showed intense amino acid mobilization during coffee grain endosperm formation (Reis et al. 2009). Furthermore, the glutamine synthetase activity in coffee plants shows no response to high N application rates by fertigation (Table 1). These results might indicate that glutamine synthetase activity in coffee also represents the rate-limiting step in N assimilation.

The peak of urease activity in coffee leaves was observed at 126 days after anthesis (rapid grain expansion stage). Urease is known to catalyze urea assimilation after uptake into plant cells, which hydrolyze urea in the cytosol to CO_2 and ammonia. Ammonia or ammonium is subsequently fixed by glutamine synthetase. Curiously, the highest urease activity coincides with the peak of glutamine synthetase during rapid grain expansion (126 days after anthesis). Reis et al. (2009) observed the nickel concentration in coffee leaves increase during grain development stage. Urease is a nickel-dependent enzyme that could play an important role in optimal N-use efficiency by recycling urea-N generated from arginase action on arginine (Polacco et al. 2013). Urea metabolism in coffee plants is still not well understood. In addition, the effects of long-term assessment of N assimilation enzymes, nutritional status of perennial crops, and monitoring soil properties upon urea application in agricultural are still somewhat unclear and further investigation is required.

Nitrogen metabolism enzymes showed higher values during anthesis and grain development stages. We argue that this is because the evaluation periods are coincident with the periods of intense metabolism and nutrient demand from the sinks (flowers and fruits). The first is the late phase of flower development (Carelli et al. 1989), when the flower buds rapidly expand and show a sharp increase in dry matter, requiring rapid metabolite transport from the nearest photosynthesizing leaves (Barros et al. 1982). The second is the late phase of fruit development, during formation of seed endosperms, which are powerful sinks of carbohydrates and minerals (Canell 1975). Higher N requirement during the formation of the fruit is because it consists of a repository of amino acids and proteins. The increase of N fertilization rates by fertigation increased coffee yield, which ranged from 2,340 to 4,320 kg ha^{-1} when applied to 400 kg ha^{-1} of N. The yield corresponding to 90 % of the relative production in response to N rates was obtained with the application of 415 kg ha^{-1} N (Neto et al. 2011).

The results of the present study suggest that both N metabolism enzymes, and the content of nitrate and ammonium in coffee were directly related to the phenological cycle. The N fertigation rates are correlated with the N and amino acid content (Table 1). The nitrate reductase and urease showed highest activities before anthesis, and during grain filling. Glutamine synthetase showed highest activities during rapid grain expansion. The data also indicate that there is merit in gaining greater understanding of N metabolism in coffee plants grown under high-intensive systems. There is therefore merit in conducting further research on how monitoring the N assimilation enzymes might be used to improve fertilizer management of coffee in commercial orchards.

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