The influence of nitrogen in nutrient solution on growth, nutrient uptake and enzymatic activity of *Anacardium othonianum* Rizz.

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The availability of nutrients directly affects plant growth and development, with nitrogen being one of the most necessary nutrients in metabolism in general. Using the hypothesis that *Anacardium othonianum* Rizz. can be physiologically affected by different doses of nitrogen, this study aimed to evaluate aspects of growth, nutrient absorption and enzymatic activity during the production of seedlings of this species in hydroponic cultivation. The doses of 0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mmol l^{-1} of N were tested. At 120 days after transplanting the seedlings into the nutrient solution, it was observed that doses higher than 10.0 mmol l^{-1} of N may constitute an excess, negatively affecting the number of leaves and leaf area. The enzymes glutamine synthetase and nitrate reductase showed greater activity in seedlings subjected to 2.5 mmol l^{-1} of N. Doses higher than this negatively affected the activity of these enzymes, indicating that *A. othonianum* Rizz. may be a species sensitive to ammonia. Alternatively, the absence of N (0.0 mmol l^{-1}) stimulated root mass accumulation, absorption of K, Mg and B ions, as well as nitric oxide synthesis. The present study contributes to obtain healthy seedlings and to the knowledge of the metabolism aspects of an important Cerrado fruit tree.

Keywords: *Anacardium orthonianum*, hydroponics, fruit trees, mineral nutrition, nitrogen metabolism.

THE Brazilian Cerrado (savannah) has a great diversity of fruit tree species with economic potential. Among these is *Anacardium othonianum* Rizz., whose fruit is similar but smaller than that of common cashew tree *Anacardium occidentale* L. This species is named after the Brazilian botanist Othon Xavier de Brito Machado especially important due to the use of its fruits in the food industry and tree in the reforestation of degraded areas. However, the large-scale production of this fruit tree is limited by the lack of knowledge about its physiology and nutritional requirements, and the effects that allow its establishment in the field. With the knowledge of the nutritional requirements, it is possible to provide adequate nutrition for the species, making it less susceptible to diseases and microorganisms, as well as to water stress and several other abiotic factors[1,2].

Among the nutrients, nitrogen plays an essential role during the early stages of fruit tree seedlings development. The absorption of N alters the pH of the rhizosphere. It alkalizes the rhizosphere when absorbed as nitrate and acidifies when absorbed as ammonia. This mechanism affects the absorption of other nutrients[3]. However, the positive response of plants to N fertilization, especially in tropical soils, is compromised by large volatilization losses and leaching[4]. There are environmental impacts and high production costs in these systems characterized by high temperature and rainfall, since under these conditions the levels of denitrification and also of leaching are increased[5]. In most agricultural production systems, approximately 50–75% of applied N is lost and not used by the plants[6]. Thus, it is important to improve the efficiency of nitrogen use, which would lead to reduced costs and increased production[7].

Knowing the N requirements of plants is essential for their development, since this nutrient is involved in the synthesis of several organic compounds, including amino acids, proteins, enzymes and nucleic acids[8,9]. Furthermore, any type of stress can alter N availability, assimilation and metabolism in plants as well as the activity of some enzymes essential for N metabolism, including glutamine synthetase (GS) and nitrate reductase (NR)[10].

NR is considered a key element in the process of N assimilation and use in plants, as it is the first enzyme in the nitrate assimilation pathway[11]. NR catalyses the transfer of electrons from 2 nicotinamide adenine dinucleotide phosphate (NADPH) to produce nitrite from nitrate; nitrite is then reduced to NH_4^+ by the enzyme nitrite reductase. Alternatively, NR catalyses the reduction of an electron from nitrite to form nitric oxide (NO) using NADPH as the electron donor, constituting an alternative physiological function of this enzyme in plants[12],

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especially under stress conditions. The role played by NR in the regulation of NO homeostasis occurs through the diaphorase/dehydrogenase domain of this enzyme, where a truncated haemoglobin (THB1) can recover NO by its dioxygenase activity, or by the NO-forming nitrite reductase (NOFNiR) responsible for the synthesis of NO from nitrite[13].

The availability of nitrogen affects the levels of transcription and activity of NR and GS[14,15], an enzyme that may be related to the maintenance of essential nitrogen (N) flows and internal N sensing during critical stages of plant development. In plants, this enzyme is essential in catalysing the ATP-dependent reaction that allows conversion of glutamate to glutamine using ammonium derived from the primary uptake of N and several of its internal recycling routes[16].

As studies on the metabolic effects of N availability on Cerrado plants are meagre and using the hypothesis that 'caju-do-cerrado' (*A. othonianum* Rizz.) seedlings respond to different doses of nitrogen the present study aimed to evaluate the growth, nutrient absorption and enzymatic activity during production of seedlings of this species in the nutrient solution.

Materials and methods

The experiment was conducted between June and November 2014 in a greenhouse at the Plant Tissue Culture Laboratory of the Goiano Federal Institute, Rio Verde Campus, Brazil.

The 'caju-de-cerrado' fruits were collected from Gameleira farm in the municipality of Montes Claros, state of Goiás, Brazil, at the following geographical coordinates: 16.09436°S–51.21617°W at 385 m amsl altitude; 16.10698°S – 51.27012°W at 412 m amsl; 16.11594°S–51.27737°W at 404 m amsl; 16.13295°S–51.29675°W at 595 m amsl and 16.13266°S–51.30228°W at 609 m amsl. The voucher specimen of the plant material has been deposited in the Jataiense Herbarium, Federal University of Goiás, Jataí Campus, Brazil under collection number 3793. After collection, the fruits were manually pulped in running water to obtain the seeds. The surface moisture of the seeds was removed by drying with paper towels at room temperature. The seeds were treated with Vitavax-Thiram fungicide (active ingredients; carboxin 200 g l^{-1} + thiram 200 g l^{-1}) using 300 ml of fungicide per 100 kg of seeds. The seeds were then dried to 13% moisture level by direct contact with silica gel in plastic trays (35 × 30 × 8 cm). Next, the seeds were packed in plastic bags and stored in a biochemical oxygen demand chamber at 10°C.

Sowing was performed in plastic trays (50 × 35 × 8 cm) containing washed sand as substrate. At 30 days after sowing, when the plants had 3–4 fully expanded leaves, the seedlings were transplanted to 8 litre hydroponic pots containing half-strength Hoagland nutrient solution modified according to the concentration of each treatment and kept in the pots for 30 days for adaptation. After this period, the plants were treated with seven doses of N (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mmol l^{-1}).

During the experiment, plants were cultivated under a mean irradiance of 584.75 µmol m^{-2} s^{-1}, and the nutrient solution was maintained under constant aeration with compressed air. The pH was adjusted daily to 5.5 ± 0.5 by adding HCl or NaOH, as needed, and the nutrient solution was replaced whenever a 30% depletion of the initial electrical conductivity was reached.

At 120 days after the seedlings were transplanted to the treatment solutions, the following plant growth characteristics were determined: stem length, root length, number of leaves, number of nodes, stem diameter and leaf area (of the fourth leaf and whole plant) in addition to the levels of N, P, K, Mg, Ca, S, B, Fe, Mn, Mo, Cu, Zn and nitric oxide, and the activities of GS and NR.

Growth characteristics

The stem length was determined by measuring the region between the root collar and apical meristem of the main stem, and root length was determined by measuring the longest part of the primary root with a ruler. The stem diameter was evaluated at the root collar of the seedling with the use of a digital caliper. The total leaf area and fourth leaf area were obtained from the integration of leaf images in ImageJ, a free, open-source image processing software program (http://rsbweb.nih.gov/ij/download.html)[17].

Nutritional content

The 'caju-do-cerrado' plants were collected and separated into leaves, stems and roots. The different parts of the plants were dried in a forced-air oven at 65°C until they reached a constant weight. The plant parts were ground in a Willey mill equipped with a 20-mesh sieve, and the levels of N, P, K, Mg, Ca, S, B, Fe, Mn, Mo, Cu and Zn nutrients were determined according to the methodology proposed by Malavolta *et al.*[18].

Nitric oxide content

The NO content was determined using the methodology described by Zhou *et al.*[19], which uses the Griess reagent. Each sample of 0.6 g of leaves was macerated with a mortar and pestle, homogenized with 3 ml of 50 mM acetic acid buffer (pH 3.6) containing 4% zinc diacetate and filtered. Subsequently, the material was centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant was added to 1 ml of the Griess reagent, and samples were incubated at room temperature for 30 min. The absorbance was read

at 540 nm in a UV spectrophotometer (Evolution 60S VIS model – Thermo Scientific, USA). The NO content was obtained by comparison with the standard curve for $NaNO_2$. The results obtained were expressed as nmol of NO per gram of fresh mass (nmol g^{-1} FM).

Enzymatic activities

Glutamine synthetase: Fresh leaf samples, each weighing 1.0 g, were macerated in liquid nitrogen. The enzyme extracts were obtained using the extraction buffer (0.05 M imidazole-HCl, pH 7.2, containing 0.5 mM EDTA and 1.0 mM dithiothreitol) plus a solution of 36 µmol ATP, 90 µmol $MgSO_4$, 12 µmol hydroxylamine, 184 µmol L-glutamate and 100 µmol imidazole-HCl. The final volume was 2 ml, and pH was 7.2. The samples were incubated in a water bath at 30°C for 30 min. The GS activity was determined as described by Rhodes and Stewart[20]. After incubation, aliquots of 0.8 ml of the samples were added to 1.2 ml of ferric chloride (10%), TCA (24%) and HCl (6N), 1 : 1 : 1, forming a yellowish-brown complex as a precipitate. The mixture was then centrifuged at 500 rpm, and the supernatant was analysed calorimetrically to determine λ-glutamyl hydroxamate formation. The absorbance was read at 540 nm in a UV spectrophotometer (Evolution 60S VIS model), and enzymatic activity was determinedby comparing the reading obtained with the standard curve. GS activity was expressed in µmol of glutamyl hydroxamate per minute per gram of protein (µM of λ-GH min^{-1} g^{-1} protein).

Nitrate reductase: The NR activity was evaluated using the method described by Radin[21]. Leaf samples were collected between 9:00 and 10:00 AM, stored in plastic bags and placed in a polystyrene box with ice. Then for each sample, 100 mg of fresh mass was weighed, macerated and placed in a test tube containing 3 ml of phosphate buffer (pH 7.4, 50 mM) + KNO_3 (200 mM). The samples were vacuum-filtered for 5 min, and the test tubes with leaf material were transferred to a water bath at 33°C for 30 min and wrapped with aluminum foil to avoid exposure to light.

The reaction was stabilized with the addition of 1 ml of 1% sulphanilamide in 2 N HCl, after which 1 ml of 0.05% naphthalenediamine was added. The absorbance was read using a spectrophotometer at 540 nm, and enzyme activity was determined based on the amount of nitrite (NO_2^-) produced, comparing the values obtained with the standard curve. The results were expressed in µmol of nitrite per hour per gram of fresh mass (µmol NO_2^- h^{-1} g^{-1} FM).

Total soluble sugars: The total soluble sugars (TSS) were determined in triplicate using the phenol–sulphuric acid method and spectrophotometry at 490 nm wavelength. The values were expressed as soluble sugar content (% sugars per gram of fresh mass), with D-glucose as the standard (standard curve: $y = 0.0185x - 0.0273$, $R^2 = 0.9968$).

Experimental design and statistical analysis: The experimental design was a randomized block design with four replications, with each experimental unit consisting of two pots with two plants per pot. Growth data, nutritional content and metabolic data were subjected to analysis of variance and regression, and the regression models were chosen based on the highest coefficients of determination, on the significance of the regression coefficients and using the *t*-test at 5% probability level. Statistical tests were performed using the SISVAR® software[22].

Results

Growth

The nitrogen doses evaluated modified aspects of the growth of *A. othonianum* Rizz., so that visual characters

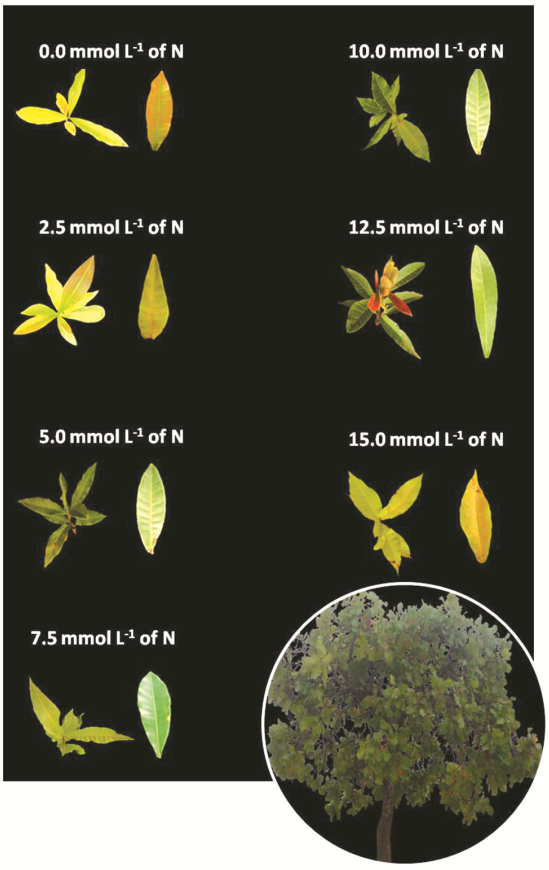

Figure 1. Visual characters observed in the aerial parts and leaves of 'caju-do-cerrado' tree seedlings (*Anacardium othonianum* Rizz.) grown under different doses (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mmol l^{-1}) of nitrogen in the nutrient solution.

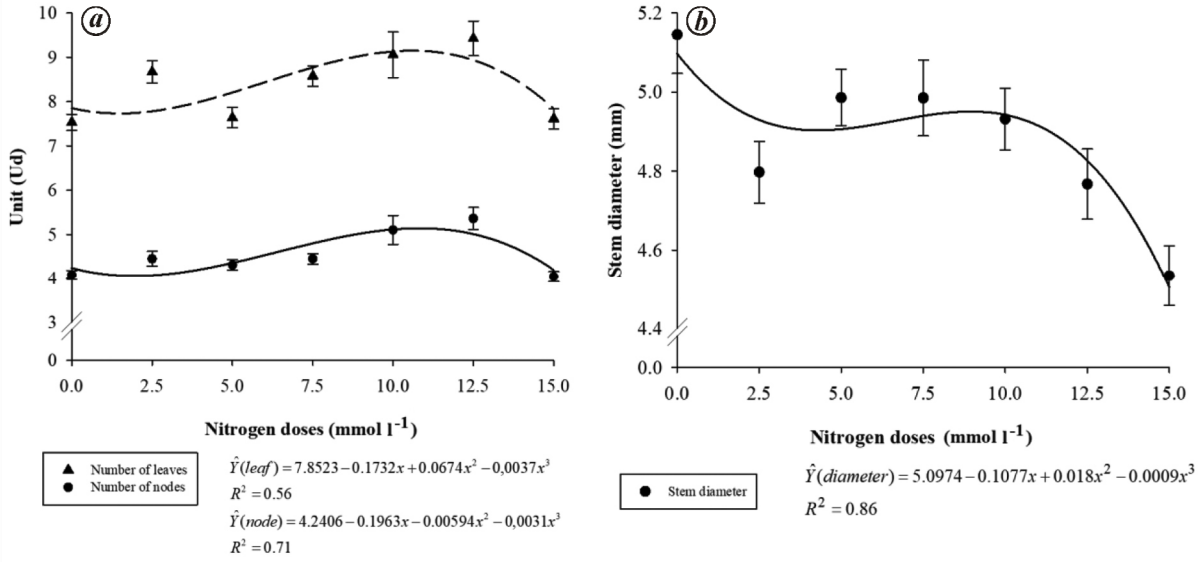

Figure 2. The number of leaves, number of nodes and stem diameter of 'caju-do-cerrado' tree seedlings grown under different doses (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mmol l^{-1}) of nitrogen in the nutrient solution.

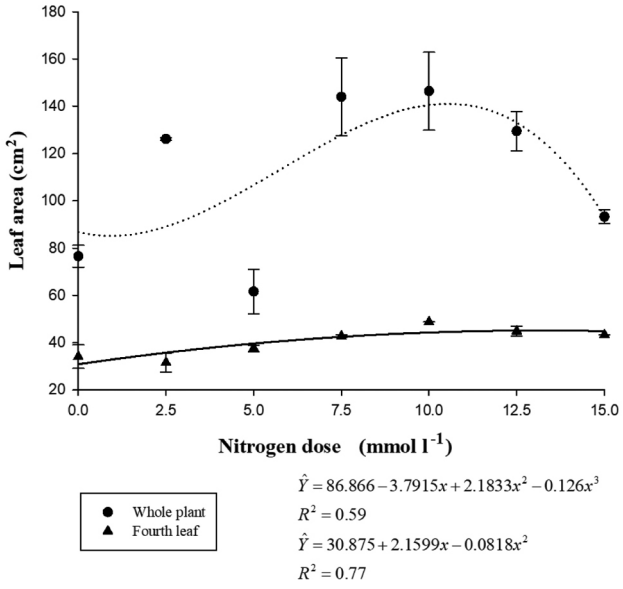

Figure 3. Leaf area (cm^2) of the plant and area of the fourth leaf of 'caju-do-cerrado' tree seedlings grown under different concentrations of nitrogen in the nutrient solution.

of deficiency and also of toxicity could be observed. Plants grown in the absence of N (0 mmol l^{-1}) or low availability of this nutrient (2.5 mmol l^{-1}) developed few leaves, which were small and chlorotic. The same was verified under the highest dose tested (15 mmol l^{-1}) (Figure 1).

Except for the stem and root lengths, which reached mean values of 11.19 and 10.95 cm respectively, the growth characteristics of *A. othonianum* Rizz. were influenced by different concentrations of N in the nutrient solution. The number of leaves and nodes reached maximum values of 9.18 units and 5.14 units respectively, when exposed to 10.7 and 10.8 mmol l^{-1} doses of N (Figure 2 *a*). The largest stem diameter value of 5.09 mm was observed at the dose of 0 mmol l^{-1} of N, and the smallest diameter of 4.49 mm was observed at the highest dose, viz. 15 mmol l^{-1} of N (Figure 2 *b*).

The leaf area of the whole plant and area of the fourth leaf were influenced by doses of N available for seedlings of *A. othonianum* Rizz. in the nutrient solutions (Figure 3). The highest average value of leaf area for the whole plant, 141.09 cm^2, was obtained at the dose of 10 mmol l^{-1} and the smallest area (86.86 cm^2) was observed at 0 mmol l^{-1} of N. The highest mean value of area for the fourth leaf (45.12 cm^2) occurred in plants exposed to 13.4 mmol l^{-1} of N. At higher doses, a decrease in the area of the fourth leaf was observed, and similar to the case of total leaf area, the lowest average area for the fourth leaf (30.87 cm^2) was found at 0 mmol l^{-1} of N.

Macronutrient content

The levels of nitrogen in the leaves and roots were influenced by the concentration of nitrogen in the nutrient solution (Figure 4 *a*). However, nitrogen content in the stem was not influenced by different doses of N, reaching a mean value of 3.64 g kg^{-1}. The maximum estimated nitrogen value in the leaves was 3.16 g kg^{-1} and in the roots it was 5.29 g kg^{-1}, at the estimated doses of 9.7 and 14.4 mmol l^{-1} N respectively (Figure 4 *a*).

The P levels in leaves and roots were affected by nitrogen doses (Figure 4 *b*). In the stem, P content was not influenced by the doses of N, with a mean value of 1.47 g kg^{-1}. The highest value of P in the leaves was

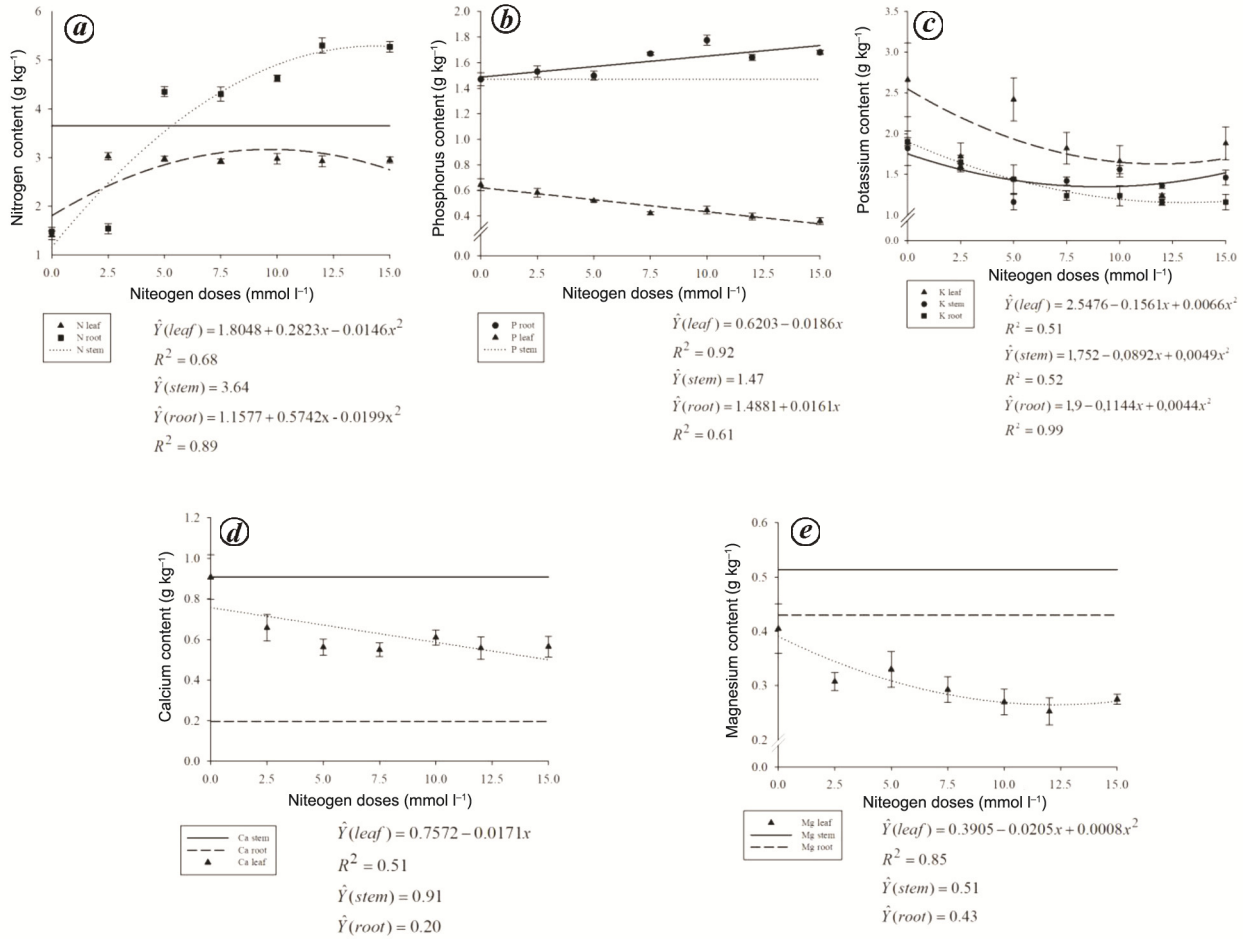

Figure 4. Macronutrient (N, P, K, Ca, Mg) content of 'caju-do-cerrado' tree seedlings grown under different doses (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mmol l^{-1}) of N in the nutrient solution. ***a***, N content; ***b***, P content; ***c***, K content; ***d***, Ca content; ***e***, Mg content of leaves, stems and roots.

0.62 g kg^{-1} at the dose of 0 mmol l^{-1} N, and in the roots it was 1.72 g kg^{-1} at the dose of 15 mmol l^{-1} N.

The different concentrations of N also influenced K content (Figure 4 *c*). The highest K levels were obtained in the absence of N in the solution (0 mmol l^{-1}), with 2.54, 1.75 and 1.90 g kg^{-1} in leaves, stems and roots respectively. The increase of N doses in the nutrient solution decreased the K levels at doses of 11.9, 9.1 and 13.0 mmol l^{-1} of N for the leaves, stems and roots respectively.

The Ca content in the leaves influenced the different doses of N in the nutrient solution (Figure 4 *d*). The highest estimated leaf calcium content was 0.75 g kg^{-1} at the dose of 0 mmol l^{-1} of N. Increasing the N doses in the nutrient solution decreased the Ca content in the leaves. The mean levels of calcium in the stems and roots were 0.91 and 0.20 g kg^{-1} respectively.

The Mg content of the stems and roots was not significantly modified by the different doses of N in the nutrient solution (Figure 4 *e*). The mean Mg levels obtained in the stems and roots were 0.51 and 0.43 g kg^{-1} respectively. However, Mg content in the leaves was influenced by the N doses, reaching the highest estimated value of 0.391 g kg^{-1} in the absence of N (0 mmol l^{-1}). The increase in the doses of N in the nutrient solution decreased the Mg content in the leaves up to the dose of 12.8 mmol l^{-1} of N.

The sulphur contents of leaves, stems and roots were not influenced by the different doses of nitrogen in the nutrient solution, reaching values of 0.02, 0.08 and 0.12 g kg^{-1} respectively.

Micronutrient content

The micronutrient levels of Fe, Mn, Mo, Cu and Zn were not significantly altered by the different doses of N in the nutrient solution in any of the plant parts evaluated. The mean values obtained for Fe, Mn, Mo, Cu and Zn were 571.18, 122.32, 0.13, 12.29 and 38.71 mg kg^{-1} respectively, in the leaves, 425.39, 53.61, 0.13, 16.89 and 43.43 mg kg^{-1} respectively, in the stems and 1210.32,

134.75, 0.14, 20.54 and 42.29 mg kg^{-1} respectively, in the roots.

Boron in the leaves was the only micronutrient influenced by the N doses (Figure 5). The highest estimated leaf B content was 113.13 mg kg^{-1} at the dose of 0 mmol l^{-1} of N. The mean levels of B in the stems and roots were 73.79 mg kg^{-1} and 79.26 Mg kg^{-1} respectively.

Nitrogen metabolism enzymes, nitric oxide and total sugars

The activities of the enzymes GS and NR, as well as NO and TSS levels affected concentration of N in the nutrient solution (Figure 6). The highest activity of GS was observed at the dose of 3.2 mmol l^{-1} of N, with 2.28 µM of λ-GH min^{-1} g^{-1} protein. At the dose of 15 mmol l^{-1} of N, the activity of this enzyme was only 2.03 µM λ-GH min^{-1} g^{-1} protein (Figure 6 *a*).

For NR, greater activity was observed between doses of 0 and 2.5 mmol l^{-1} of N, with 11.625 and 10.451 µmol min^{-1} g^{-1} respectively. Observing a tendency of decreased activity at doses above 2.5 mmol l^{-1} (Figure 6 *b*).

The NO content was influenced by the N doses in the nutrient solution. The highest concentration of this compound was verified in the absence of N (0 mmol l^{-1}), with 16.107 nmol g^{-1} MF. The lowest production was 0.56 nmol g^{-1} MF for 15 mmol l^{-1} of N (Figure 6 *c*).

The highest contents of TSS were found in the absence of N with 0.71% of polysaccharides, while the lowest carbohydrate contents were observed at the dose of 15 mmol l^{-1} of N, with production of 0.61% of total sugars (Figure 6 *d*).

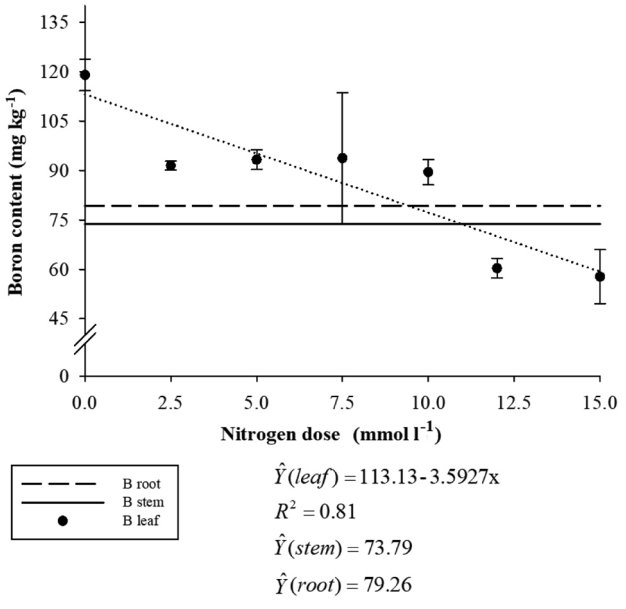

Figure 5. Boron content of leaves, stems and roots of 'caju-do-cerrado' tree seedlings grown under different doses (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mmol l^{-1}) of nitrogen in the nutrient solution.

Discussion

Growth

The increase of N concentration in the nutrient solution promoted increase followed by a decrease in the number of leaves and nodes, with increases at doses of 10.7 and 10.8 mmol l^{-1} of N and decreases at higher doses (Figure 2). This can be explained by a possible toxicity effect of excess nitrogen in the nutrient solution. One of the sources of nitrogen used in this study (in the proportion of 5% of the total supply) is ammonia. Several studies have demonstrated that many plants respond negatively to high NH_4^+ concentration showing a decrease in their growth[23,24]. These plants are classified as sensitive to NH_4^+ (ref. 25). Dias *et al.*[3] also observed a decrease in the number of leaves in *Psidium guajava* L. seedlings in doses higher than 770 mg dm^{-3} of N.

In plants of *A. othonianum* Rizz., an increase in the nitrogen dose in the nutrient solution also reduced the stem diameter, and its absence promoted an increase in the same. This can be explained as an adaptive response of this species, which, in an attempt to absorb nitrogen and make it available to the tissues, especially foliar tissues, in the absence of N, invested metabolically in the formation of conducting tissues. A similar effect also occurred in plants of *Calophyllum brasiliense* Cambèss, which showed reduced stem diameters with increasing doses of available N[26]. In *P. guajava* L., with seedlings subjected to doses higher than 667 mg dm^{-3} of N, a decrease of 0.62 mm in stem diameter was observed[3].

Fourth leaf area and total leaf were also affected by doses of N in the nutrient solution (Figure 3). Although there was a difference between the mean values, the plants subjected to doses higher than 10 mmol l^{-1} N showed a decrease in both areas. Reduction of the fourth leaf area was observed from the dose of 13.4 mmol l^{-1} of N. The increase in the leaf area of the crops has been related to the availability of nitrogen[27]. However, excess N can cause toxicity. There is a relationship between the optimal N supply and stimulation of leaf area expansion, and leaf area expansion can even be used for estimating the N assimilation requirements of plants. Moreover, there is a relationship between N and cytokinin because this plant hormone regulates cell growth and differentiation, and nitrate regulates the expression of isopentenyl transferase, essential for cytokinin synthesis[28].

Macronutrients content

The maximum nitrogen content was found in the roots. This is because roots are the plant organ directly responsible for the uptake of N present in the nutrient solution. The highest N uptake by the roots was observed in plants treated with a dose of 14.4 mmol l^{-1} of N. At higher doses,

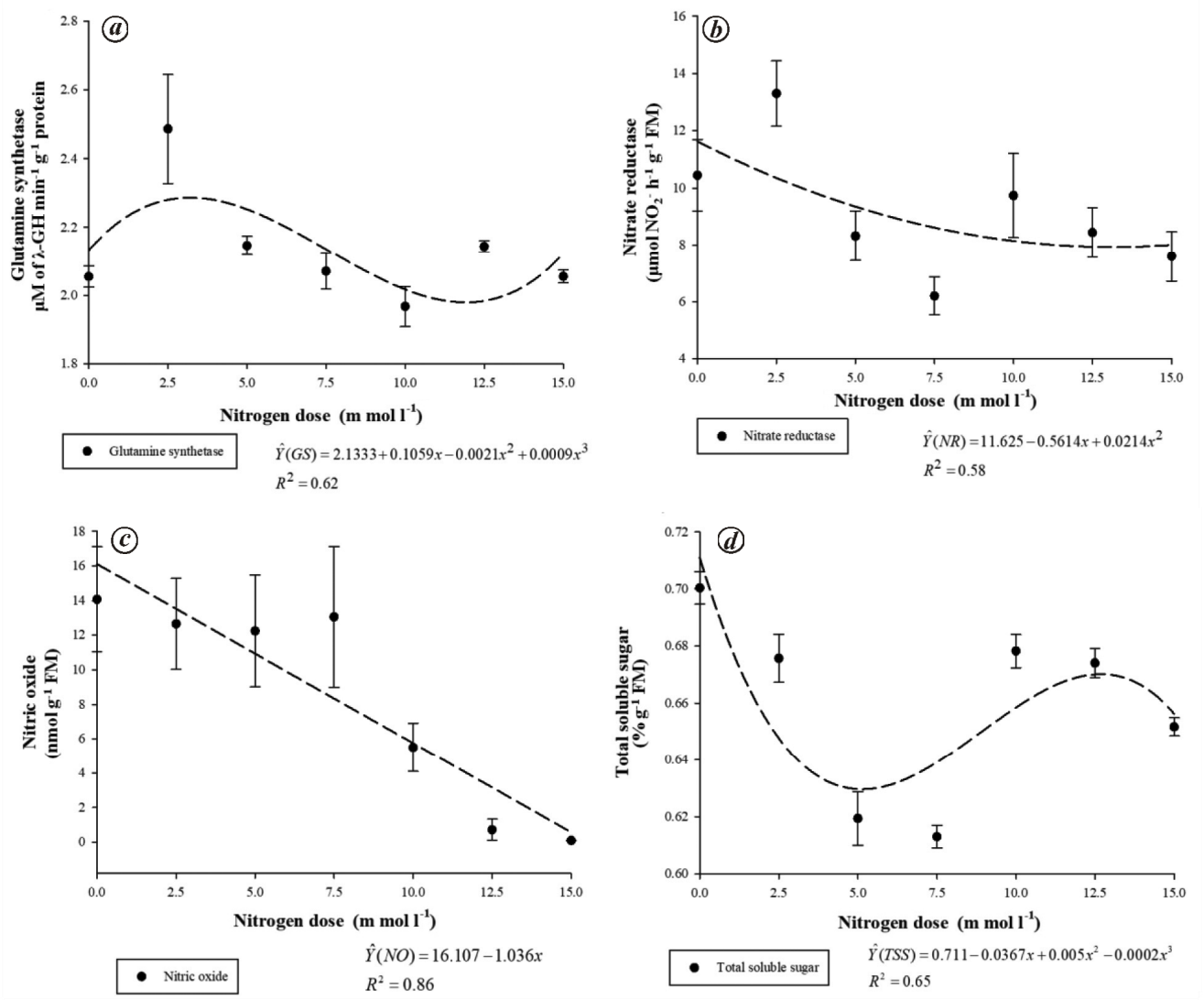

Figure 6. Activities of enzymes glutamine synthetase (GS) (μM λ-GH min^{-1} g^{-1} protein) and nitrate reductase (NR) (μmol NO_2^- h^{-1} g^{-1} FM), and nitric oxide (NO) (nmol g^{-1} FM) and total soluble sugar (TSS) contents of 'caju-do-cerrado' tree seedlings grown under different doses (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mmol l^{-1}) of nitrogen in the nutrient solution.

a decrease in N uptake by the roots was observed. This effect is an indication that doses of N above 14.4 mmol l^{-1} are excessive for *A. othonianum* Rizz. plants. Excess nitrogen in plants can affect growth and yield[29], making them more susceptible to diseases. In some fruit trees, application of this nutrient in high concentrations is common to promote increase of the mean weight. Determining the optimal concentration of N is important to avoid groundwater pollution and poor fruit quality[30]. In this work, in addition to roots, the leaves were influenced by different concentrations of N in the nutrient solution.

The phosphorus content of the seedlings of *A. othonianum* Rizz. was influenced by different doses of N available in the nutrient solution. This result is expected because P, together with N and K, is among the nutrients required in large quantities by the plants, given the global consumption of these fertilizers, which in 2012 was 109 Mt (million metric tonnes) of N, 41 Mt of phosphate (P_2O_5) and 29 Mt of K (K_2O)[31]. In addition, inorganic phosphate is a component of many cellular molecules that play an essential role in maintenance and structure, as well as in the primary and secondary metabolism[32]. However, for *A. othonianum* Rizz. plants, the more highly accumulated macronutrients are Ca and N, while P is only fourth in the accumulation scale[33]. The highest levels of K in leaves, stems and roots were observed in the absence of N in the nutrient solution. With the increasing doses of N, a decrease in K levels was observed. The high concentration of K in the absence of N can be explained by the need of the plants for adequate amounts of K^+ in the cytoplasm, since it is essential for N metabolism, especially for the incorporation of mineral nitrogen through

nitrate reductase[34]. Probably in *A. othonianum* Rizz., K^+ could be required for stomatal closure, NR metabolism and NO synthesis as a response to stress suffered by these plants in the absence of N in the solution. The potassium ion also plays an important role in the primary and secondary metabolism as well as in the regulation of cellular transport[32].

The highest Mg content in the leaves was observed in plants grown in the absence of N. An increase in N availability led to a decrease in the leaf Mg content. Magnesium is involved in nitrogen metabolism, being present at the centre of the chlorophyll molecule. A magnesium chelatase inserts Mg^{2+} into protoporphyrin IX, the tetraphyrrol precursor of chlorophyll[35]. In the leaves and fruits of *Musa paradisíaca* L. cv. D'Angola, nitrogen fertilization did not influence the accumulation of Mg, with mean values of 7.49 and 8.19 g plant^{-1} respectively[36].

In the seedlings of *A. othonianum* Rizz., only Ca content in the leaves was influenced by the nitrogen doses. In *M. paradisíaca* L. cv. D'Angola, no plant organ was influenced by different concentrations of N[36]. In the present study, highest calcium content was observed in the stems of *A. othonianum* Rizz. This may have occurred due to redistribution of calcium in the phloem, since it is considered a low-mobility nutrient[29].

N doses did not affect the sulphur content in leaves, stems and roots of *A. othonianum* Rizz. seedlings. Sulphur is present in the amino acids cysteine and methionine, and is a component of enzymes involved in nitrogen metabolism, including nitrate and nitrite reductases. The positive relationship between N and S with increasing plant biomass and yield has been discussed in other studies[37,38].

Micronutrients content

The levels of micronutrients, including Fe, Mn, Mo, Cu and Zn, in the leaves, stems and roots of *A. othonianum* Rizz. seedlings were not influenced by the doses of N present in the nutrient solution. The effects of nitrogen application on the micronutrients content of fruit tree species have not been widely studied. However, in cereal species, including *Oryza sativa* L., application of nitrogen fertilizers has been shown to increase Fe, Zn, Cu and Mn levels in leaves, stems and grains[39].

The B content of leaves decreased with the increase of N concentration available to the seedlings. In contrast, the B content of stems and roots was not influenced by the doses of N in the nutrient solution. This result was not expected because boron is a micronutrient that plays an essential role in plant development, and the combination of N and B can stimulate the growth of plants, as observed in the fruits of *Carica papaya* L., in which fertilization with both nutrients promoted increased fruit diameter and length[40]. In contrast, in plants of *Brassica juncea* (L.) Czern, application of nitrogen promoted increase in biomass but did not affect boron uptake[41].

Nitrogen metabolism enzymes and nitric oxide

In the present study, the activities of nitrogen metabolism enzymes, including GS and NR, influenced different doses of N in the nutrient solution for *A. othonianum* Rizz. seedlings. In the absence of N, the activities of these enzymes were detected, even if at low levels. The activities of these enzymes were not expected under this condition; however, NR could be acting on nitrate originating from amino acid catabolism. Likewise, GS could be acting on glutamate derived from protein degradation. Studies have shown that, in situations of salt and water stress, GS activities tend to increase due to increase in protease activity and amino acid catabolism[42]. This effect was detected in plants of *A. occidentale* under salt stress conditions by Viégas *et al.*[43].

The plants subjected to a dose of 2.5 mmol l^{-1} of N showed highest activities of both GS and NR, indicating that the initial supply of N in the solution at low concentrations may have been enough to stimulate N reduction to nitrite and condensation of glutamate into ammonia. The main inorganic source of N absorbed by higher plants is NO_3^-, and its reduction to NO_2^- is catalysed by NR. However, GS is responsible for N recycling, assimilating ammonium released by various metabolic processes of the plant[44]. Studies have shown that nitrate functions as a signalling molecule in the expression of NR genes, and that the supply of nitrate in plants grown in the absence of N stimulates the induction of NR expression[45]. Thus, enzymes, including NR and GS, are essential in nitrogen assimilation and metabolism (Figure 7). Nitrogen metabolism comprises of a complex network of sugars, organic acids, amino acids and other chemical substances[32]. At doses higher than 2.5 mmol l^{-1} of N, a reduction in GS activity was observed in the leaf tissues of *A. othonianum* Rizz., which may indicate that it is an ammonia-sensitive plant. The concentration of ammonia in the total N provided triggered toxicity effects from 2.5 mmol l^{-1}, since in NH_4^+-tolerant plants, there is generally a higher activity of GS and less free accumulation of NH_4^+ in the tissues[46]. The sensitivity of *A. othonianum* Rizz. to ammonia is also signalled by the depletion of potassium in the tissues, as N is supplied to the seedlings. Ammonia competes with K^+ for absorption cell sites[29].

Plants of *A. othonianum* Rizz. grown in the absence of N had the highest NO content. It is possible that the absence of N in the nutrient solution stimulated the activation of metabolic pathways related to stress, thus increasing NO synthesis. In the mitochondria of *Arabidopsis thaliana*, NO levels are controlled by the external influence of NAD(P)H dehydrogenase and activity of the alternative oxidase[47]. The same process may occur in *A.*

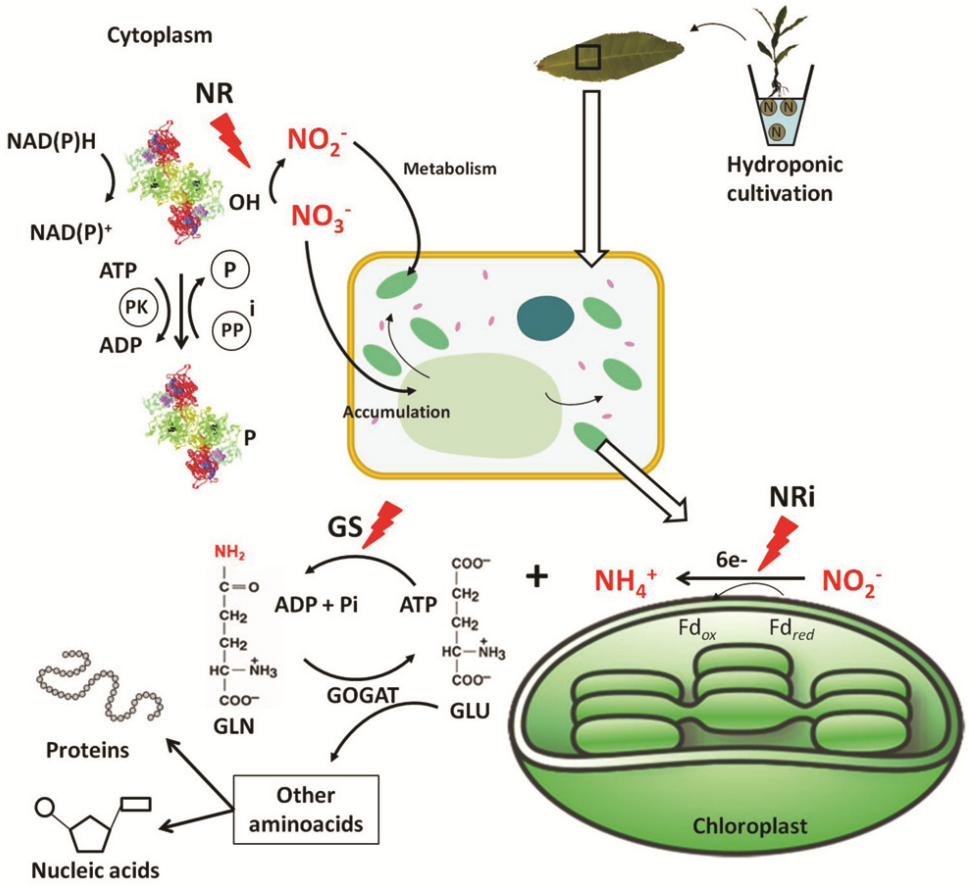

Figure 7. Representation of nitrate assimilation in the leaves of *A. othonianum* Rizz. From the solution, roots absorb nitrate (NO_3^-), which is transported to the cytosol of the plant cell by transporters. Excess concentrations of nitrate can be accumulated in vacuoles. In the cytosol, NO_3^- is reduced to nitrite (NO_2^-) by the enzyme nitrate reductase (NR), which is activated by dephosphorylation and using NAD(P)H as the electron donor. Nitrite is rapidly transported to the chloroplast stroma. Using the energy supplied by six ferredoxins (Fd), nitrite reductase (NiR) converts nitrite to ammonium (NH_4^+). Ammonium then combines with glutamate (GLU) to form glutamine (GLN), using energy from the hydrolysis of ATP and action of glutamine synthetase (GS). Other amino acids are formed from glutamine and can then be used in the structure of proteins and nucleic acids.

othonianum Rizz., in which the possible activation of the alternative oxidase may play a role in the conversion of nitrite to NO in the absence of N in the nutrient solution. Nitric oxide is considered a biological messenger, playing an important role in the regulation of various physiological processes in plants, including growth, development and responses to biotic and abiotic stress factors[48]. However, the lowest NO synthesis was observed in plants subjected to a higher dose of N, viz. 15 mmol l^{-1}, indicating that high concentrations of N do not induce the production of reactive nitrogen species, including NO, in *A. othonianum* Rizz. plants, and it is possible that other stress pathways are activated under conditions of toxicity from excess N.

In this study, the absence of N induced the highest synthesis of soluble sugars. This may have occurred because plants are able to adapt to different C/N conditions through the specific partitioning of C and N sources, and the cellular adjustment to their availability[49]. Such behaviour was also observed by Brunetto *et al.*[50] in *Vitis vinifera* buds, with a decrease of soluble carbohydrates at all doses of N offered. Loaiza *et al.*[51] also observed a negative effect of increase in the application rate of N on the content of soluble carbohydrates in *Lolium perenne* L. Neumann *et al.*[52] verified a linear decrease in the carbohydrate content present in maize for silage, as the availability of N for the crop increased. These data confirm that the synthesis of soluble sugars can be affected by different concentrations of N in the growth substrates.

Conclusion

Symptoms of excess N, including a reduction in the number of leaves and in the leaf area of the whole plant and of the fourth leaf, were observed. Activity peaks for NR and GS were found at the dose of 2.5 mmol l^{-1} of N, but doses higher than thus negatively affected the activity of

these enzymes, so that the plants of *A. othoniaum* Rizz. showed sensitivity behaviour to ammonia. Similarly, concentrations of NO and soluble sugars were also affected by the increase in available N, being the highest concentrations for these compounds, observed in the seedlings submitted to the absence of N.

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