

## Biochemical response of Tachi-branco (*Tachigali vulgaris* L.G. Silva & H.C. Lima) in function of sources and doses of nitrogen

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### Abstract

This study aimed to evaluate the effects of application of nitrogen sources and doses in the biochemical response of seedlings of *Tachigali vulgaris* L. G. Silva & H. C. Lima. The seedlings were grown in pots containing 3 dm<sup>3</sup> of soil. Nitrogen sources such as urea [(NH<sub>2</sub>)<sub>2</sub>CO], ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>), ammonium sulfate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>] and calcium nitrate [Ca (NO<sub>3</sub>)<sub>2</sub>] were tested in five doses of 0, 75, 150, 225 and 300 mg dm<sup>-3</sup>. They were applied as a solution in four portions, 25, 50, 75 and 100 days after transplanting. The experimental unit consisted of a vase and a randomized block design (RBD) in factorial scheme (4 × 5), corresponding to 4 sources and 5 doses, with four replications, totally 80 vessels. The biochemical analyzes were performed at the end of the experiment and nitrate levels, nitrate reductase enzyme activity, free ammonium, total soluble amino acids, total soluble proteins, carbohydrates, sucrose and chlorophylls were determined. There was a reduction in the concentration of protein in leaf and root, with the application of ammonium nitrate and calcium nitrate. The conversion of nitrate into the root in amino acids shown to be dependent to the oxidation of carbohydrates, causing the reduction of the concentration of this solute increases with the supply NO<sub>3</sub><sup>-</sup>. The results showed that for production of *Tachigali vulgaris* seedlings the dose of 150 mg dm<sup>-3</sup> of N, applied in a piecemeal manner, at 25, 50, 75 and 100 days of transplanting with the ammonium nitrate fertilizer can be recommended.

**Keywords:** Forest species, Organic solutes, Photosynthetic pigments.

**Abbreviations:** ERO\_ Reactive oxygen species, DM\_ dry matter.

### Introduction

The Tachi-branco (*Tachigali vulgaris* L. G. Silva & H. C. Lima, Leguminosae Caesalpinioideae) is a species that gathers promising characteristics for energy plantations in the Brazilian Amazon and bordering regions (Carpanezzi et al., 1983). Although the available information is scarce, its wood is considered to be good characteristics for firewood and charcoal (Ducke, 1949), since it has characteristics similar to eucalyptus wood, such as the yield of weight and volume in the carbonization process and higher calorific value of charcoal (Vale et al., 2002). In addition to this, there are high values of annual increase, reaching 2.5 m year and 3.4 cm year in height and diameter, respectively, in full sun (Yared, 1990). It is estimated a rotation of 5 to 10 years for plantations with energetic purposes. The production of forest seedlings in quality and quantity is one of the most important phases for the establishment of forest stands with native species. It is conditioned by the use of quality seeds, appropriate cultivation substrates and adequate fertilization, (Scheer et al., 2010). The commercial substrates do not always provide satisfactory amounts of nutrients, and when enriched with fertilizers their efficiency is increased. Associated with the type of container and substrate, the application of nutrients, especially macronutrients in the

growth of the seedlings, has aroused the interest of several forest researchers. Several works have been conducted to understand the best form of fertilization in forest species for correct recommendation. The use of chemical fertilizers is; therefore, a usual practice in forest nurseries, functioning as a valuable tool in controlling the size and vigor of seedlings (Ribeiro et al., 2001). As a key component of many macromolecules, including proteins and nucleic acids, nitrogen is essential for normal plant growth and development (Sinfield et al., 2010). In plant metabolism, it participates as a constituent of proteins, coenzymes, nucleic acids, cytochrome, chlorophyll, pigments and by-products (Silva et al., 2011). When there is a deficiency of this nutrient in the growth substrate, it is translocated from the older leaves to the younger parts of the plant. In addition, it affects leaf initiation and expansion rates, leaf size and leaf senescence intensity (Schroder et al., 2000). Despite the importance of this nutrient (Davies, 2000), there are few studies on plants involving this chemical element. The nitrogen (N) in ammoniacal or nitric form influences several physiological and biochemical processes in plants, such as photosynthesis and enzymatic activities of N metabolism (Borgognone et al., 2013). Development and morphology

(Zhu et al., 2000) depend on species and plant variety (Lasa et al., 2002). In many production systems, nitrogen availability is almost always a limiting factor, influencing plant growth more than any other nutrient (Bredemeier and Mundstock, 2000). Thus, it has been sought to reduce soil nitrogen losses, given its importance and high mobility to improve the absorption and metabolization of N within the plant, maximizing the efficiency of its use. The current scientific research is focused on the economic use of fertilizers, being of the utmost importance to the evaluation of factors that interfere in the processes of the nitrogen cycle as well as the factors associated to the technology of use of the nitrogen sources, to minimize the losses of the soil-plant (Cantarella, 2007). There is a permanent need for actions that lead to a substantial increase in the efficient use of nitrogen. However, it mainly depends on actions that allow greater technological advances for greater efficiency of nitrogen fertilizations. This is fundamental when it is sought to increase productivity with sustainability (Lopes et al., 2007). The present work aimed to evaluate the effects of the application of different sources and nitrogen rates on the biochemical response of *Tachigali vulgaris* L. G. Silva & H. C. Lima.

## Results

### Nitrate concentration

The nitrate concentration showed a quadratic adjustment in response to levels of nitrogen sources, ammonium nitrate and calcium nitrate in leaf and ammonium sulfate and ammonium nitrate in the root (Fig.1). In leaf, The nitrate concentration showed similar behavior to N supply, more ammonium nitrate and nitrate, ranging from only 12.4% in the mean calcium nitrate (0.13 mmol NO<sub>3</sub><sup>-</sup>/kg DM) and ammonium (0.11 mmol of NO<sub>3</sub><sup>-</sup>/kg DM), demonstrating the presence of NH<sub>4</sub><sup>+</sup>. In the present study, it did not affect the accumulation of nitrate in leaves. In root, quadratic adjustments were observed; however, with the concavity of the curve different, showing that the maximum nitrate concentration would be observed at higher nitrogen levels than those studied to ammonium nitrate and ammonium sulfate. The other sources were not significant for the adjustment of the regression model ( $p > 0.05$ ).

### Activity of reductase of nitrate

The activity of reductase of nitrate in leaf showed some variation, considering the mean values, ranging from 0.34 to 0.17 μmol de NO<sub>2</sub><sup>-</sup> g<sup>-1</sup> h<sup>-1</sup> with a incremente of dose 0 to 300 mg dm<sup>-3</sup> of nitrogen, or the maximum dose of N has a negative effect on the enzyme activity as shown in (Fig. 2). Through the regression equation we found that the maximum value for the activity of RNO<sub>3</sub><sup>-</sup> in leaf is 1.31 μmol of NO<sub>2</sub><sup>-</sup> g<sup>-1</sup> h<sup>-1</sup> by providing dose of 170 mg dm<sup>-3</sup> of N. From this dose, the increase in the supply of nitrogen promoted decrease of enzyme activity. The activity of nitrate reductase in the root (Fig. 2) showed a quadratic effect and increased with increasing N doses, using ammonium nitrate, confirming that the presence of NO<sub>3</sub><sup>-</sup> soil favors the activity of the enzyme.

### Free ammonium

It was possible to adjust the regression models for all tested nitrogen fertilizer sources, for the free ammonium variable in the roots (Fig. 3). As observed in leaf, ammonium nitrate also presented positive adjustment to the linear model. The urea,

ammonium sulphate and calcium nitrate showed quadratic effect of ammonium accumulation depending on the doses of N. The maximum dose accumulation found for the sources that showed quadratic effect such as 261.38 (171.30 mmol of NH<sub>4</sub><sup>+</sup> kg<sup>-1</sup> DM), 242 (98.41 mmol of NH<sub>4</sub><sup>+</sup> kg<sup>-1</sup> DM) and 278.17 mg dm<sup>-3</sup> (70.12 mmol of NH<sub>4</sub><sup>+</sup> kg<sup>-1</sup> DM) for urea, ammonium sulfate and calcium nitrate, respectively. However, the nitrate concentrations were also higher in the roots than in the leaves, which did not happen for *T. vulgaris* seedlings, where the maximum concentration of NO<sub>3</sub><sup>-</sup> in leaf (0.201 mmol of NO<sub>3</sub><sup>-</sup> kg<sup>-1</sup> DM) was bigger than the maximum concentration in root (0.063 mmol de NO<sub>3</sub><sup>-</sup> kg<sup>-1</sup> DM). These results may indicate that the sheets are the preferred sites of reduction of NO<sub>3</sub><sup>-</sup>, because the reductase activity of the nitrate enzyme is relatively high, which explains the higher nitrate concentration of the leaves in relation to the concentration of the roots.

### Total soluble aminoacids

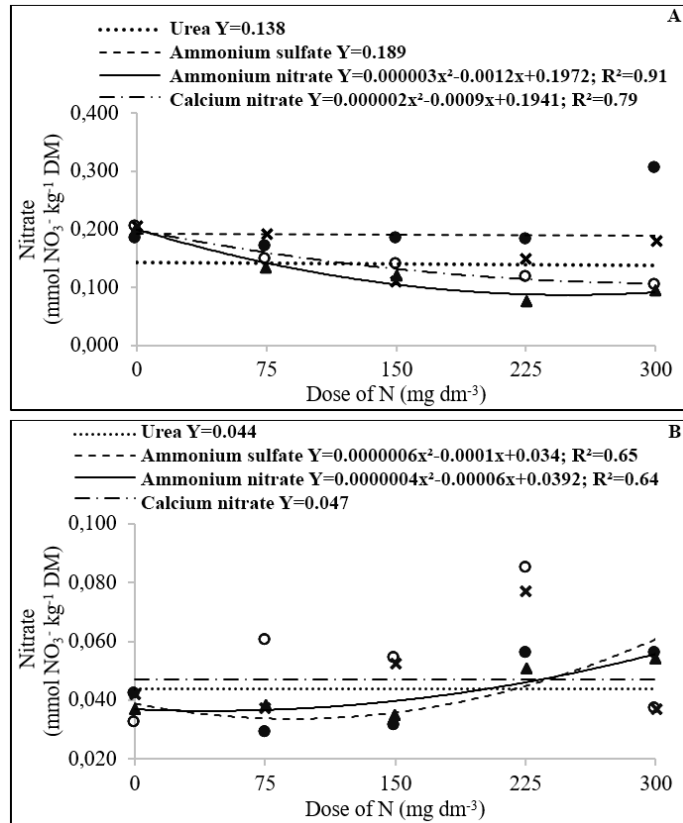
In leaves, the ammonium sulfate and ammonium nitrate sources provided adjustment of the quadratic model as a function of N levels and calcium nitrate source set to the positive linear model. As for urea, there was no significance ( $p > 0.05$ ) for adjusting the regression model (Fig. 4). For the accumulation of aminoacids in the root, urea, ammonium sulfate and ammonium nitrate were significant for the adjustment of the regression model, which is quadratic in the three sources.

### Total soluble proteins

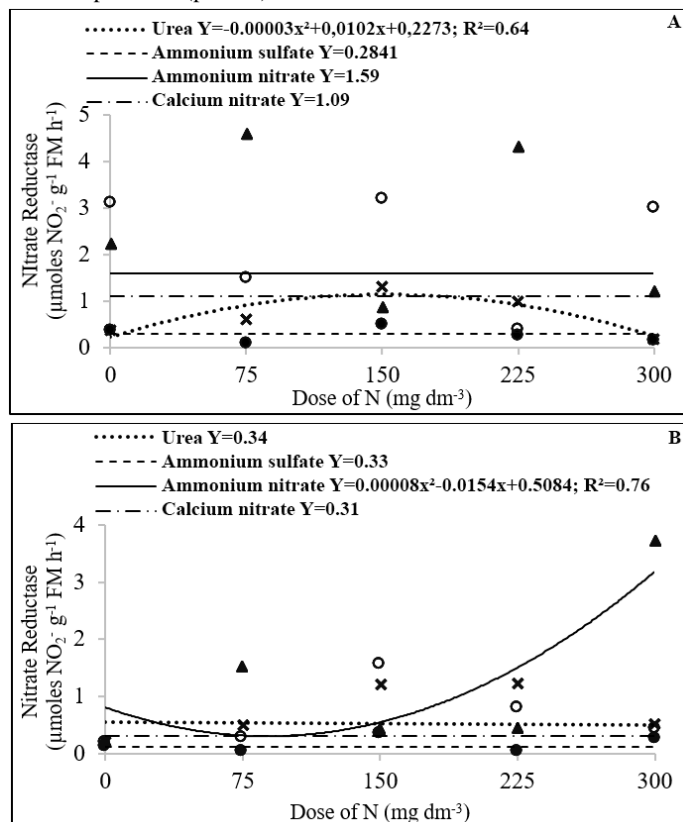
The data of protein accumulation in sheet for the significant interaction of the factors (F x D) ( $p \leq 0.05$ ), adjusted to the quadratic regression model according to the N levels, with a tendency to decrease due to the increase of the dose, only for ammonium nitrate and calcium nitrate (Fig. 5). In the case of protein content in the root, the main effect of doses showed significance for setting the regression equation to curve following the same trend observed for the sheet.

### Carbohydrates

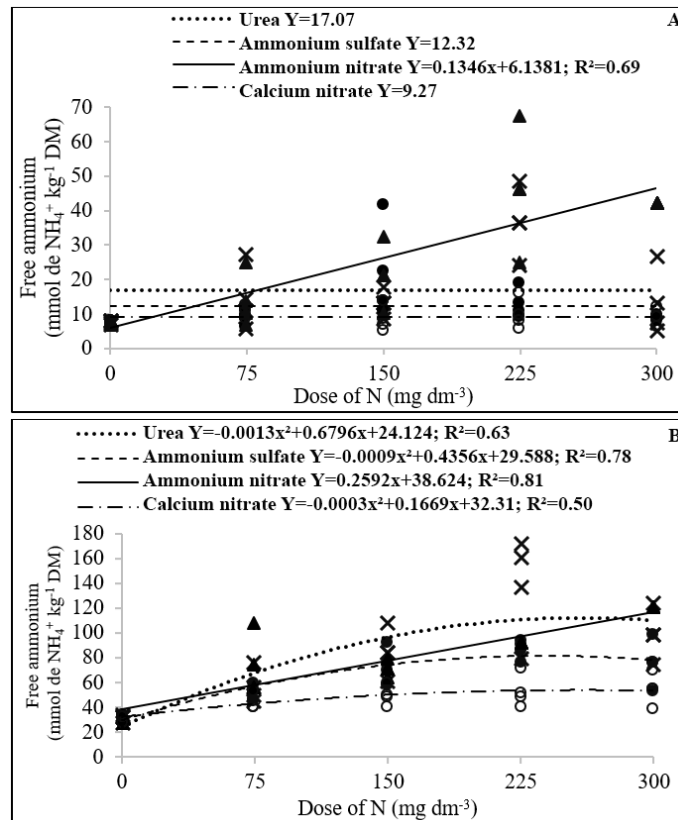
For the accumulation of carbohydrates in the leaf, there was a significant effect for the interaction between factors (F x D). Regarding the sources urea and ammonium nitrate, a quadratic effect with a tendency to decrease the accumulation of carbohydrates with increasing doses and negative linear effect on calcium nitrate was observed. It depends on the N rates as shown in (Fig. 6). The N rates of ammonium sulfate showed no significant response correspond to accumulation of carbohydrates in the sheet to fit the regression model ( $p > 0.05$ ). The analysis of the total content of soluble carbohydrates in the roots (Fig. 6) showed decreased content due to the increase of N rates for the application of urea, similar to behavior observed in the leaves. There was negative linear effect for this source in the setting of the regression model. The other sources were not significant for the adjustment of the equation ( $p > 0.05$ ). Comparing treatment without fertilizer and the one with the highest dose (300 mg dm<sup>-3</sup>), the contents of total carbohydrates decreased approximately 1.6 times, reaching 0.37 mmol of GLU g<sup>-1</sup> DM, while the seedlings without nitrogen showed 0.60 mmol de GLU g<sup>-1</sup> DM.



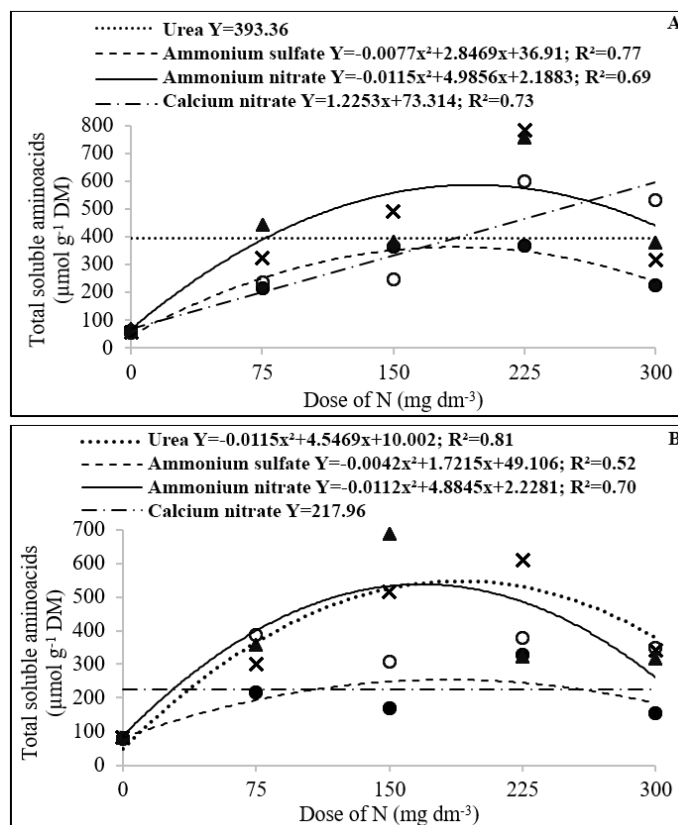
**Fig 1.** Concentration of nitrate accumulated in leaves (A) and roots (B) of *T. vulgaris* seedlings, respectively, according to the sources and nitrogen at the end of the experiment ( $p \leq 0.05$ ).



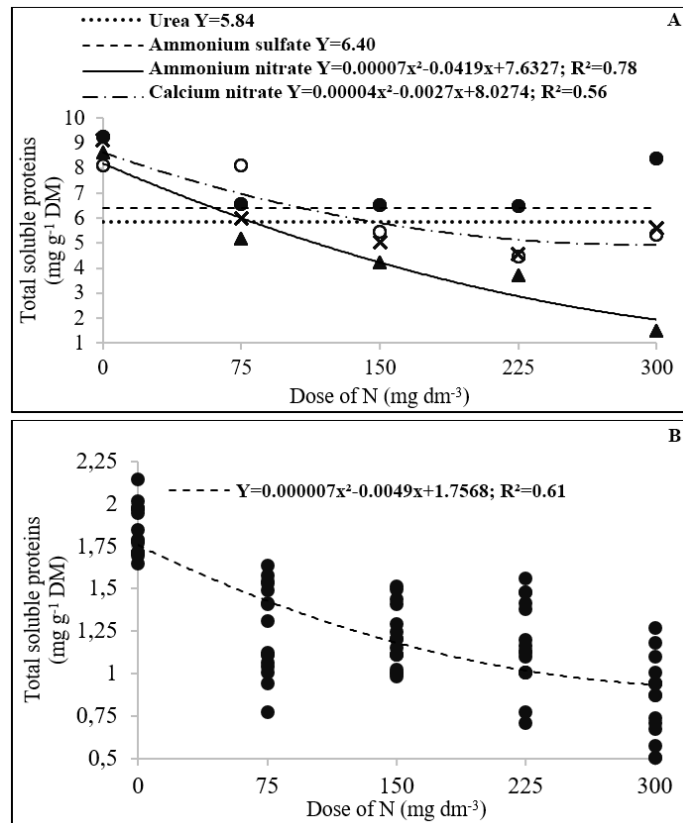
**Fig 2.** Nitrate reductase enzyme activity in leaves (A) and roots (B) of *T. vulgaris* seedlings (fresh matter - FM), respectively, according to the sources and nitrogen at the end of the experiment ( $p \leq 0.05$ ).



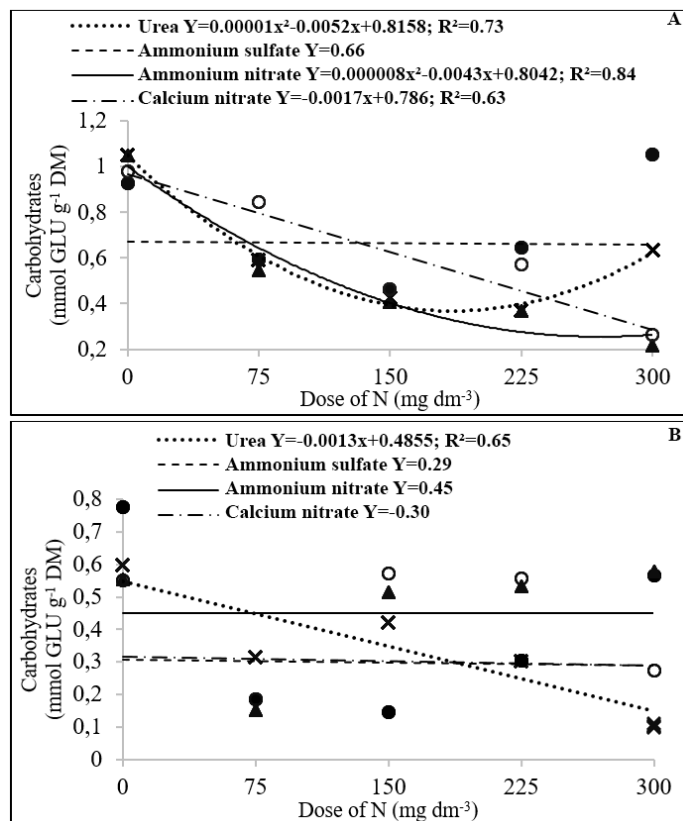
**Fig 3.** Accumulated free ammonium concentration in leaves (A) and roots (B) of *T. vulgaris* seedlings, respectively, according to the sources and doses of nitrogen at the end of the experiment ( $p \leq 0.05$ ).



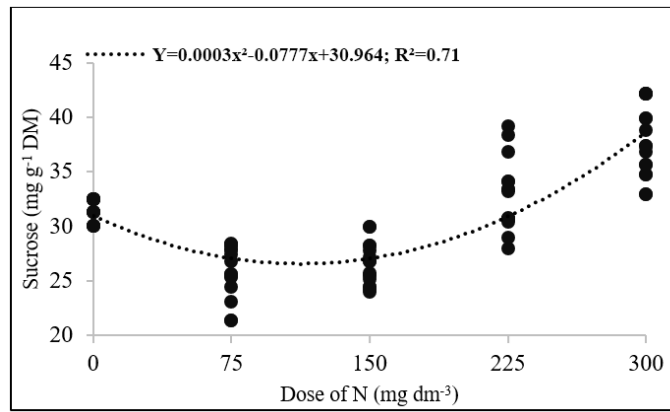
**Fig 4.** Total soluble aminoacids concentration in leaves (A) and roots (B) of *T. vulgaris* seedlings, respectively, according to the sources and doses of nitrogen at the end of the experiment ( $p \leq 0.05$ ).



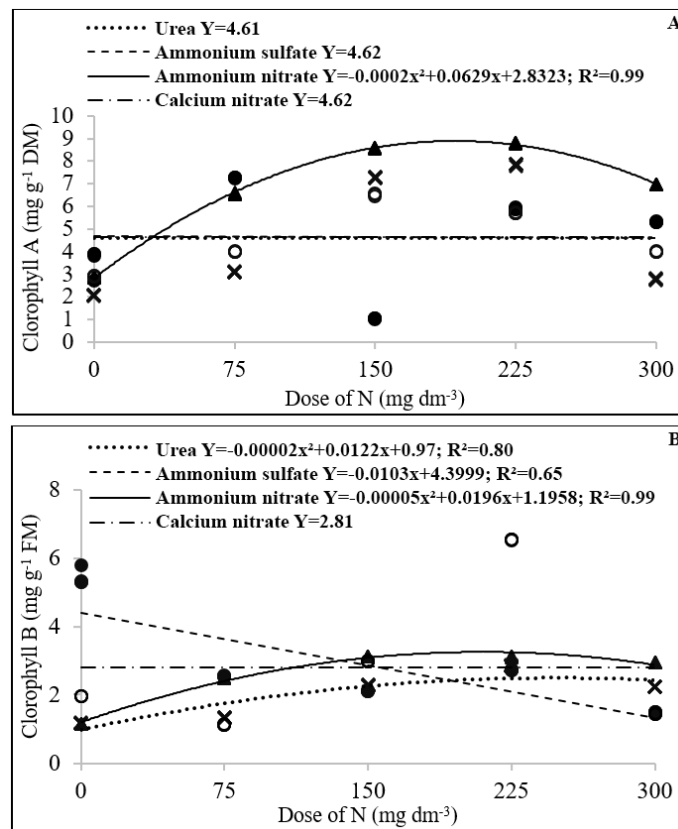
**Fig 5.** Concentration of soluble proteins in leaves (A) of *T. vulgaris* seedlings as a function of nitrogen sources and doses, and in roots (B) as a function of nitrogen doses, at the end of the experiment ( $p \leq 0.05$ ).



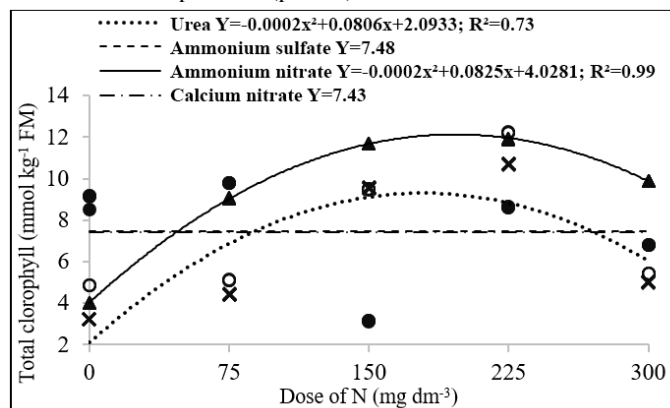
**Fig 6.** Concentration of carbohydrates in leaves (A) and roots (B) of *T. vulgaris* seedlings, respectively, according to the sources and doses of nitrogen at the end of the experiment ( $p \leq 0.05$ ).



**Fig 7.** Concentration of sucrose in leaves of *T. vulgaris* seedlings as a function of nitrogen doses at the end of the experiment ( $p \leq 0.05$ ).



**Fig 8.** Chlorophyll content A (A) and B (B), respectively, in leaves (fresh matter – FM) of seedlings of *T. vulgaris* according to the sources and doses of nitrogen at the end of the experiment ( $p \leq 0.05$ ).



**Fig 9.** Content of total chlorophyll in leaves (fresh matter – FM) of seedlings of *T. vulgaris* according to the sources and doses of nitrogen at the end of the experiment ( $p \leq 0.05$ ).

## Sucrose

For the sucrose content of leaves, the interaction between nitrogen sources and doses was not significant ( $p>0.05$ ). However, the main effect of N rates was verified on the accumulation of sucrose in the leaves (Fig. 7). The N provided the maximum accumulation of sucrose up to 300 mg dm<sup>-3</sup>.

## Chlorophylls A and B

The N dose of 157.25 mg dm<sup>-3</sup> by applying ammonium nitrate caused the highest values of *Chla*. Under this treatment the *Chla* content reached to 7.78 mg g<sup>-1</sup> DM, which is an increase of about 170% compared to the treatment without fertilization, which showed approximately 2.88 mg g<sup>-1</sup> DM of *Chla* in average. For chlorophyll content B (*Chlb*), there was a quadratic effect on the basis of urea and ammonium nitrate doses and negative linear effect for the doses of ammonium sulfate. The regression model for calcium nitrate showed no significance ( $p>0.05$ ). The application of ammonium sulfate indicates that the fertilizer in question adversely affected *Chlb* levels. Doses of maximum efficiency with urea ammonium nitrate sources and were of 227 (2.83 mg g<sup>-1</sup> DM) and 196 mg dm<sup>-3</sup> (3.13 mg g<sup>-1</sup> DM), respectively.

## Total chlorophyll

The effect of N rates tested on the total chlorophyll content (*Chlt*) was set in the quadratic model for urea and ammonium nitrate, indicating that the species responded positively to the application of N for this variable. Similar behavior was found for contents of *Chla* and *Chlb* for most of the sources of N. The maximum efficiency doses found to *Chlt* were 201.5 (10.71 mg g<sup>-1</sup> DM) and 206.25 mg dm<sup>-3</sup> (11.91 mg g<sup>-1</sup> DM), for urea and ammonium nitrate, respectively (Fig. 9). There was no significant effect on N doses of ammonium sulphate and calcium nitrate on this variable.

## Discussion

The absence of fertilization or the addition of N at high doses has been considered limiting, causing changes in the metabolism of the plants during the evaluation period.

For the activity of nitrate reductase enzyme (RNO<sub>3</sub><sup>-</sup>), the interaction between nitrogen sources and doses was significant ( $p\leq 0.05$ ) (Table 3). The leaf was responded to urea while root to ammonium nitrate. No significant interaction between factors (F × D) in the setting of the regression model was observed other sources in leaf and root ( $p>0.05$ ).

Application of nitrogen, with largest experimental doses (225 and 300 mg dm<sup>-3</sup>) of urea, may have caused increased excretion of H<sup>+</sup> ions by roots of *T. vulgaris* seedlings. Consequently, acidification of the soil (pH) and decreased availability of molybdenum may have been occurred. This acidification observed for absorbing NH<sub>4</sub><sup>+</sup> can affect the availability and the biological activity of various nutrients in the root zone (Vian and Kiehl, 2010). The concentration of ammonium in leaves showed a positive linear adjustment only for the ammonium nitrate, providing NO<sub>3</sub><sup>-</sup> more than NH<sub>4</sub><sup>+</sup>. Increasing N rates can elevate ammonium concentration in leaves (Fig 3). The other sources were not significant for the adjustment of the regression model ( $p>0.05$ ). However, it is observed that the application of urea increased this variable higher than the other sources, when

the average ammonium accumulation for each source analyzed.

There are various symptoms caused by excess ammonia, which may occur in various plants. Some of them symptoms are reduced plant growth, short and stunted roots (Jampeetong et al., 2012), chlorosis, necrosis, stunting and wilting of leaves and brown color of root and stem, showing signs of senescence (Neuberg et al., 2010).

The increased concentration of free amino acids occurs primarily as a result of the increase in the biosynthesis of these compounds (Oliveira et al., 2006). Furthermore, protein degradation allows the recycling of free amino acids, leading to increase concentration of these compounds (Beltrão et al., 2007). In this study, we also observed the increase in the total free amino acid content and reduced total soluble protein content. It is believed that the ROS cause damage to the photosystem by the fact that inhibits protein synthesis and consequently D1 synthesis, the major protein of PSII (Silva et al., 2011). It explains that the increase in protein content as a function of N can be directly related to the synthesis of structural and enzymatic proteins, which are responsible for synthesis of other proteins and metabolic intermediates that plant can store them for use upon their metabolic needs. However, for *T. vulgaris* species, decrease of the protein content indicates a similar behavior of plants under oxidative stress. It may be related to production of ROS and; therefore, damage to the photosystems denaturation and inhibition of protein synthesis. Carbohydrates regulate the rate of CO<sub>2</sub> fixation and /or synthesis or hydrolysis of starch both in the chloroplast (Gorai, 2010). In general, the carbohydrate content is used as a parameter to identify abiotic stress nature, such as water stress. According to (Lyse et al., 2012) under water stress of the willing carbohydrates in the cytoplasm, they are used in the activation of drought tolerance mechanisms, regardless of the mechanism in question. Usually in plants under stress, it is common to increase the accumulation of carbohydrates in the leaf and root. However, according to the results found in this study, with reduction of carbohydrate content in function of N doses (Marschner, 2012), nitrogen alters the composition of plants much more than any other nutrient and simultaneously to increase in nitrogen concentration is drastic reduction of the amount of reserved carbohydrates. The increase in carbohydrate content may also occur at seedlings without fertilization and fertilization with the highest dose was 30%. Sucrose was recognized as the main sugar to act in maintaining cell turgor and regulating the osmotic adjustment (Farrant et al., 2009). Regarding the chlorophyll A content (*Chla*), only the application of ammonium nitrate provided significant effect to the adjustment of the regression model through regression analysis. There is the quadratic effect on levels of *Chla* depend on the N levels, resulting in the decrease of the curve near the higher fertilizer doses (Fig 8), which could be viewed from the unfolding of the interaction.

The use of N in chlorophyll production can be a plant's strategy to ensure adequate capture of light energy by photosystems (Carneiro et al., 2015). Leaves with a high nitrogen content have higher chlorophyll content, which results in improved photosynthetic capacity (Silva et al., 2011).

## Materials and methods

### Site description and substrate preparation

The experiment was conducted in a greenhouse of the Institute of Agricultural Sciences at the Federal Rural

University of Amazonia in Belém, Pará, in the period from April to July 2015. The soil used as substrate was yellow latosol. It was characterized by analysis conducted by the Instituto Brasileiro de Análises (IBRA) (Table 1). After 30 days, the soil was fertilized with macronutrient via solution in the following doses: P = 300 mg dm<sup>-3</sup>, K = 100 mg dm<sup>-3</sup> and S = 40 mg/dm<sup>3</sup>, having as sources NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, KCl and K<sub>2</sub>SO<sub>4</sub>. And more, a micronutrient solution, in the following doses: B = 0,81 mg dm<sup>-3</sup> (H<sub>3</sub>BO<sub>3</sub>), Cu = 1,33 mg dm<sup>-3</sup> (CuSO<sub>4</sub>·5H<sub>2</sub>O), Mo = 0,15 mg dm<sup>-3</sup> [(NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O], Mn = 3,66 mg dm<sup>-3</sup> (MnCl<sub>2</sub>·H<sub>2</sub>O) e Zn = 4,0 mg dm<sup>-3</sup> (ZnSO<sub>4</sub>·7H<sub>2</sub>O) (Alvarez et al., 2006). After that, the substrate was placed in plastic pots with a capacity of 3 dm<sup>3</sup>/vessel.

### Plant materials

The seeds of Tachi-branco (*Tachigali vulgaris*) were obtained from the seeds of forest sector of Embrapa Amazônia Oriental, harvested headquarters located in the municipality of Paragominas-PA (2 ° 59 '51" S, 47 ° 21' 13" W) and germinated in seedbeds, with washed sand as substrate. After 30 days of the start of germination, 2 seedlings were transplanted per pot, with capacity of 3 dm<sup>3</sup> of soil. After another 30 days, one thinning was carried out, leaving only one plant per pot.

### Treatments and experimental design

Nitrogen sources tested were urea [CO(NH<sub>2</sub>)<sub>2</sub>] with 45% of N; ammonium sulfate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>] with 21% N and 23% sulfur; ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) having 33.5% N (half from nitrate form and half from ammonium); and calcium nitrate [Ca(NO<sub>3</sub>)<sub>2</sub>], having 15.5% N and 19% calcium. The fertilizers were applied in five doses of 0, 75, 150, 225 and 300 mg dm<sup>-3</sup> N in the form of solution in four equal portions at 25, 50, 75 and 100 days after transplanting to vessels.

The experimental unit consisted of a vase containing 3 dm<sup>3</sup> of soil, with a seedling. The experiment constitutes a randomized block design (RBD) in factorial scheme (4 × 5), corresponding to 4 sources of nitrogen and 5 doses, with four replications, totaling 80 vessels. The plant material to carry out the biochemical analysis was collected at the end of the experiment, 100 days after the start of treatment.

### Biochemical analyzes

Biochemical analyzes were performed on Biodiversity Studies Laboratory in Higher Plants (EBPs), located at the Federal Rural University of Amazonia (UFRA), Belém, Pará. Therefore, we determined the levels of nitrate (NO<sub>3</sub><sup>-</sup>) (Cataldo et al., 1975); reductase enzyme activity of nitrate (RNO<sub>3</sub>) (Hageman; Hucklesby, 1971); free ammonium (NH<sub>4</sub><sup>+</sup>) (Weatherburn, 1967); soluble amino acids (TSAA) (Peoples et al., 1989); total soluble protein (PST) (Bradford, 1976); carbohydrates (Dubois et al., 1956); sucrose (Van Handel, 1968) and; chlorophylls (Lichthenthaler, 1987).

### Statistical analysis

Analysis of variance was performed depend on the significance of the F-test for the interaction between factors Sources × nitrogen. Developments were conducted to evaluate the effect of doses (quantitative) within each nitrogen source (qualitative) for through polynomial regression of first and second degrees. In cases where the

interaction nitrogen sources × nitrogen levels was not significant (p > 0.05) the factors were analyzed separately. The effects due to nitrogen sources were compared by Tukey's test. The effect of nitrogen doses was assessed through polynomial regression of first and second degrees. The 5% significance level was applied for all statistical tests. For regression equations, the significance of the coefficients and the coefficient of determination (R<sup>2</sup>) were also calculated.

### Conclusion

With application of ammonium nitrate and calcium nitrate, there was a reduction in the concentration of protein in leaf and root, which may be related to the formation of reactive oxygen species (ROS) by excess NO<sub>3</sub><sup>-</sup>, causing damage to photosystems and inhibition of protein synthesis. The accumulation of amino acids in the leaf and root was positively influenced by the application of N, occurring maximum accumulation with the source of nitrate ammonium in doses upcoming of 225 mg dm<sup>-3</sup>. The increase of amino acid content in the leaf and root can be the plant's strategy to cope with detoxification of ROS. The conversion of nitrate into amino acids in root depends on the oxidation of carbohydrates, causing the reduction of the concentration of this solute with the NO<sub>3</sub><sup>-</sup> supply. Based on the results, for the production of *Tachigali vulgaris* seedlings the dose of 150 mg dm<sup>-3</sup> of N, applied in a piecemeal manner, at 25, 50, 75 and 100 days with the ammonium nitrate fertilizer is recommended.

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