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REGULAR ARTICLE

Oxidant and antioxidant compounds, gas exchange and growth of young *Schizolobium parahyba* var. *amazonicum* plants under high boron and calcium concentrations

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ABSTRACT

Boron (B) and Calcium (Ca) unbalance in plants during early stages can generate oxidative stress and consequently to interfere negatively on growth and quality of seedlings. This study aims to evaluate the gas exchange and measure the biochemical responses, responding how high concentrations of B and Ca can affect the growth and quality of young *Schizolobium parahyba* plants. The experimental design used was completely randomised with four treatments [1 - 25 μM B + 5 mM Ca (control); 2 - 25 μM B + 50 mM Ca (Ca high); 3- 250 μM B + 5 mM Ca (B high) and 4 - 250 μM B + 50 mM Ca (B and Ca high)]. Negative impacts on gas exchange, photosynthetic pigments and total glutathione were obtained, besides increases in hydrogen peroxide and electrolyte leakage were verified in plants treated with B and Ca high, indicating oxidative stress. Thus, application 250 μM B combined with 50 mM Ca promoted disorders in plant metabolism, decreasing the growth and quality of young *Schizolobium parahyba* plants.

Keywords: Antioxidant metabolism; Gas exchange; Macronutrient; Micronutrient; Reactive oxygen species

INTRODUCTION

Paricá (*Schizolobium parahyba* var. *amazonicum* (Huber ex Ducke) Barneby) is a forestry species with large economic potential and there is demand for uniform seedlings and with adequate quality aiming to improve the assimilation of nutrients, more specifically boron (B) and calcium (Ca).

B is a micronutrient essential for growth and development in higher plants, mainly during the early stages of growth, because participates formation of the cell wall, root elongation, carbon metabolism and cell membrane integrity (Martinez-Cuenca et al., 2015). In addition, Ca is a macronutrient that acts on the cell wall structure, during cell division, and as secondary messenger during stomatal closing in plants (White and Broadley, 2003; Dayod et al., 2010).

Nutritional imbalances frequently cause oxidative stress, which result in membrane damages and cell death (Kaya and Ashraf, 2015). B and Ca under inadequate levels affect the biochemical and physiological behaviours, mainly during early stages of plant growth, with consequences on the establishment, quality and survival of seedlings in field conditions (Lautner et al., 2007; Lehto et al., 2010). In *Vitis vinifera* excess B promoted oxidative stress due to high production of hydrogen peroxide (H_2O_2) (Gunes et al., 2006). On other hand, higher plants can produce antioxidant compounds, such as total glutathione (GSH) and ascorbate (ASC), which it act during the elimination of reactive oxygen species (ROS) (Molassiotis et al., 2006). Gas exchanges also are negatively affected in *Ocimum basilicum* plants treated with B high (Landi et al., 2013a) and in *Lysionotus pauciflorus* and *Boea hygrometrica* exposed to Ca high (Li et al., 2014).

The appropriate balance to B and Ca in forest species may be decisive for the better growth and development of seedlings, which these elements have an intrinsic

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relationship in plant metabolism. Our hypothesis is that the B and Ca unbalance in *S. parahyba* plants during early stages can generate oxidative stress and consequently to interfere negatively on growth and quality of seedlings.

This study aims to evaluate the gas exchange and measure the biochemical responses, responding how high concentrations of B and Ca can affect the growth and quality of young *Schizolobium parahyba* plants.

MATERIALS AND METHODS

Location and growth conditions

The experiment was carried out in the Universidade Federal Rural da Amazônia located in Paragominas, Brazil (2°55'S and 47°34'W). Seedlings were grown in a greenhouse under environmental control to temperature and humidity, the minimum, maximum, and median temperatures were 24°C, 33°C, and 27.5°C, respectively, and relative humidity of 65/85%.

Plants, containers and acclimation

Seeds of *S. parahyba* were sterilized for 3 min in a 1% of sodium hypochlorite (NaCIO), being scarified to increase speed of the germination. These seeds were placed into 1.2-L containers to germinate (0.15 m in height and 0.10 m in diameter) occupied with substrate mixture composed of sand and vermiculite in a 3:1 proportion. The ionic force started at 25 %, and it was reformed to 50 % and 100 % at regular intervals over three days. Subsequently these periods, the nutritive solution persisted with the total ionic force (100%) from 20th until 35th day after experiment implementation.

Experimental design

The experimental design used was completely randomised with four treatments [1 - 25 μM B + 5 mM Ca (control); 2 - 25 μM B + 50 mM Ca (Ca high); 3- 250 μM B + 5 mM Ca (B high) and 4 - 250 μM B + 50 mM Ca (B and Ca high)]. The B and Ca concentrations were chosen in agreement with Hoagland and Arnon (1950). This study used five replicates and 20 experimental units, being one plant per container considered one unit experimental.

Treatments and nutrient solution

One young plant was preserved in each pot, during plant conduction. The seedlings were supplied with nutrient solution containing the following macronutrients and micronutrients: 5.71 mM KNO_3, 2.85 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1.43 mM $NH_4H_2PO_4$, 3.21 mM $MgSO_4 \cdot 7\ H_2O$, 0.71 mM KCl, 1.42 mM KH_2PO_4, 1.42 μM $MnSO_4 \cdot H_2O$, 1.42 μM $ZnSO_4 \cdot 7H_2O$, 0.35 μM $CuSO_4 \cdot 5H_2O$, 0.35 μM $NaMoO_4 \cdot 5H_2O$, 215 μM $NaEDTAFe \cdot 3H_2O$. To simulate B and Ca treatments, H_3BO_3 was used at concentrations of 25 μM and 250 μM B, while $CaCl_2$ was used at concentrations 5 and 50 mM Ca during 25 days (35th day until 60th day). On 60th day after experiment implementation, all plants were physiologically evaluated and leaf, stem and root tissues were collected for biochemical analysis.

Evaluation of gas exchange

The net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured on the adaxial surface of fully expanded leaves that were collected from the middle region of the plant using an infrared gas analyser (model LCPro$^+$; ADC BioScientific). The efficiency in the instantaneous use of water (WUE = P_N/E) was estimated according to Ma et al. (2004). To instantaneous carboxylation efficiency (P_N/C_i) was used the formula described previously by Aragão et al. (2012). Gas exchange was mensured in all plants under constant conditions of CO_2 concentration, photosynthetically active radiation, air-flow rate and temperature in a chamber set at 360 μmol mol^{-1} CO_2, 800 μmol photons m^{-2} s^{-1}, 300 μmol s^{-1} and 28 ºC, respectively, between 10:00 and 12:00 h.

Determination of photosynthetic pigments

The chlorophyll and carotenoid determinations were performed using 40 mg of leaf tissue. The samples were homogenised in the dark with 8 mL of 90% methanol (Nuclear). The homogenate was centrifuged at 6.000 × g for 10 min at 5 ºC. The supernatant was removed, and the chlorophyll *a* (Chl *a*) and *b* (Chl *b*), and carotenoid (Car) and total chlorophyll (total Chl) contents were quantified using a spectrophotometer (model UV-M51; Bel Photonics) according to the methodology of Lichtenthaler and Buschmann (2001).

Extraction and determination of oxidant and antioxidant compounds

The extraction of Hydrogen peroxide (H_2O_2), total glutathione (GSH), and ascorbate (ASC) from leaf and root tissues was made using the methodology described by Wu et al. (2006). To measure H_2O_2 was used the methodology by Velikova et al. (2000). To total GSH was used methodology described by Wu et al. (2006). The methodology to measure the ASC in the plant tissue was defined by Cakmak and Marschner (1992).

Determination of electrolyte leakage

Electrolyte leakage (EL) was measured using the method described by Gong et al. (1998) with minor modifications.

Growth and quality of seedlings

The height and stem diameter of the seedlings were measured 60 days after experiment implementation. The plant material was separated in leaves, stem and roots,

washed with deionised water (Malavolta et al., 1997). Leaves, roots and stems were then dried in a forced circulation oven at 65°C until constant weight to determine their dry matters. The Dickson quality index (DQI) was determined using the equation: DQI = TDM/[(H/SD)+ (SDM/RDM)]. Where: TDM = total dry matter (g), SD = stem diameter (mm), H = height (cm), SDM = shoot dry matter (g), RDM = root dry matter (g). For analysis of concentration and accumulation of B and Ca in plant tissues, the samples were extracted by dry digestion (incineration) for the analysis of B and nitric perchloric digestion for the Ca. And determination B was according to the colorimetric method and Ca were determined by atomic absorption spectrophotometry, both methods described by Malavolta et al. (1997).

Data analysis

The data were subjected to one way analysis of variance, and significant differences between the means were determined using the Scott-Knott test at a probability level of 5%, being chosen this test due to your precision, if compared other statistical tests. The procedure involved in Scott-Knott test to determinate the significance between treatments is based on maximum likelihood estimator, and not LSD as in other tests (Duncan, Dunnett, SNK and Tukey). Standard deviations were determined for each treatment. The statistical analyses were performed with Assistat software.

RESULTS

B and Ca contents

The higher availability of B and Ca (250 μM B + 50 mM Ca) was responsible for significant increases in content and accumulation B in leaf (Table 1). In relation to B content, the treatment 250 μM B + 5 mM Ca promoted higher B content in stem and root. On Ca content, the treatment with high B and Ca (250 μM B + 50 mM Ca) presented not differences to leaf and stem, comparison with control treatment (25 μM B + 5 mM Ca). The treatment with 250 μM B + 50 mM Ca caused a significant increase (Table 1) in the Ca accumulation in the leaf (20%), stem (39%) and root (15%), respectively.

Consequences of B and Ca supplies on H_2O_2 and EL

In H_2O_2 were showed significant increases promoted by the B and Ca supplies (Fig. 1 A and B). The effects more intense were observed in plants that received 250 μM B + 50 mM Ca with increases of 35% and 40% in leaf and root, if compared with 25 μM B + 5 mM Ca treatment. To EL were observed similar behaviors in leaf and root, being detected to 250 μM B + 50 mM Ca treatment increases of 12% and 22% in leaf and root, respectively, when compared with 25 μM B + 5 mM Ca (Fig. 1 C and D).

Interference of B and Ca on gas exchange

The P_N decreased significantly with applications combined of 250 μM B + 50 mM Ca, being verified a reduction of 44%, compared with 25 μM B + 5 mM Ca (Fig. 2 A). To g_s was observed a decrease of approximately 35% (Fig. 2 B) in the plants that received 250 μM B + 5 mM Ca comparing with control (25 μM B + 5 mM Ca). Results linked to E shown that plants had an increase of 13% (non-significant) with the high availability of B and Ca in the solution (Fig. 2 C). The C_i was decreased by 14% in treatment that used 250 μM B + 50 mM Ca, however was not sufficient to differ 25 μM B + 5 mM Ca treatment (Fig. 2 D). The P_N/C_i also dramatically reduced with 250 μM B + 50 mM Ca (34%), when compared with 25 μM B + 5 mM Ca (Fig. 2 E). Similar behavior was observed in WUE, reduced 51% with 250 μM B + 50 mM Ca (Fig. 2 F).

Reductions promoted by high B and Ca on chlorophylls

The CHL a presented significant reductions, being the most severe occurred when there was an increase in availability of B and Ca (250 μM B + 50 mM Ca), with decrease

Table 1: Content and accumulation of B and Ca in young *S. parahyba* plants subjected to different of B and Ca supplies. Columns with different letters next to treatments indicate significant differences from the Skott-Knott test (*P*<0.05). Values described corresponding to means and standard deviations from five repetitions.

Tratamentos	B content (mg kg^{-1})			B accumulation (mg g^{-1})		
	Leaf	Stem	Root	Leaf	Stem	Root
25 μM B+5 mM Ca	127.89±12.04c	30.36±2.93a	36.69±3.32c	623.75±57.53c	118.43±9.65b	73.33±4.53c
25 μM B+50 mM Ca	81.92±8.03d	26.57±1.16b	28.50±2.37d	400.64±38.52d	86.25±6.49c	54.02±4.93d
250 μM B+5 mM Ca	178.12±16.88b	32.69±2.97a	47.99±1.39a	877.45±82.59b	131.53±9.04b	91.23±5.87b
250 μM B+50 mM Ca	240.33±18.62a	29.25±2.76a	42.35±1.01b	1419.73±129.91a	167.06±13.58a	116.72±10.89a
	Ca Content (g kg^{-1})			Ca accumulation (g g^{-1})		
	Leaf	Stem	Root	Leaf	Stem	Root
25 μM B+5 mM Ca	5.08±0.10a	2.19±0.20a	7.33±0.41a	25.26±2.46b	8.55±0.81b	14.73±1.05b
25 μM B+50 mM Ca	5.08±0.44a	2.33±0.20a	7.25±0.31a	24.69±2.39b	7.56±0.71c	13.71±0.97b
250 μM B+5 mM Ca	5.00±0.35a	1.75±0.11b	6.33±0.10b	24.72±2.45b	7.04±0.68c	12.07±1.19c
250 μM B+50 mM Ca	5.17±0.41a	2.08±0.10a	6.17±0.27b	30.46±2.99a	11.91±1.08a	16.97±1.23a

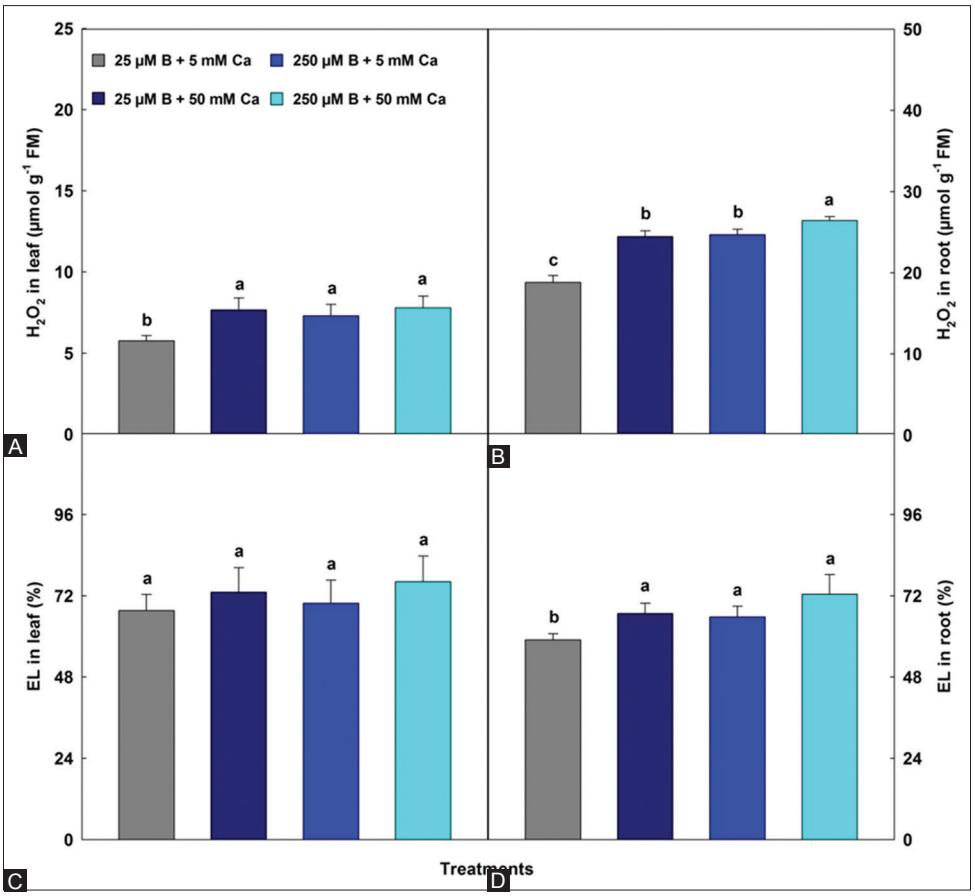

Fig 1. Hydrogen peroxide in leaf (A) hydrogen peroxide in the root (A), electrolyte leakage in leaf (C) and electrolyte leakage in root (D) in young *S. parahyba* plants subjected to different of B and Ca supplies. Bars with different letters indicate significant differences from the Skott-Knott test (P<0.05). Bars represent the mean values and error bars represent the standard deviations from five replicates.

of 42% compared with control plants (Fig. 3 A). The concentration of CHL b decreased 7% (non-significant) after the excessive application of B and Ca (Fig. 3 B). Total CHL rate had a significant reduction of 32% when applied 250 µM B + 50 mM Ca compared with control treatment, while applications with 250 µM B + 5 mM Ca, and 25 µM B + 50 mM Ca presented reductions of 27% and 15%, respectively (Fig. 3 C). The CAR in plants submitted to high B and Ca (250 µM B + 50 mM Ca), suffered the strong increase (147%) comparison with control treatment (Fig. 3 D).

ASC and GSH total had different behaviors after B and Ca supplies

There were significant changes in the ASC content in the leaves and roots with the combinations of B and Ca (Fig. 4 A and B), being observed increase of 187% and 85% in leaf and root after application of 250 µM B + 50 mM Ca when compared to control. The combination of B and Ca caused a reduction in GSH levels in the leaf and root, showing greater reduction when B and Ca were in excess, with 24% and 55% in leaf and root compared to control (Fig. 4 C E D).

B and Ca levels on growth

High B and Ca supplies applied resulted in negative effects on plant growth. In relation to stem diameter was not verified significant modifications promoted by the B and Ca supplies. Total dry matter had significant decreases when applied 250 µM B + 50 mM Ca treatment, being 21% and 14% in leaf and total, respectively, compared with 25 µM B + 5 mM Ca (Table 2). The quality index of S. parahyba seedlings decreased with the application of high B and Ca, but all seedlings are above standard of quality established by Dickson et al. (1960) for seedlings.

DISCUSSION

Contents and accumulation of B and Ca increased in treatments exposed to high B and Ca levels in nutrient solution, because the transpiration rate was not affected. These nutrients are normally distributed in plants tissues, being both (B and Ca) transported mainly by the mass flow. Siddiqui et al. (2013) observed that the application together B and Ca were more effective than isolated in the improvement of plant growth.

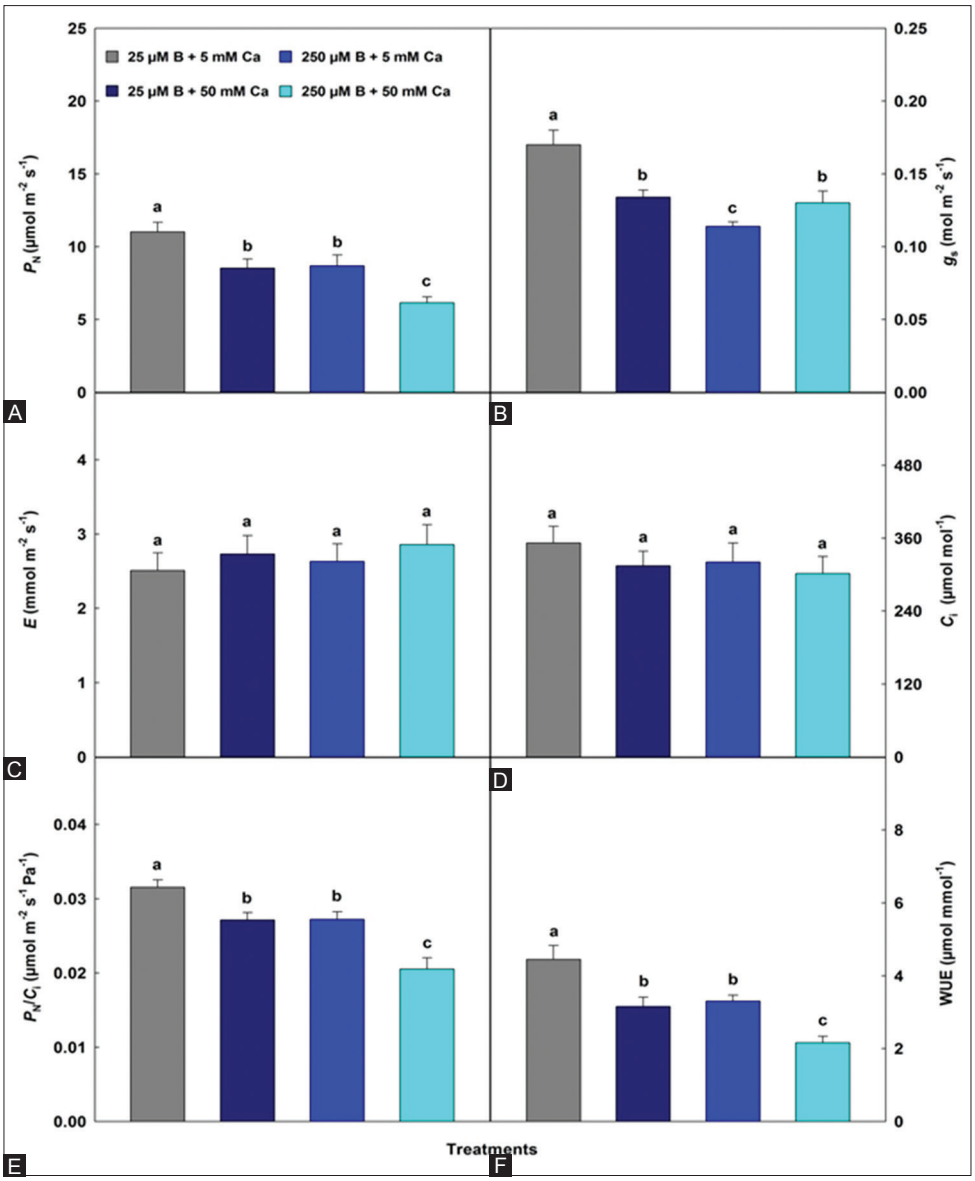

Fig 2. Net photosynthetic rate (A), stomatal conductance (B), transpiration rate (C) intercellular CO2 concentration, (D) efficiency of instantaneous carboxylation (E), and water use efficiency (F) in young *S. parahyba* plants subjected to different of B and Ca supplies. Bars with different letters indicate significant differences from the Skott-Knott test (P<0.05). Bars represent the mean values and error bars represent the standard deviations from five repetitions.

Table 2: Stem diameter, dry matter and Dickson quality index (DQI) in young *S. parahyba* plants subjected to different of B and Ca supplies. Columns with different letters next to treatments indicate significant differences from the Skott-Knott test (P<0.05). Values described correspond to means and standard deviations from five repetitions.

Treatments	Stem diameter (cm)	Dry matter (p plant^{-1})				DQI
		Stem	Leaf	Root	Total	
25 µM B+5 mM Ca	6.58±0.65a	3.92±0.37a	4.97±0.46a	2.01±0.17a	10.90±0.51a	1.13±0.19a
25 µM B+50 mM Ca	6.87±0.50a	3.85±0.24a	4.87±0.47a	1.99±0.13a	10.71±0.44a	1.24±0.17a
250 µM B+5 mM Ca	6.81±0.70a	3.42±0.35a	4.94±0.36a	1.90±0.16a	10.26±0.43a	1.23±0.25a
250 µM B+50 mM Ca	5.91±0.72a	3.71±0.55a	3.90±0.34b	1.75±0.21a	9.36±0.32b	0.99±0.12a

The H_2O_2 were increased in the leaves and roots, and these results are related to oxidative stress caused by the B and Ca excess in solution (Esim et al., 2012; Blasco et al., 2014.). Siddiqui et al. (2013) studying *Raphanus sativus* submitted the interaction of B and Ca toxic observed similar behavior to this study. To EL, increases were caused by the H_2O_2 accumulation; however this negative effect was more evident in the roots. Excess

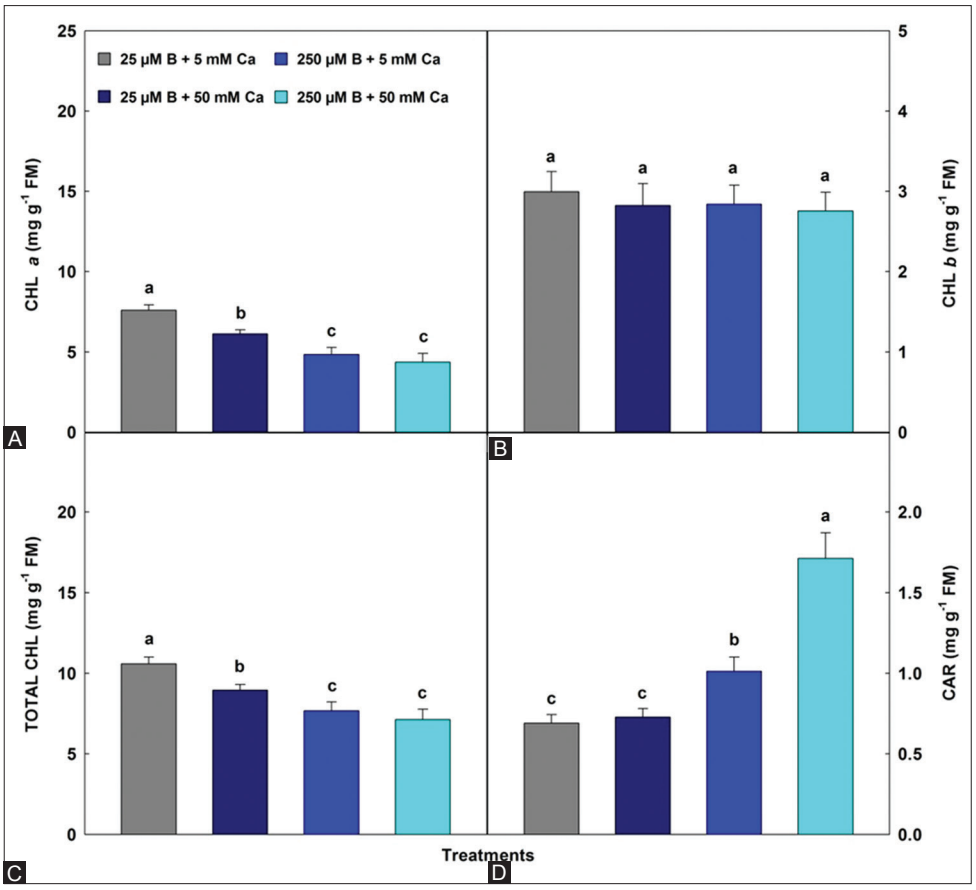

Fig 3. Chlorophyll a (A), chlorophyll b (B), total chlorophyll (C) and carotenoids (D) in young *S. parahyba* plants subjected to different of B and Ca supplies. Bars with different letters indicate significant differences from the Skott-Knott test (P<0.05). Bars represent the mean values and error bars represent the standard deviations from five repetitions.

ROS frequently induces increases in EL (Arbona and Gómez-Cadenas, 2012).

The decrease in P_N is explained by the damages in the chloroplasts due to oxidative stress caused by the combined excess of B and Ca (Landi et al., 2013b and Wang et al., 2010). Chloroplasts are affected by the oxidative stress caused during nutritional imbalance. Hossain et al. (2015) studying the excess B in *Brassica napus* obtained similar results to this study. In addition, Wang et al. (2010) reported that the excess of Ca in *Camellia sinensis* can cause rupture in chloroplast membrane, damages in photosystem II, lower photosynthetic rate and decrease in light capitation.

Reduction in g_s was detected in response to excess of B and Ca, because the high B in leaf directly affects the stomatal mechanism (Apostol and Zwiazek, 2004). Mattiello et al. (2009) studying the effect of excess B in *E. grandis* clones, observed decrease in g_s, corroborating with the results of this study. Cabañero and Carvajal (2007) also observed this behavior in *Capsicum annuum* L. plant with the excess Ca.

In relation to E *and* C_i, these variables presented not significant changes, corroborating with the study by Huang et al. (2014) who also did not found modification in E of *Citrus sinensis* under B excess. Papadakis et al. (2004) studying the B toxicity effects in *Citrus* plants observed decreased in P_N/C_i, similar with results detected in this research. Reduction in WUE is related to decrease in P_N caused during excessive nutrient supply. Hosaini et al. (2009) applied 30 mg kg^{-1} of B in the soil and observed that the WUE decreased in *Brassica napus* plants.

The decrease in CHL *a*, and Total CHL occurred due to lower biosynthesis of precursors of chlorophylls during high B and Ca concentrations. B when in excess provides increases of the phenolic compounds by the action of peroxidase enzyme, and high levels of Ca affect absorption and Ca and Mg distributions in plant, both actions result in the chlorophyll degradation (Pandey, 2013; Seth and Aery, 2014; Jiazhi et al., 2014.). Similar results were described by Landi et al. (2013b) working with *Cucurbita pepo* with B excess. Jiazhi et al. (2014) observed that the high Ca reduced the pigments levels in *Camellia sinensis* plants, corroborating with the results of this study.

The increase in CAR was due to protection mechanism against the stress caused by the high availability of B and

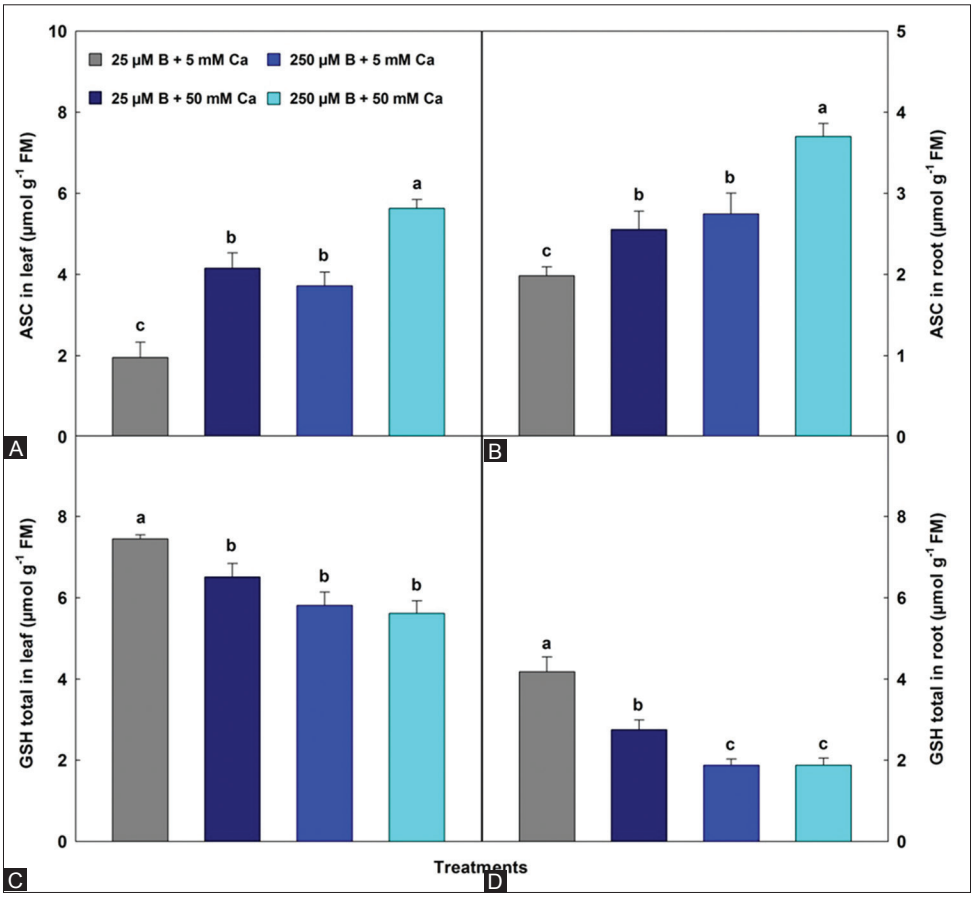

Fig 4. Ascorbate in leaf (A), ascorbate in root (B), glutathione total in leaf (C) and glutathione total in root (D) in young *S. parahyba* plants subjected to different of B and Ca supplies. Bars with different letters indicate significant differences from the Skott-Knott test (P<0.05). Bars represent the mean values and error bars represent the standard deviations from five repetition.

Ca. In agreement with Nisar et al. (2015), this pigment has an essential role in photoprotection, in which this protects the photosynthetic apparatus. These results corroborate the study of Cervilla et al. (2012) when studied the B effect in *Solanum lycopersicum* plants and Danieli et al. (2002) working with *Diospyros kaki* plants under high Ca.

The ASC increases in leaf and root tissues are related to the oxidative stress. Similar results were observed by Cervilla et al. (2007) studying B toxicity in *Solanum lycopersicum* plants. To GSH total were verified decrease and this effect was caused by the increase in B treatment, which contributed to inefficient performance of the antioxidant system (Silva et al., 2016). In agreement with Ruiz et al. (2003) the B toxicity inhibits the conversion of cysteine for GSH, this fact is considered the main reason of the phytotoxicity in *Helianthus annus* plants. et al. (2009) obtained similar behavior in *Citrus sinensis* plants in response to B toxicity.

High availability B and Ca promoted decreases the plant growth, represented by lower values of SD, LDM, SDM, RDM, TDM and DQI, this fact can be explained by the imbalance nutritional. Ramos et al. (2013) evaluating the Ca/B ratio in *Eucalyptus citriodora* plants detected that the B application without a suitable supply of Ca can lead to an imbalance between these nutrients affecting plant nutrition and growth. Turan et al. (2009) studying the effect of these nutrients in *Triticum aestivum* plants observed results similar corroborating with this study.

Negative impacts on gas exchange, photosynthetic pigments and total glutathione were obtained, besides increases in hydrogen peroxide and electrolyte leakage were verified in plants treated with B and Ca high, indicating oxidative stress. Thus, application 250 µM B combined with 50 mM Ca promoted disorders in plant metabolism, decreasing the growth and quality of young *Schizolobium parahyba* plants.

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Author contributions

Lobato E.M.S.G. was the advisor of this project and planned all phases of this research. Callegari D.M., Silva B.C., Sousa P.R., Lobato A.K.S. conducted the experiment in the greenhouse and performed physiological, biochemical and morphological determinations, as well as interpreted the results and wrote the manuscript.

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