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Original Article

Proline but not Glutathione Actively Participates in the Tolerance Mechanism of Young *Schizolobium parahyba* var. *amazonicum* Plants Exposed to Boron Toxicity

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Abstract

Glutathione, a peptide frequently associated with the antioxidant mechanism of plants against reactive oxygen species, and proline, an amino acid whose function is related to cellular homeostasis, can both contribute to improve plant tolerance under situations of abiotic stress, such as boron toxicity. Aims of this research were to (i) quantify the oxidant and antioxidant compounds, (ii) evaluate the photosynthetic pigments, (iii) determine amino acids and PRO, and (iv) determine whether GSH and PRO contribute to the tolerance mechanisms in young *Schizolobium parahyba* var. *amazonicum* plants under B toxicity. This experiment tested five boron levels (25, 50, 100, 150 and 250 µM B), being evaluated physiological and biochemical variables. The values reported to proline levels presented significant variation for treatments with 50, 100, 150 and 250 µM B, with increases for the 150 and 250 µM B levels, being 45.2 and 52.4%, respectively. This study found that boron toxicity promoted similar behaviours in both the leaves and root, which included progressive increases in hydrogen peroxide, electrolyte leakage, amino acids and proline, and decreases in total glutathione, chlorophyll *a*, chlorophyll *b* and total chlorophyll, confirming that proline but not glutathione actively participates in the tolerance mechanism of young *Schizolobium parahyba* plants exposed to boron toxicity.

Keywords: amino acid, antioxidant system, boron, chlorophyll, micronutrient, peptide

Introduction

The tree *Schizolobium parahyba* var. *amazonicum* (Huber ex. Ducke) Barneby, is a specie frequently found in tropical environments from Brazil, more specifically in the Amazon region. The botanical characteristics are rapid growth, erect trunk and few ramifications (Rosa, 2006), additionally it has economic importance because of its wood proprieties (Silva *et al*., 2013).

Boron (B) is an essential micronutrient to vascular plants (Koshiba *et al*., 2009) because this element has structural and physiological functions linked to cell wall stabilization (Brown *et al*., 2002), starch transport in chloroplasts (Silva *et al*., 2008) and lignification (Hansch and Mendel, 2009).

Stress caused by the excessive supply of B to plants frequently induces changes on the oxidant system (Molassiotis *et al*., 2006; Wang *et al*., 2011), including the overproduction of reactive oxygen species (ROS) such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) (Landi *et al*., 2013). The oxidative damage is characterized by the accumulation of high levels of ROS and insufficient detoxification promoted by antioxidant enzymes, such as catalase and glutathione peroxidase (Gill and Tuteja, 2010).

The chlorophylls are responsible for the photochemical and biochemical reactions during light absorption (Streit *et al*., 2005), whereas carotenoids play an important role related to photoprotection against excessive sunlight (Cazzonelli, 2011), both pigments work simultaneously in the photosynthetic machinery (Croce *et al*., 2001). However, the excessive B supply represents a problem for photosynthetic pigments, with a consequent decrease in chlorophyll contents (Papadakis *et al*., 2004; Tepe and Aydemir, 2011).

Glutathione (GSH) is the thiol tripeptide that is most abundant in eukaryotes (Cameron and Pakrasi, 2010) and is frequently associated to defence against ROS in plants (Foyer and Noctor, 2005; Mullineaux and Rausch, 2005), due to its ability to conjugate toxic substances that will be subsequently transported to the vacuole, thus avoiding damage in the cells (Klein *et al*., 2006; Yazaki, 2006). The availability of GSH is also linked to the production of phytochelatins, which are used to alleviate the toxicity by nutrients and heavy metals (Yadav, 2010).

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Proline (PRO) is an amino acid synthesized during nitrogen metabolism with functions related to osmoprotection (Costa *et al*., 2011) and cellular homeostasis (Szabados and Savouré, 2010); it can contribute to improve plant tolerance under situations of abiotic stress, such as B toxicity.

Our hypothesis is that total GSH and PRO can work together simultaneously or separately in tolerance mechanism of *Schizolobium parahyba* plants exposed to progressively increasing boron levels. Thus, the aims of this research were to (i) quantify the oxidant and antioxidant compounds, (ii) evaluate the photosynthetic pigments, (iii) determine amino acids and PRO, and (iv) determine whether GSH and PRO contribute to the tolerance mechanisms in young *Schizolobium parahyba* var. *amazonicum* plants under B toxicity.

Materials and Methods

Location and growth conditions

The experiment was performed in the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55'S and 47°34'W). The study was conducted in a greenhouse without environmental controls, and the minimum, maximum, and median temperatures were 22 °C, 35 °C, and 26.5 °C, respectively. The relative humidity during the experimental period varied between 70% and 90%, and the photoperiod was set to 12 h of light. During the measurement period (12:00 h), the amount of photosynthetically active radiation varied between 551 and 1,666 μmol m^{-2} s^{-1}.

Plants, containers and acclimation

Seeds of *Schizolobium parahyba* var. *amazonicum* (Huber ex Ducke) Barneby were placed to germinate in sand and subsequently transferred to hydroponic containers. The 15-days-old seedlings with similar aspects and sizes (10 cm height and three leaves) were selected and placed in 3.0-L containers (0.20 m in height and 0.14 m in diameter). Each container was filled with 2.8 L of Hoagland and Arnon nutrient solution (1950) modified in relation to the nutritional requirements of this species. The ionic force started at 25% and was modified to 50 and 100% in regular intervals of seven days, after this period the nutrient solution continued with total ionic force. Subsequently, 45-day-old plants were subjected to different B levels.

Plant conduction and boron treatments

During plant conduction, one young plant was placed in each pot. The treatments received macronutrients and micronutrients from the nutrient solution as follows: 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 $NaMoO_4{\cdot}5H_2O$, and 215.0 μM NaEDTAFe·$3H_2O$, and a boron supply limited to 25 μM H_3BO_3. To simulate the boron treatments, H_3BO_3 was also used in concentrations of 25, 50, 100, 150 and 250 μM B. The different boron levels were applied to the young plants for 15 days, which were changed at 07:00 h over 5-day intervals and had their pH adjusted to 5.5 ± 0.2 via the addition of HCl or NaOH. On the 15th day after the treatments were started, all the plants were harvested and analysed.

Extraction of oxidant and antioxidant compounds

Hydrogen peroxide (H_2O_2) and total glutathione (total GSH) were extracted from the leaf and root tissues as described by Wu et al. (2006). An extraction mixture was prepared by homogenizing 500 mg of fresh matter in 5 mL of 5% (w/v) trichloroacetic acid. Subsequently, the samples were centrifuged at 15,000 x g for 15 min at 3 °C, and the supernatant was collected.

Hydrogen peroxide determination

For H_2O_2 detection, 200 μL of supernatant and 1,800 μL of reaction mixture (2.5 mM potassium phosphate buffer [pH 7.0] and 500 mM potassium iodide) were combined, and the absorbance was measured at 390 nm (Velikova *et al*., 2000).

Total glutathione quantification

For total GSH detection, 200 μL of supernatant and 1,800 μL of reaction mixture (containing 100 mM phosphate buffer [pH 7.6] and 0.60 mM 2-nitrobenzoic acid) were combined, and the absorbance was measured at 412 nm (Wu *et al*., 2006).

Determination of chlorophyll content

The determination of the photosynthetic pigments was carried out with 40 mg of leaf tissue. The samples were homogenized in the dark in the presence of 8 mL of methanol at 90% (Nuclear). Subsequently, the homogenate was centrifuged at 6.000 *g* for 10 minutes at 5 °C. The supernatant was removed and chlorophylls *a* (CHL *a*) and *b* (CHL *b*), total chlorophylls (total CHL) and carotenoids (CAR) were quantified using a Bel Photonics spectrophotometer (UV-M51), according to the methodology of Lichtenthaler and Buschmann (2001).

Electrolyte leakage

Electrolyte leakage was measured according to the method described by Gong *et al.* (1998), with minor modifications. Fresh leaves (200 mg) were cut into pieces 1 cm long and were placed in containers with 8 mL of distilled deionised water. The containers were incubated in a water bath at 35 °C for 30 min, and the initial electrical conductivity of the medium (EC_1) was measured. The samples were boiled at 95 °C for 20 min to release the electrolytes. After the samples were cooled, the final electrical conductivity (EC_2) was measured (Gong *et al*., 1998). The percentage of electrolyte leakage was calculated using the formula EL (%) = (EC_1 / EC_2) x 100.

Amino acids and proline

The amino acids and proline were extracted using 20 mg of powdered dry leaf matter, being tissue dried in oven at 65° C for 72 h, it incubated in 2 mL of deionized distilled water at 100 °C for 30 min. After being homogenized, the solution was centrifuged at 2000 × *g* for 5 min at 20 °C and the supernatant was removed. The quantification of the total soluble amino acids was performed at 570 nm according to Peoples *et al.* (1989), and L-asparagine + L-glutamine (Sigma Chemicals) was used as a standard. The quantification of PRO was performed after measuring the absorbance at 520 nm according to Bates *et al.* (1973) based on L-proline (Sigma Chemicals) as the standard.

Experimental design

The experiment was carried out in an entirely randomized design with five boron levels (25, 50, 100, 150 and 250 μM B).

The experiment was assembled with five replicates for a total of 25 experimental units, with one plant in each unit.

Data analysis

The data were subjected to an analysis of variance, and significant differences between the means were determined using the Skott-Knott test at a probability level of 5% (Steel *et al.*, 2006). Standard deviations were calculated for each treatment. The statistical analyses were performed using SAS software.

Results

Consequences of boron toxicity on the H2O2 and total GSH concentration

The B content in the leaves was increased after progressive B treatments (Fig. 1), and a variation of 45% under the application of 250 µM B was found compared with that of the control (25 µM). The H_2O_2 levels in the leaves and root presented similar behaviours with significant changes being found for treatments with 150 and 250 µM B. Significant increases of 59.8 and 60.7% in the leaf compared with those in the control were shown in treatments using 150 and 250 µM B boron exposure (Fig. 2 A), respectively. For the root, significant increases were 16.6 and 27.1% (Fig. 2 B) in the treatments

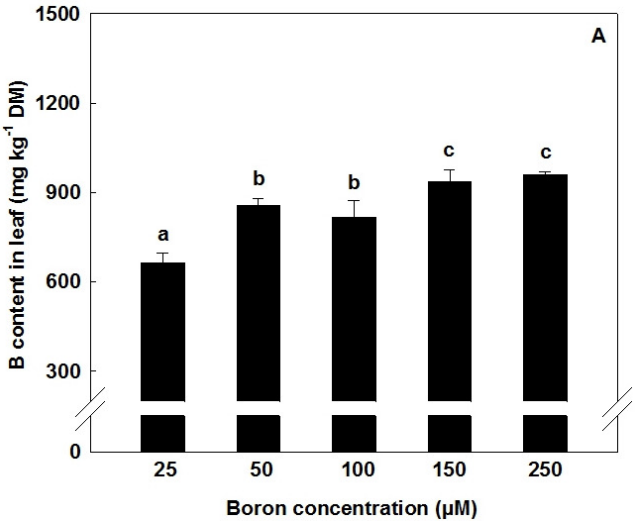

Fig. 1. Boron content in leaf of young *Schizolobium parahyba* var. *amazonicum* plants subjected to boron toxicity. Different letters for boron levels indicate significant differences from the Scott-Knott test (P<0.05). Columns represent the mean values from 5 repetitions, and bars represent the standard deviations

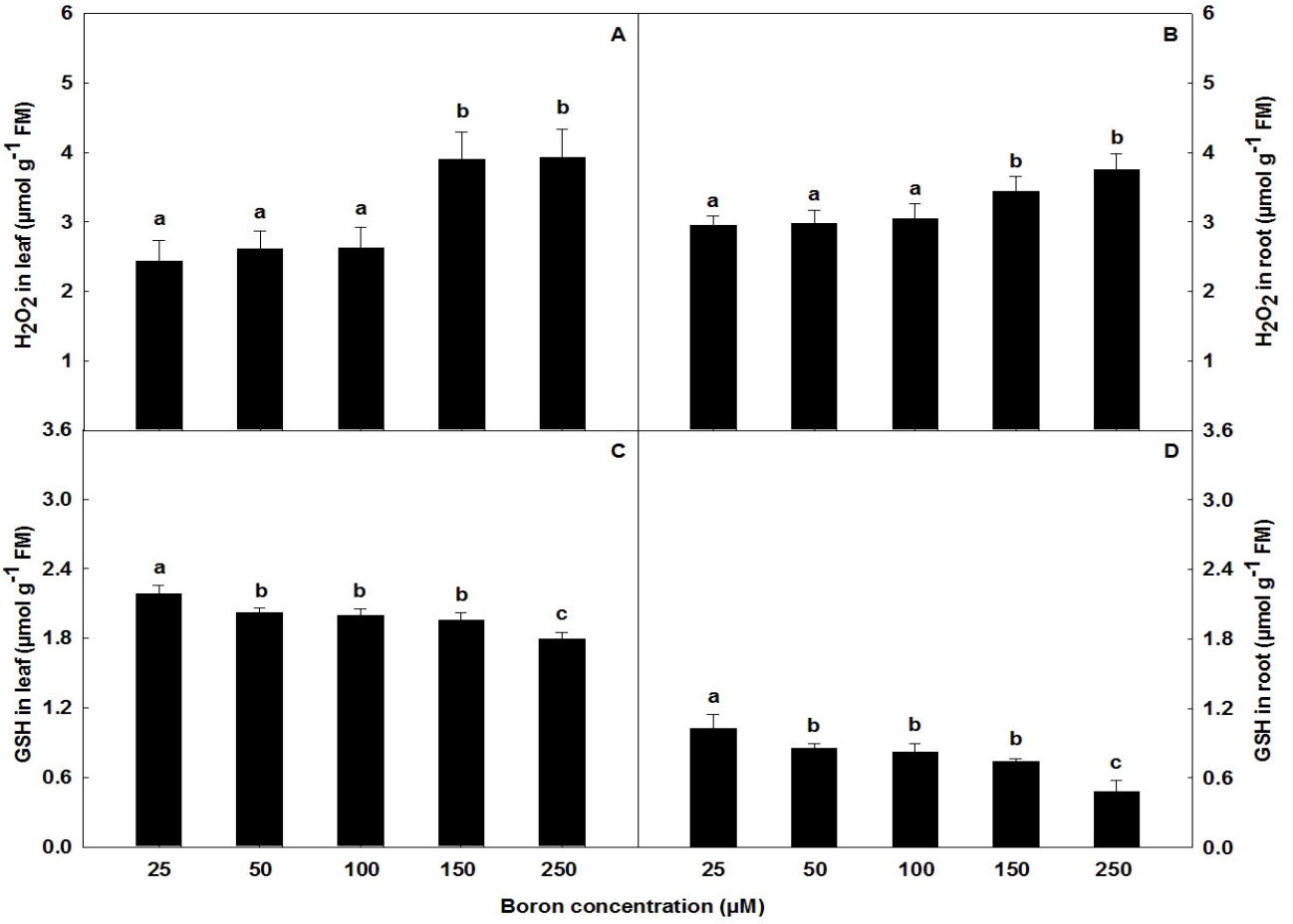

Fig. 2. Hydrogen peroxide in the leaf and root (A – B) and total glutathione in the leaf and root (C –D) of young *Schizolobium parahyba* var. *amazonicum* plants subjected to boron toxicity. Different letters for boron levels indicate significant differences from the Scott-Knott test (P<0.05). Columns represent the mean values from 5 repetitions, and bars represent the standard deviations

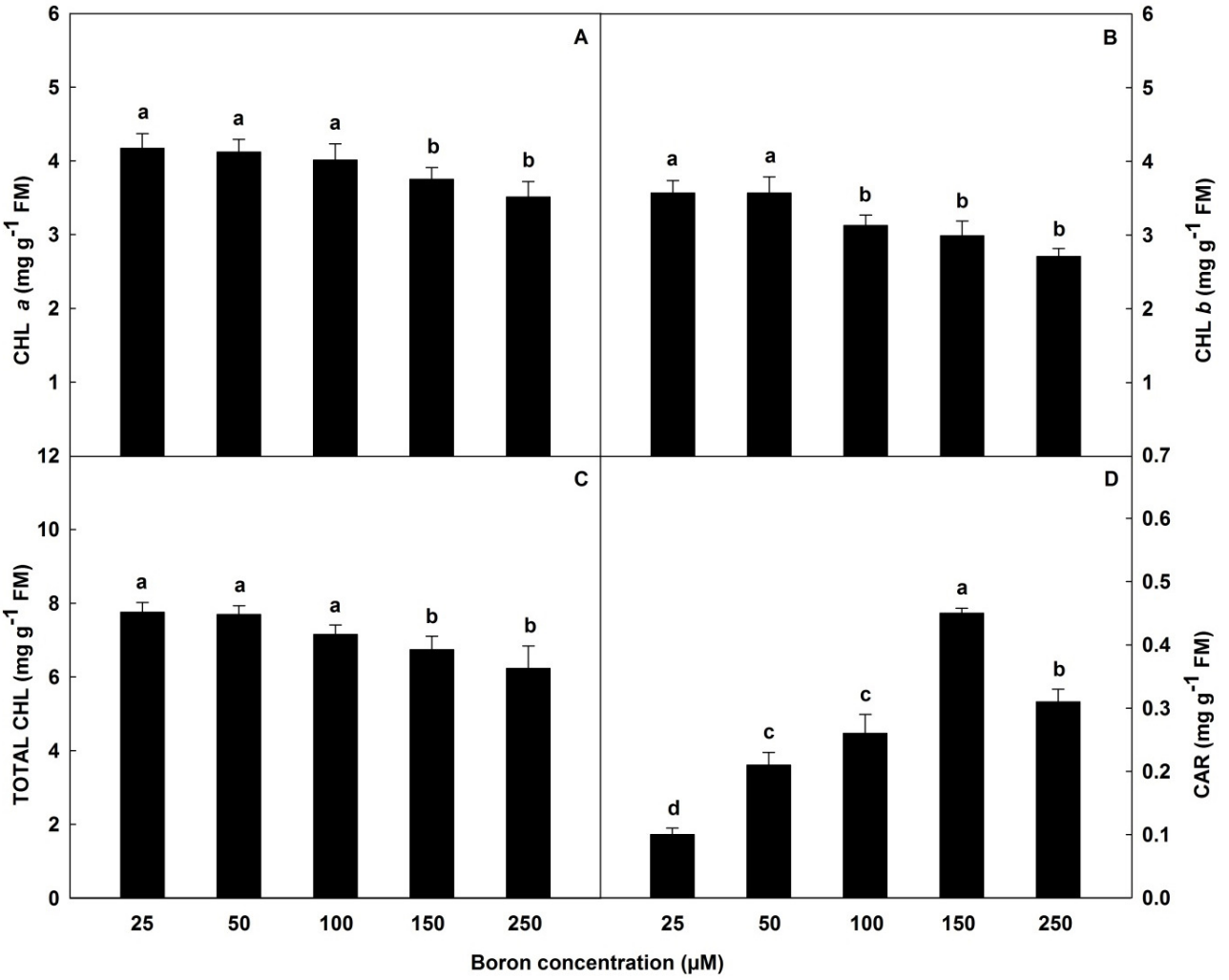

Fig. 3. Chlorophyll *a* (A), chlorophyll *b* (B), total chlorophyll (C) and carotenoids (D) of young *Schizolobium parahyba* var. *amazonicum* plants subjected to boron toxicity. Different letters for boron levels indicate significant differences from the Scott-Knott test (P<0.05). Columns represent the mean values from 5 repetitions, and bars represent the standard deviations.

submitted to levels of 150 and 250 µM B, respectively, compared to those in the control. The total GSH levels presented significant alterations in both tissues, with a verified reduction of 17.9% for leaves exposed to 250 µM B (Fig. 2 C) compared to that of the control (25 µM B). In the root, the total GSH levels revealed significant reductions under the levels of 50, 100, 150 and 250 µM B (Fig. 2 D). A decrease of 53.4% was shown for the level of 250 µM B compared with that of the control.

Changes in concentrations of chlorophylls and carotenoids after boron toxicity

The CHL a contents presented significant changes for treatments with 150 and 250 µM B (Fig. 3 A), with decreases of 10.1 and 15.8%, respectively, compared with control. The CHL b levels suffered interference in treatments exposed to 100, 150 and 250 µM B (Fig. 3 B), with decreases of 12.3, 16.2 and 24.1%, respectively, when compared with 25 µM B. The total CHL presented significant changes under the 150 and 250 µM B levels (Fig. 3 C), with decreases of 13.0 and 19.6%, respectively. The CAR levels of plants submitted to the progressive increase in boron levels presented significant interferences in treatments under 50, 100, 150 and 250 µM B (Fig. 3 D), with increases of 110, 160, 350 and 210%, respectively, if compared to control treatment.

Interference of boron toxicity on electrolyte leakage, amino acids and proline

The progressive increase in boron levels of 50, 100, 150 and 250 µM B presented significant differences in values found for EL (Fig. 4 A), which increased with the increase in boron levels. The highest value was obtained for the 250 µM B level, which was an increase of 8.6% when compared with control. The amino acid contents in the leaves exposed to increases in boron levels (Fig. 4 B), demonstrated significant modifications in the treatments with 50, 100, 150 e 250 µM B, with increases of 30.0, 47.3, 49.1 and 50.8%, respectively, if compared to 25 µM B. The values found in the PRO levels presented significant variation for treatments with 50, 100, 150 and 250 µM B (Fig. 4 C), with increases for the 150 and 250 µM B levels, being 45.2 and 52.4%, respectively.

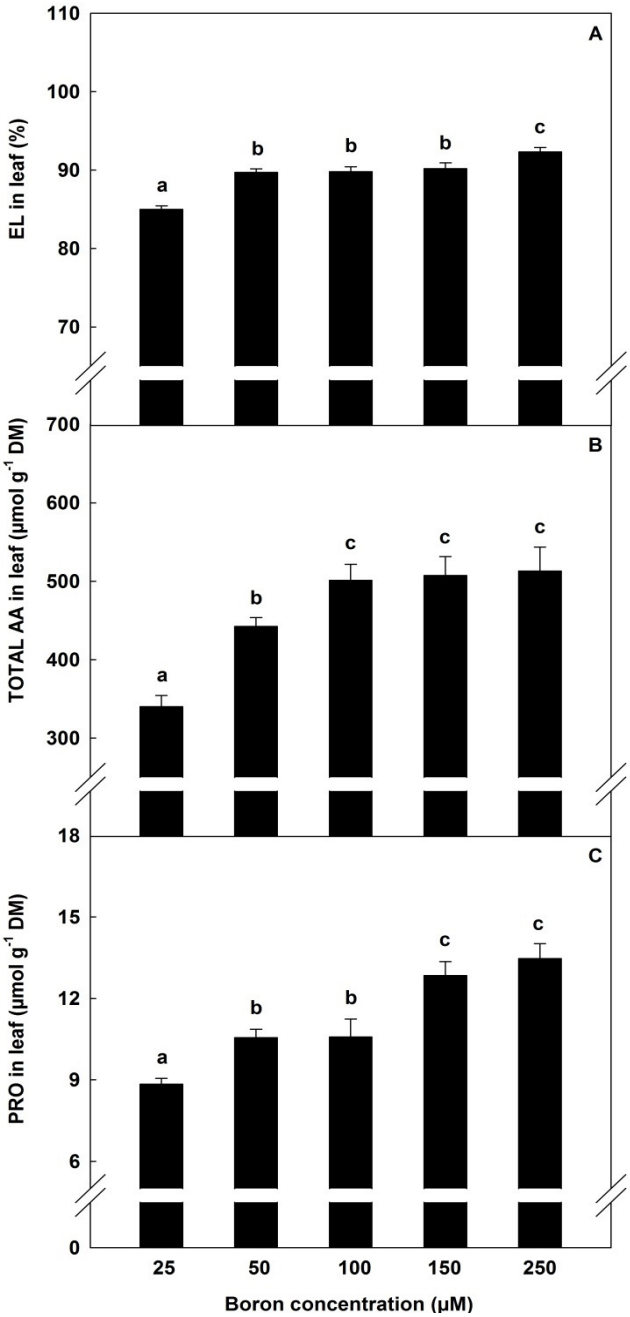

Fig. 4. Electrolyte leakage (A), total amino acids (B), and proline (C) of young *Schizolobium parahyba* var. *amazonicum* plants subjected to boron toxicity. Different letters for boron levels indicate significant differences from the Scott-Knott test ($P<0.05$). Columns represent the mean values from 5 repetitions, and bars represent the standard deviations.

Discussion

The increased B contents in the leaves after treatments with B indicate that this element was absorbed and transported to the leaves; it also reveals that the progressive B levels tested in this study induced similar behaviour related to accumulations in the leaf tissue. B is a micronutrient fundamental to the growth and development of trees, such as *Schizolobium parahyba*, because it contributes to the lignification of the stems (Hansch and Mendel, 2009).

The H_2O_2 levels in the leaf and root presented increases that were related to the excessive production of ROS, which resulted from the toxicity caused by the excessive supply of B (Gunes *et al*., 2006). H_2O_2 is one of the more stable ROS and overproduction frequently occurs in plants during stress conditions (Gill and Tuteja, 2010). Molassiotis *et al*. (2006) obtained similar results working with *Malus domestica* plants cultivated in different B concentrations.

The decrease of total GSH into both tissues can be associated to insufficient actuation of this compound in the antioxidant system in response to excess B. The boron-induced stress promoted the formation of increased amounts of H_2O_2, which suggests that total GSH does not contribute on non-enzymatic defence to remove or/and attenuate the cell oxidative damage (Wang *et al*., 2011; Dresler and Maksymiec, 2013). Li *et al*. (2012) also described a decrease in the total GSH levels in *Oryza sativa* plants.

The decrease in CHL *a* levels in plants of *S. amazonicum* var. *parahyba* subjected to excess B can be related to the minor biosynthesis of this pigment influenced by the limited absorption and transport of nitrogen (N) to the leaves. Cervilla *et al*. (2009) described that B toxicity reduces the assimilations of N forms, such as NO_3^- and NH_4^+ interfering also in the N organic concentration. This macronutrient is essential to CHL synthesis because the basic structure of this pigment is composed of one magnesium (Mg) ion in the centre of a porphyrin ring containing four N atoms (Streit *et al*., 2005). Kent *et al*. (2004) studying the behaviour of *Pinus banksiana* plants under B toxicity also observed a decrease in the CHL *a* level.

The reduction in CHL *b* must be the result of alterations in the functional state of the thylakoid membranes of the chloroplasts occasioned by the high B levels. Abiotic stresses, such as toxicity, can promote the disorganization of the thylakoids, which negatively affects the chloroplast structures and reduces the production of photosynthetic pigments, such as CHL b (Papadakis *et al*., 2004; Wang *et al*., 2011). Decrease in the CHL *b* level were described by Han et al. (2009) working with *Citrus grandis* under the stress of B in nutrient solution.

The reduction in total CHL levels in plants under excess B was induced by the simultaneous effects related to decreases in the amounts of CHL *a* and CHL *b*. Fávaro *et al*. (2011) reported decrease in CHL level in *Corymbia citriodora* plants exposed to B toxicity.

The increase in CAR concentration induced by B toxicity suggests that these pigments contributed to the protection mechanism of chlorophylls in young *S. amazonicum* plants. In agreement with Valladares *et al*. (2003), the CAR act as protection for the photosynthetic apparatus against the oxidation occasioned during stress conditions, such as B toxicity. Seth and Aery (2014) found increase in CAR studying the B effect in *Vigna radiata* plants.

The EL in the leaves presented an increase subsequent to B toxicity, and this effect is related to the accumulation of H_2O_2 in the cell. The H_2O_2 is responsible for the rupture and increase in membrane permeability, with the consequent liberation of cell contents and ions to the external medium. In agreement with Jambunathan (2010), the formation of H_2O_2 and OH^- occasioned higher rates of EL because they are highly reactive and interfere with the activities of the antioxidant enzymes. Apostol and Zwiazek (2004) observed an increase in EL when evaluating different B concentrations on the growth of *Pinus banksiana*.

The total AA accumulated in the leaf must be probably associated to protease enzyme activity and concomitantly to the inhibition of the protein biosynthesis rate, due to the high B levels. According to Paula *et al.* (2013), the increases in protease activities promote the breakdown and consequent decrease of proteins, increasing the total amount of AA, which are used in the synthesis of other AA, such as PRO. Cervilla *et al.* (2009) found similar results evaluating the effects of the B toxicity in *Solanum lycopersicum* plants.

The PRO concentration was maximized, which is related to the activity of this amino acid in the detoxification process of ROS and its role in protecting membranes against lipid peroxidation (Hong *et al.*, 2000; Cervilla *et al.*, 2012). PRO is frequently associated as an ROS antagonist that attenuates the oxidative stress that can cause cell death. This compost protects the protein structure against denaturation and stabilizes the cell membranes during interaction with phospholipids (Cervilla *et al.*, 2007). Contreras *et al.* (2011), evaluating the B and NaCl effects in *Solanum lycopersicum* plants, verified results similar to those found in this research.

Conclusions

This study found that B toxicity promoted similar behaviours in both leaf and root tissues, including progressive increases in H_2O_2, EL, AA and PRO and decreases in GSH, CHL *a*, CHL *b* and total CHL. These results confirm that PRO but not GSH actively participates in the tolerance mechanism of young *Schizolobium parahyba* plants exposed to B toxicity.

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