

## Biochemical metabolism of two cultivars of cowpea treated with 24-Epibrassinolide and subjected to saline stress

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### Abstract

We aimed to evaluate the changes in biochemical metabolism generated by salt stress and to investigate the effect of brassinosteroids in mitigating of this stress on two cultivars of *Vigna unguiculata* L. We used a completely randomized experimental design in a 2 x 3 x 3 factorial scheme, using two cultivars of cowpea (BRS Guariba and BR3 Tracuateua - moderately tolerant and sensitive to salinity, respectively), three concentrations of brassinosteroids (0, 0.2 and 0.4  $\mu$ M Br) and three concentrations of NaCl (0, 50 and 100 mM NaCl), with four replicates. The following evaluations were carried out: relative water content, electrolyte leakage, nitrate levels, nitrate reductase activity, free ammonium, total soluble amino acids, soluble proteins, glycine betaine and proline. The results showed that salinity at 100 mM affected the nitrate reductase enzyme activity, the relative water content, total soluble amino acids and soluble proteins for cultivars BR3 Tracuateua, and BRS Guariba, but the 24-epibrassinolid attenuated the effects of salinity for these variables. The concentration of 0.2  $\mu$ M of Br increased 55% and 20% in proline and glycine betaine contents, respectively, in both of cultivars. The plants under stress saline and 0.2 mM of 24-epibrassinolid, presented 42% and 58% reductions in electrolyte leakage of BR3 Tracuateua and BRS Guariba cultivars, respectively. The concentrations of ammonium were slightly varied. Therefore, the application of 0.2  $\mu$ M of 24-epibrassinolid caused a greater acclimatization of the cultivars, being the BR3 Tracuateua (sensitive to salt) cultivar more expressive in most treatments.

**Keywords:** BRS Guariba, BR3 Tracuateua, hormone, nitrogen, salinity.

**Abbreviations:** Br\_Brassinosteroids; DAS\_Day after sowing; CRD\_Completely randomized design; cv\_Cultivate; EBL\_Active form of brassinosteroids; EL\_Electrolyte leakage; TW\_Turgid weight; FW\_Fresh weight; DW\_Dry weight; RWC\_Relative water content.

### Introduction

The plants are affected by various of stress combinations in nature (Suzuki et al., 2014), like biotic and abiotic stress factors, limiting agricultural productivity, causing the loss of plant biomass and negative effects on growth, development, and consequently production of the vegetable (Nascimento et al., 2011). Among the abiotic stresses, salinity is among those that cause more damages to crops by reducing the osmotic potential of the soil, causing the physiological drought of plants invalidating a number of metabolic processes, as well as economic performance of yields (Major and Sales, 2012).

The cowpea bean is a crop of great potential and its grains are among the main sources of protein and energy for man, consisting of a rich food source with high fiber content, vitamins, minerals and a low amount of lipid (Frota et al., 2008). However, it is strongly affected by saline stress, which

promotes major metabolic disturbances and nutritional disorders in the specie (Hasegawa, 2013).

The saline soils impair the absorption of the nitrogen (N) mineral (Zahran, 1999) affecting several stages of metabolism of this element which is limiting to plant growth (Willians; Miller, 2001) and reducing protein synthesis (Frechill et al., 2003). In response to excess salts in the soil, the plants restrict the absorption and/or transport of ions from root to shoot by amplifying their biochemical mechanisms of tolerance to this adverse condition (Wei et al., 2003).

In this context, the study to find alternatives for adaptation of plants to the simultaneous action of multiple stress factors is highly demanded (Suzuki et al., 2014). The scientific evidences have pointed the activities of several hormones related to the joint response of plants to stress, including ethylene, abscisic acid, salicylic acid and brassinosteroids (Sharma et al., 2007). The brassinosteroid

(Br) are a new class of phytohormones with polyoxygenated structure endowed with pronounced growth-regulatory activity (Zullo and Adam, 2002).

This growth regulator is integrated to various physiological processes of positive responses to stress (Anuradha and Rao, 2003). Thus, the 24-epibrassinolid (EBL - active form of brassinosteroids) (Adam and Marquardt, 1986), can generally contribute positively on the physiological and biochemical parameters, improving the response to stress and change the structures and permeability of cells (Hamada, 1986).

Thus, the use of hormones to attenuate plant stresses are important tools to understand the metabolic processes involved in adapting the species. Because of its environmental, social and economic importance, study on cowpea in regions with saline soils has become fundamental in the scientific perspective. Therefore, addition of plant hormones on cultivation and value of cowpea in unfavorable areas is important.

There are many studies on the effects of salinity on the biochemical variables of cowpea. Besides, the action of the brassinosteroids to mitigate the harmful effects of this stress has been widely known. However, they do not clearly involve the processes of nitrogen metabolism. Therefore, this work focuses the nitrogen metabolism which contributes to the current knowledge on salt stress and the action of brassinosteroids on this specie of importance economic, social and environmental.

Therefore, the aim of this work was to evaluate the changes in biochemical metabolism generated by salt stress in two cowpea cultivars and to investigate the effect of brassinosteroids in mitigating the salt stress, in which, we hypothesized that the brassinosteroids will attenuate the deleterious effects of saline stress.

## Results

### *Relative water content and electrolyte leakage*

Through the results of this study it was possible to note that the RWC presented a general tendency of reduction with the increasing levels of salinity. The higher NaCl concentration significantly ( $p < 0.05$ ) reduced the water content and electrolyte leakage by 50% and 57%, respectively, in both cultivars compared to the control (Fig. 1 A).

We observed that plants under stress of NaCl 100 mM showed partial recovery of relative water content, when treated with 24-epibrassinolid. It was more evident in the concentration of 0.4  $\mu$ M, with 43% and 33% of increase ( $p < 0.05$ ) in both cultivars, compared to plants under the highest level of saline stress (Fig. 1 A).

Increasing levels of salinity promoted gradual increases in electrolyte leakage (EL) when compared to control plants under NaCl stress. So, the 100 mM salt treatment increased the EL significantly ( $p < 0.05$ ) by 97% and 62% in the leaf tissue of cultivars BR3 Tracueteua and BRS Guariba, respectively (Fig. 1 B). Plants under stress of 100 mM NaCl, presented significant reductions ( $p < 0.05$ ) of 42% and 58%, when nourished with 0.2 mM Br, for BR3 Tracueteua and BRS Guariba cultivars, respectively, when compared to plants treated with only 100 mM NaCl (Fig. 1 B).

### *Content of $\text{NO}_3^-$ and nitrate reductase activity*

There was a significant ( $p < 0.05$ ) reduction of 38% for the cv. BRS Guariba and 50% for the cv. BR3 Tracueteua in  $\text{NO}_3^-$  levels in saline stress treatments subjected to 100 mM NaCl compared to the control treatment (Fig. 2 A). The results indicated a possible attenuation of saline stress when the *V. unguiculata* plants were treated with brassinosteroids under 100 mM NaCl, showing that the nitrate levels cv. BRS Guariba was increased significantly ( $p < 0.05$ ) by 40% for the two concentrations of Br and the cv. BR3 Tracueteua by 33% for the concentration of 0.2  $\mu$ M of Br and 30% for the concentration of 0.4  $\mu$ M (Br).

In contrast, we observed that cultivar BR3 Tracueteua showed to be more expressive when submitted to 0.2  $\mu$ M Br, presenting values similar to those of the control (Fig. 2 A).

There was a reduction in the enzymatic activity of nitrate reductase as the saline stress was increased. At the concentration of 100 mM a significant ( $p < 0.05$ ) reduction of 87% for cv. BRS Guariba and 98% for cv. BR3 Tracueteua was occurred, compared to the control treatment (Fig. 2 B).

When the cultivars were treated with the two levels of the hormone they showed higher activity of nitrate reductase in relation to the NaCl concentrations. However, a higher recovery was observed in the plants treated with 0.2 and 0.4  $\mu$ M under 50 mM NaCl stress, in which the BRS Guariba was significantly ( $p < 0.05$ ) increased by 48% and 21% for two hormone levels, respectively. The increase in nitrate reductase BR3 Tracueteua was 33% and 68%, according to the level of hormones studied (Fig. 2 B).

### *Ammonium, amino acid content and soluble proteins*

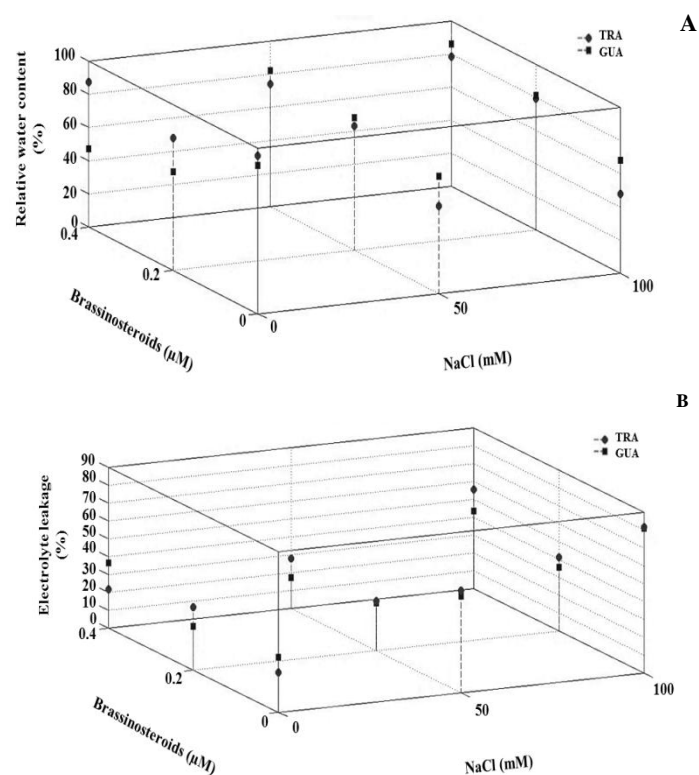
It was observed that the ammonium concentration suffered variations in leaf tissue of both cowpea cultivars under the effect of salt stress. As shown in Fig 3 A, the values found in plants under 100 mM NaCl, were slightly higher than the control - 14% and 17% to cultivate BR3 Tracueteua and BRS Guariba, concomitantly (Fig. 3 A).

Application of Br 0.2  $\mu$ M on cultivar BR3 Tracueteua showed significant increases of 36% in ammonium concentration compared to the control. In a divergent way, under the effect of the same treatment, there was 37% reduction in ammonium concentration in the leaf tissue of BRS Guariba (Fig. 3 A).

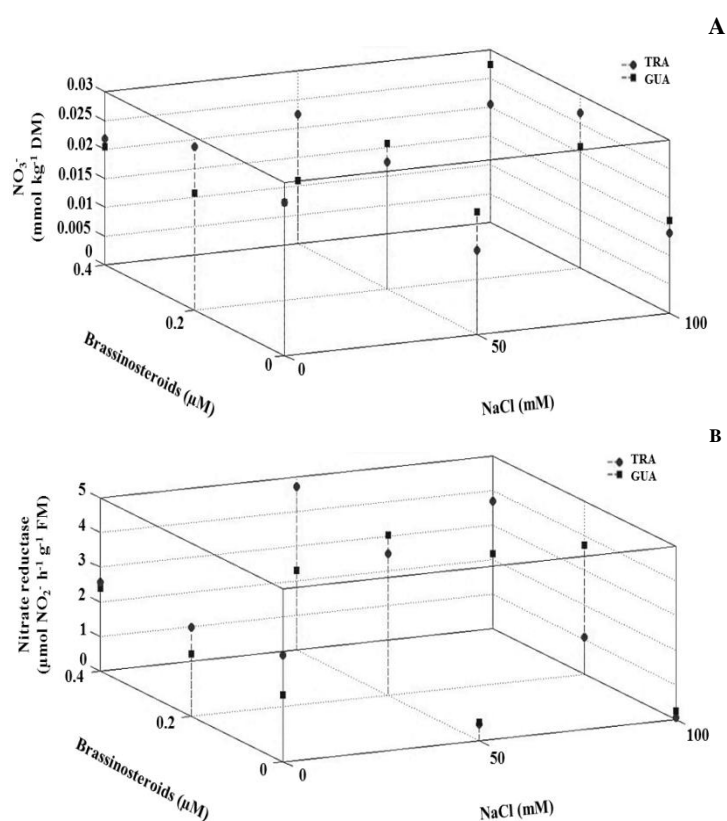
The results showed a reduction of amino acid content by 32% and 43% in BRS Guariba and 33% and 53% for BR3 Tracueteua in saline stress of 50 mM and 100 mM, respectively, compared to the control. The concentrations of brassinosteroids 0.2 and 0.4  $\mu$ M exerted great influence on the attenuation of acclimatization of salinity cultivars (Fig. 3 B).

It was observed that application of 0.2  $\mu$ M of Br on BR3 Tracueteua under stress of 100 mM NaCl causes a significant increase (72%) in amino acid content, compared to control plants. In BRS Guariba, there was slight variation in concentration of amino acid content, although they have also exhibited increase of 22% in the studied variable, as effect of the same treatment (Fig. 3 B).

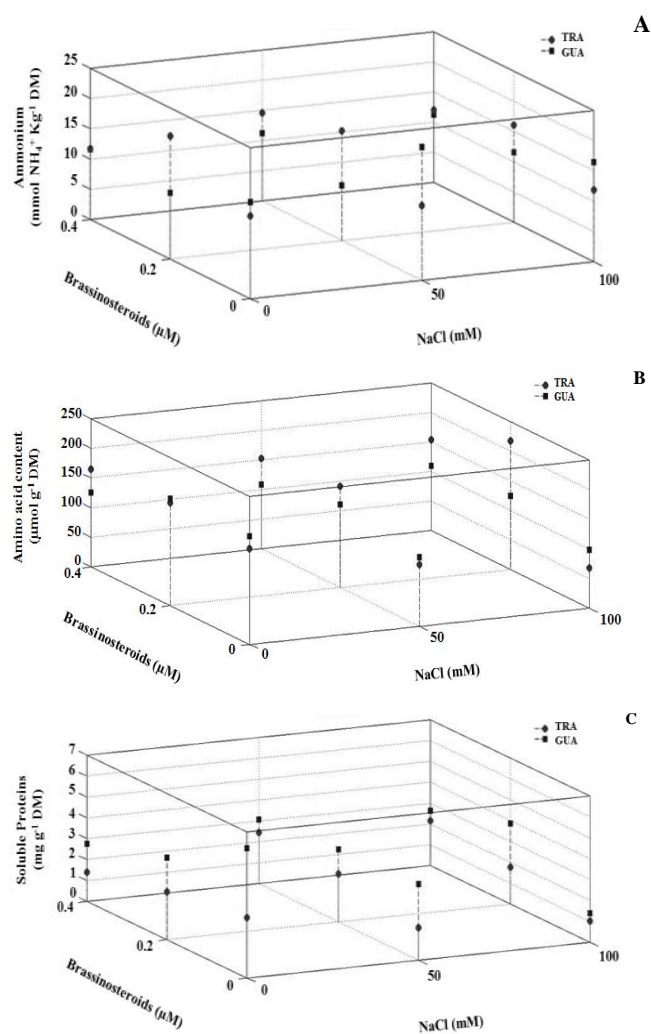
Among the results, soluble protein contents were decreased in saline concentrations of 100 mM, presenting a decrease of 66% and 77% for BR3 Tracueteua and BRS Guariba,



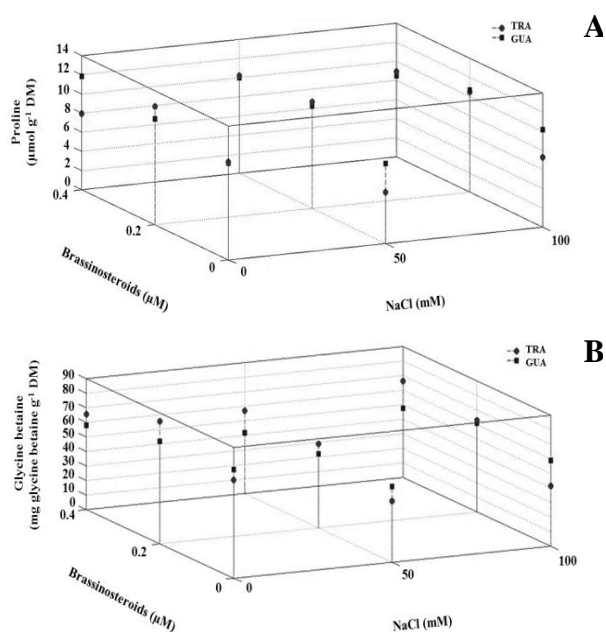
**Fig 1.** Relative water content (A) and Electrolyte leakage (B) in leaves of cultivars BRS Guariba and BR3 Tracuateua *Vigna unguiculata* (L.) Walp. grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.



**Fig 2.** Nitrate content (A) and Nitrate reductase activity (B) in leaves of cultivars BRS Guariba and BR3 Tracuateua of *Vigna unguiculata* (L.) Walp. grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.



**Fig 3.** Ammonium (A), Amido acid (B) and Soluble proteins contents (C) in leaves of cultivars BRS Guariba and BR3 Tracueteua of *Vigna unguiculata* (L.) Walp. grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.



**Fig 4.** Proline contents (A) and Glycine betaine contents (B) in leaves of cultivars BRS Guariba and BR3 Tracueteua of *Vigna unguiculata* (L.) Walp. grown in nutrient solution as a function of increasing concentrations of brassinosteroids (Br) and NaCl.

respectively, when compared to control plants. From the results found in this study, the concentrations of brassinosteroids are considered to have partially reversed effects. The leaf tissue of cv. BRS Guariba plants submitted to saline stress under treatment with 0.2  $\mu\text{M}$  of Br showed high protein content (by 64%), compared to plants under stress by 100 mM NaCl (Fig. 3 C).

However, the highest concentrations of Br (0.4  $\mu\text{M}$ ) did not show superior effects on proteins compared to 0.2 in both analyzed cultivars, under stress of 100 mM NaCl. This dosage of the hormone (0.4  $\mu\text{M}$ ) only increased 47% and 52% for BR3 Tracuateua and BRS Guariba, concomitantly (Fig. 3 C).

### Compatible osmolytes

The highest concentration of NaCl (100 mM) decreases the levels of proline and glycine betaine, where under the effect of such treatment proline concentration of leaves was 42% lower than control cultivar BR3 Tracuateua. Under the effect of the same concentration of NaCl, we observed concentration of only 11% osmolytes in leaf tissue of BRS Guariba, when compared to control plants (Fig. 4 A).

However, plants under saline stress of 100 mM treated with 0.2  $\mu\text{M}$  Br showed a considerable increase of proline and glycine betaine, by 55% and 20%, in foliar tissue of the cv. BR3 Tracuateua and BRS Guariba, respectively (Fig. 4 A).

Thus, it is considered that the concentration of 0.2  $\mu\text{M}$  induces tolerance in plants under NaCl stress. The results show that treatment with only 0.2  $\mu\text{M}$  of brassinosteroid induced increases in proline content in the leaves of both cultivars exceeding those observed in control plants (39% cv. BR3 Tracuateua and 32% cv. BRS Guariba) (Fig. 4 A).

Under saline stress of 100 mM, we observed decreases in glycine betaine content in the leaf tissue of cv. BRS Guariba by 21% when compared to that observed in the control treatment. However, the cv. BR3 Tracuateua, was considerably affected by saline stress, in which the glycine betaine content was reduced by 42%, when compared to the control treatment (Fig. 4 B).

The application of brassinosteroids induced an increase in glycine-betaine concentrations in both cultivars, showing that this hormone acted in the induction of the plant to acclimation to saline stress. However, it was more evident in cv. BRS Tracuateua at saline stress of 50 and 100 mM treated with 0.4  $\mu\text{M}$ , presenting increases of 36% and 52% in the content of this osmolyte for the concentrations of NaCl, respectively, compared to the foliar tissue of plants exclusively treated with 50 and 100 mM NaCl (Fig. 4 B).

The exclusive treatment with 0.2  $\mu\text{M}$  brassinosteroids was the one that best induced increases in glycine concentration in both cultivars. Under this concentration of Br increases in glycine concentrations were 23% (cv. BR3 Tracuateua) and 18% (cv. BRS Guariba) higher than the control plants and 53% (cv. BR3 Tracuateua) and 37% (cv. BRS Guariba) higher compared to plants under stress by 100 mM NaCl (Fig. 4 B).

### Discussion

The results suggest that the supplementation of saline solution with 24-epibrassinolide increased the tolerance of plants to saline stress, by which it promoted improvements in the water status evidenced by the increase of RWC in both cultivars studied. Possibly this hormone alters the leaf

osmotic potential, making the cell charge more negative. This could also be attributed to the influence of Br on further accumulation of compatible solutes as it was found in this research. Similar results were also found by Furtado et al. (2014), in which increase of saline level of irrigation water promoted a linear reduction of the relative water content in *Vigna unguiculata* L. (Walp.) plants, presenting a decrease of 2.36% by unit increment of the electrical conductivity of the irrigation water.

The attenuation of saline toxicity in the presence of Br was resulted from the probable reduction of  $\text{Na}^+$  content in the cellular tissues and by the possible dilution effect of the toxic salts, since the hormone increased the water content by the greater accumulation or synthesis of compatible solutes in the plant as reported in this study.

Although our study does not report the functioning of  $\text{H}^+$ -ATPase in conferring the exclusion of  $\text{Na}^+$  ions in cowpea plants, but the possible action of brassinosteroids may be attributed to high energy availability to the greater efficiency of these pumps, giving the plants a greater inducement to acclimation to salt stress by exclusion of  $\text{Na}^+$  ions of the cellular compartments.

The reduction of the nitrate concentration in plants treated with NaCl may also be related to the competition of ions  $\text{Cl}^-$  by conveyors of  $\text{NO}_3^-$  (Ogawa et al., 2000) and/or inactivation of nitrate transporters by the toxic effects of ions (Lin et al., 2002), affecting nitrogen absorption because  $\text{NO}_3^-$  is the main form that is absorbed by plants, which is reduced to ammonium by sequential reduction reactions. The sequential reduction reactions are catalyzed by the enzymes nitrate reductase, glutamine synthetase and glutamate synthase, leading to the synthesis of amino acids (Debouba et al., 2007).

The activity of nitrate reductase was directly affected by saline stress and this stress leads to water deficiency. This enzyme is reduced due to the low flow of water by the transpiratory current and the consequent decrease of nitrate flow to the leaves (Alves, 2010).

According to Moro (2012), nitrate reductase is often reduced in activity when subjected to high levels of water stress. Oliveira (2010) pointed out another characteristic of the enzyme to use of energy from photosynthesis, which regulates the supply of carbon skeletons for incorporation of nitrogen into amino acids.

The free ammonium accumulation in plant tissue in high concentrations of NaCl may be attributed to their direct uptake, nitrate reduction, deamination of nitrogenous compounds by photorespiratory cycle or by biological fixation (Horchani and Ashi-Smiti, 2010). Several authors have shown that plants under salt stress conditions promote an increase in the activity of the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) involved in ammonium assimilation, which are assimilated on the alternative route glutamate dehydrogenase (GDH) (Carvalho, 2012; Taiz and Zeiger, 2013).

The decrease in amino acid levels due to NaCl stress can be resulted from the reduction in protein synthesis and the association of mRNA (messenger RNA) with polysomes (Bagga et al., 2005). In this regard, it is considered that this reduction is closely related to the lower synthesis of proteins in stressed plants, the fact proven in this study through a considerable reduction in protein concentration.

In a study of two cultivars of cowpea in salt stress conditions, Praxedes et al. (2009) observed that there was an increase in the concentration of amino acids. According to these authors, a drain was occurred in response to accumulated excess nitrogen content due to reduced plant growth. This result; however, differs from the present study. It is considered that the experimental conditions and the phenological stage of the plants worked by the above mentioned authors have influenced the opposite results that have been obtained in this research.

In this study, the biochemical changes are occurred as the greater regulation in the synthesis of amino acids in the presence of the hormone, being inferred that the brassinosteroid contributed to the carbon and nitrogen ratio (C/N) balance in the plant in favor of the nitrogen.

To protein synthesis reduced when plants were subjected to the salt stress due to salinity to affect many metabolic pathways. It also promotes water stress, in which there is a change in the metabolic behavior of the plant degrading the proteins to amino acids. This process is an important indicator of physiological state of the plant (Demiral and Turkan, 2006).

However, possibly the concentrations of Br contributed to the synthesis of the proteins, keeping water levels in the leaves, answering with the cellular osmotic balance. In addition, some proteins that involve in hydrophilic interaction with cellular macromolecules may have been synthesized to establish the metabolism and act in the recovery from damage caused by stress (Streit et al., 2005).

The increases in proline content were possibly generated by disturbances in the metabolism of proteinases, probably to maintain the water potential of the leaf. Increase of the content of this amino acid induces the synthesis and the accumulation of compatible solutes in the cytosol, as a way to balance the Osmotic pressure promoted by the excess of toxic ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ) in the vacuole. This stabilizes the membranes and to maintain the conformation of proteins, preventing the dehydration of the cytosol (Kishor et al., 2005; Munns and Tester, 2008).

The increase of glycine betaine probably occurred to protect plant metabolism, since glycine betaine can act as a compatible solute by maintaining the water balance between the plant cell and the environment, stabilizing the macromolecules (Carlin and Santos 2009). The metabolic pathways, by which brassinosteroids act on the plant and gives the induction of tolerance to salt stress, are still not well-understood. However, from our results, it is considered that the hormonal regulation is involved in the synthesis of compatible osmolytes such as proline and glycine betaine.

## Materials and methods

### Plant material and culture conditions

The experiment was conducted from July to August 2015 in the greenhouse of Federal Rural University of Amazonia Belém, Pará, Brazil, located at 01°28'03" S; 48°29'18" W. For this study two cultivars of *Vigna unguiculata* (L.) Walp., BRS Guariba and BR3 Tracuateua (moderately tolerant and sensitive to salinity, respectively) from the Germplasm Bank of Embrapa Eastern Amazon were used. The biochemical and physiological analyzes were made in Laboratory of

Biodiversity Studies of Upper Plants (EBPS), belonging to the Institute of Agrarian Sciences (ICA) of the university campus.

### Nutrient solution, salt and brassinosteroids concentrations

At 7 days after sowing (DAS), when the plants threw the first pair of leaves, the cowpea seedlings were transplanted to 1000 mL capacity pot-vessel cups containing 500 mL nutrient solution Hoagland and Arnon (1950) with  $\frac{1}{4}$  of ionic strength in pH  $5.5 \pm 0.5$ . After the second pair of leaves, the solution was modified to  $\frac{1}{2}$  of ionic strength. The 24-epibrassinolid was purchased of the Sigma. It was dissolved in ethanol ( $1 \text{ mg mL}^{-1}$ ) diluted in this same solution.

The plants were subjected to the treatment with brassinosteroids (Br) in concentrations of 0.2 and 0.4  $\mu\text{M}$ , and remained under this condition for 7 days for acclimation. After this period, the plants were subjected to the saline treatment (NaCl) in the concentration of 50 and 100 mM for 7 days. Treatments with doses of zero refers to the control treatment.

### Collection and storage of plant material

The collection of the plants occurred at 24<sup>th</sup> DAS, 04:30 AM. The determination of the relative water content was performed *in vivo*. On that occasion, primary leaves fully expanded from each of the repetitions, fresh, were reserved for determining the activity of nitrate reductase (NR) and electrolyte leakage (EL).

Subsequently, the leaves were wrapped in aluminum paper and stored in a freezer at  $-80^\circ\text{C}$ . For the determination of biochemical analysis, the material was taken to the oven with ventilation of forced air of  $65^\circ\text{C}$  for 48 h. After drying, the dry weight of the sheet was determined. The dried material was ground in mill to obtain a fine powder being properly stored in Falcon tubes until used in the assays.

### Relative water content and Electrolyte leakage

The RWC was calculated according Slavick (1974), using the following formula:

$$\text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Electrolyte leakage was determined by Blum and Ebercon (1981). 100 mg of leaf was weighed separately, where they were stored in Falcon tubes with 10 mL of deionized water, remaining undisturbed for 6h (room temperature;  $25^\circ\text{C}$ ). After that period, the electrical conductivity was measured (L1). Subsequently, the Falcon tubes were heated in water bath at  $100^\circ\text{C}$  for 1 hour and then the second reading was held (L2). EL was estimated by the equation:

$$\text{EL(\%)} = (\text{L1/L2}) \times 100$$

### Experimental design

A completely randomized experimental design (DIC) was used in a  $2 \times 3 \times 3$  factorial scheme, being two cowpea cultivars. Three brassinosteroid concentrations (0, 0.2 and 0.4  $\mu\text{M}$  Br) and three concentrations of NaCl (0, 50 and 100 mM NaCl), composed of 4 replicates.

## Nitrogen compounds

### Content of $\text{NO}_3^-$ and nitrate reductase activity

With the aid of a stainless steel driller foliar discs were collected *in vivo*, according to the method described by Hageman and Hucklesby (1971). Nitrate levels were determined according to Cataldo et al. (1975), in a reaction vial containing 100  $\mu\text{L}$  of extract and 200  $\mu\text{L}$  salicylic acid 5% solution (w/v) in concentrated sulfuric acid.

### Content of Ammonium

Free ammonium was determined using the method by Weatherburn (1967).

### Content of Soluble amino acid

For the determination of total soluble amino acid, the method used was the second Peoples et al. (1989).

### Content of Soluble protein

Determined by the method described by Bradford (1976).

### Content of Proline

For this, we used the method described by Bates et al. (1973).

### Content of Glycine betaine

The method utilized was according to Grieve and Grattan (1983).

### Statistical analysis

The data were subjected to statistical analysis using analysis of variance (ANOVA) in Sisvar program version 5.4, and the averages compared by Tukey test at 5% probability.

### Conclusion

The concentrations of brassinosteroids reversed the effect of saline stress on plants and the pathways of hormonal action were involved in the better efficiency of nitrogen metabolism by cultivars, in which the use of 0.2  $\mu\text{M}$  of 24-epibsinolide enabled the acclimation of *Vigna unguiculata* L. under saline stress, being the BR3 Tracuateua cultivar more expressive in most treatments.

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