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Original Article

Mitigation of the effects of salt stress in cowpea bean through the exogenous aplication of brassinosteroid

Mitigação dos efeitos do estresse salino em feijão-caupi através da aplicação exógena de brassinosteroide

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Abstract

Cowpea (*Vigna unguiculata* (L.) Walp.) is a legume widely cultivated by small, medium and large producers in several Brazilian regions. However, one of the concerns for the production of cowpea in Brazil in recent years is the low rainfall activity in these regions, which generates the accumulation of salts on the surface. The objective of this work was to evaluate the effects of salt stress on growth parameters and enzyme activity in cowpea plants at different concentrations of brassinosteroids. Experiment was developed in a greenhouse using a completely randomized experimental design in a 3 x 3 factorial scheme. The treatments consisted of three levels of brassinosteroids (0, 3 and 6 μM EBL) and three levels of salt stress (0, 50 and 100 mM NaCl). Growth factors (height, diameter and number of leaves) decreased in the saline condition. With the presence of brassinosteroid the height did not increase, but the number of leaves did, mainly in the saline dosage of 100 mM NaCl. In the variable membrane integrity, brassinosteroid was efficient in both salinity dosages, the same not happening with the relative water content, where the saline condition did not affect the amount of water in the vegetable, with the application of brassino it remained high, decreasing only at dosage 100 mM NaCl. The nitrate reductase enzyme was greatly affected in the root system even with the application of increasing doses of brassino. Therefore, brassinosteroids as a promoter of saline tolerance in cowpea seedlings was positive. The concentration of 3μM of EBL provided the most satisfactory effect in tolerating the deleterious effects of the saline condition. The same cannot be concluded for the concentration of 6μM of EBL that did not promote tolerance to some variables.

Keywords: biomass, enzyme, legume, phytohormones, salinity.

Resumo

O feijão-caupi (*Vigna unguiculata* (L.) Walp.) é uma leguminosa amplamente cultivada por pequenos, médios e grandes produtores em diversas regiões brasileiras. No entanto, uma das preocupações para a produção de feijão-caupi no Brasil nos últimos anos é a baixa atividade pluviométrica nessas regiões, o que gera o acúmulo de sais na superfície. O objetivo deste trabalho foi avaliar os efeitos do estresse salino nos parâmetros de crescimento e atividade enzimática em plantas de feijão-caupi em diferentes concentrações de brassinosteróides. O experimento foi desenvolvido em casa de vegetação com delineamento experimental inteiramente casualizado em esquema fatorial 3 x 3. Os tratamentos consistiram de três níveis de brassinosteróides (0, 3 e 6 μM EBL) e três níveis de estresse salino (0, 50 e 100 mM NaCl). Os fatores de crescimento (altura, diâmetro e número de folhas) diminuíram na condição salina. Com a presença do brassinosteroide a altura não aumentou, mas o número de folhas sim, principalmente na dosagem salina de 100 mM NaCl. Na variável integridade de membrana, o brassinosteróide foi eficiente em ambas as dosagens de salinidade, o mesmo não ocorrendo com o teor relativo de água, onde a condição salina não afetou a quantidade de água na hortaliça, com a aplicação de brassino manteve-se elevada, diminuindo apenas na dosagem de NaCl 100 mM. A enzima nitrato redutase foi bastante afetada no sistema radicular mesmo com a aplicação de doses crescentes de brassino. Portanto, o uso de brassinosteroides como promotor de tolerância salina em plântulas de feijão-caupi foi positivo. A concentração de 3μM de EBL proporcionou o efeito mais satisfatório em tolerar os efeitos deletérios da condição salina. O mesmo não pode ser concluído para a concentração de 6μM de EBL que não promoveu tolerância a algumas variáveis.

Palavras-chave: biomassa, enzima, leguminosa, fitormônio, salinidade.

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1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is a grain legume of great importance for human diet and is one of the main sources of proteins for low-income populations, especially in Central and South America, and in Africa (Oliveira et al., 2015). In Brazil, the regions that most consume this legume are the North and Northeast, and the projection of total cowpea production in these two regions for the 20/21 harvest is around 35.3 thousand tons (Pereira et al., 2016).

One of the concerns for the production of cowpea in Brazil, especially in the Northeast region, is the low average rainfall and high evaporation of water from the soil, which contribute to the accumulation of salts on its surface and is enhanced in irrigated areas with poor drainage management, and insufficient or low-quality water for irrigation, or even the combination of these factors (Ribeiro et al., 2016).

Photosystem II is a salt-sensitive component of the photosynthetic machinery, since it can cause reductions in the efficiency of this photosystem and in the electron transport chain, resulting in a decrease in photosynthetic activity and consequently in plant growth (Parihar et al., 2015).

For Marques et al. (2011), the salinity in the early development of seedlings, alters their metabolism, leading to inhibition of the reserves mobilization and disturbances in the membrane system of the embryonic axis.

Management practices can be adopted to maximize tolerance to saline conditions, and among these practices, the application of plant regulators stands out (Rostami and Movahedi, 2016). The application of some phytohormones can help the plant to overcome the harmful effects of salt stress and other abiotic stresses (Liu et al., 2018).

Among the phytohormones, the brassinosteroids (BRs) are associated with the adaptation of plants to environmental stress through stimuli in enzymatic complexes that act in the elongation of stems and roots, increase in biomass, seed germination and protection from reactive oxygen species because it is involved in the activation of protective mechanisms against oxidative stresses, by causing changes in the fatty acid composition of cell membranes (Freitas et al., 2015). Increased plant growth and development rates (Wei and Li, 2016). They participate in the osmotic adjustment (turgescence), followed by the accumulation of nutrients (Hasanuzzaman et al., 2013).

The application of brassinosteroids may increase the resistance of cowpea plants in situations of salt stress, thus, their use may represent an important method to increase crop productivity. Due to these facts, the objective was to evaluate the effects of salt stress on growth parameters and enzymatic activity in cowpea plants at different concentrations of brassinosteroids.

2. Material and Methods

2.1. Plant material and growing conditions

The experiment was carried out in a greenhouse condition at the Institute of Agrarian Sciences – ICA, at the Federal Rural University of Amazonia on the geographic coordinates 01° 27' 21" S, 48° 30' 16" W and an average altitude of 10 meters.

Seeds of the "Tumucumaque" cultivar, which is classified as a salt stress tolerant cultivar, from the EMBRAPA Germplasm Bank were used. The seeds were soaked in the brassinosteroid solution for 6 hours at room temperature. Soon after, the seeds were sown in adapted leonard-type pots, containing washed and sterilized sand as substrate. Three seeds were placed in each pot, and daily irrigations were carried out with distilled water. After 11 days of growth, thinning was carried out, leaving only one plant per pot.

Five days after sowing, the seedlings started to receive weekly 800 mL of Hoagland nutrient solution (Hoagland and Arnon, 1950), with a quarter of ionic strength. The solution was changed every seven days keeping the pH at 5,8 ± 6.0 using NaOH or HCl solution. In the third application of nutrient solution, it was necessary to change the ionic strength to half strength.

2.2. Exposure of plants to NaCl and brassinosteroids

The nutrient solution with sodium chloride (NaCl) was added, for the first time, to the plants after 33 days after sowing (DAS) at concentrations of 0, 50 and 100 mM NaCl. The 24-epibrassinolide was dissolved in ethanol (1mg/ml) and distilled water to concentrations of 0, 3 and 6 µM in which it was used in seed soaking and 1 ml was applied every seven days on the plant neck, for hormone absorption by the roots.

2.3. Experimental design and statistical design

A completely randomized experimental design was used with a 3 x 3 factorial scheme, totaling 9 treatments with 5 replicates each, with a total of 45 experimental units. Each experimental unit consisted of 1 plant. The program used for statistical analysis was the Sisvar v. 5.6, where the Tukey test was performed at a 5% probability.

2.4. Material collection and storage

Plant collection took place at 52° DAS, where fresh leaf and root material were removed to determine electrolyte leakage (EL), relative water content (RWC) and to determine nitrate reductase activity (RN). The plants were separated into shoots and roots, wrapped in aluminum foil, and stored in a freezer at -80ºC. For the dry weight evaluation and for biochemical analysis, the remaining fresh material was taken to a forced air ventilation oven at 65° for 48 hours till constant weight. Next, the dry mass of roots and leaves was determined.

2.5. Biometric evaluation

The biometric evaluation was performed before the collection of the plant material, right at the 51 DAS. The shoot height (cm) was measured with a millimeter ruler, from the region of the plant neck to the insertion of the last pair of expanded leaves. The diameter of the stem (mm) was determined with a digital caliper, in the stem region at the level of the substrate. The number of leaves was determined by weekly counting the leaves from the start of the of the salt stress treatments to the end of the experiment.

2.6. Biochemical analysis

2.6.1. Relative Water Content (RWC)

For the CRA, 10 leaf discs were collected using a stainless-steel leafcutter and immediately weighed on an analytical scale to obtain the fresh weight (FW), then placed in a petri dishe containing 35 mL of distilled water and left at room temperature (25°C) for a period of 12 hours to saturate.

Soon after, the discs were placed on sheets of filter paper to remove excess water and immediately weighed to determine the turgid weight (TW). Then, they were stored in paper bags and taken to a forced air ventilation oven at 72°C, for 48 h till constant weight. The now dry discs were weighted to obtain their dry weight (DW). The C.R.A was calculated according to Slavíck (1974) (Equation 1).

$$\mathbf{RWC} = \left(\mathbf{FW} - \mathbf{DW}\right) / \left(\mathbf{TW} - \mathbf{DW}\right) \times \mathbf{100} \quad (1)$$

2.6.2. Electrolyte leakage (membrane integrity)

The EL was determined accordingly to Blum and Ebercon (1981), with modifications. 100 mg of the leaves were weighed separately, followed by a triple washing with deionized water. Then, the plant material was transferred to test tubes, to which 10 ml of deionized water were added. The closed vials were left to stand at room temperature (25°C) for 6 hours, with stirring every 20 minutes.

After this period, with an electrical conductivity meter, the electrical conductivity of the solution was measured, determining the first reading (L1). Afterwards, the tubes were closed again and heated in a water bath at 100°C for 1 hour. After cooling the test tubes to room temperature, the electrical conductivity of the extract was measured again, obtaining the second reading (L2) (Equation 2).

$$\mathbf{EL}\ \left(\%\right) = \left(\mathbf{L1} / \mathbf{L2}\right) \times \mathbf{100} \quad (2)$$

2.6.3. Nitrate reductase

Leaf discs were collected with a stainless-steel leafcutter, in vivo, according to the method described by Hageman and Hucklesby (1971). Approximately 200 mg of the leaf discs were weighed and placed in falcon test tubes, wrapped with aluminum foil containing phosphate buffer 0.1 mM potassium; pH 7.5; 50 mM KNO3; and 1% isopropanol (v/v).

After this step, the tubes were properly sealed with a plastic stopper and wrapped with Parafilm™, then vacuumed for 2 minutes. After vacuumed, they were taken to a water bath at 30°C for 30 minutes. In a common test tube, 2.0 mL of buffer + 1.0 mL of the reaction extract + 1.0 mL of 1% sulfanilamide + 1.0 mL of 0.02% NNEDA were added, leaving it to rest for 15 minutes. Absorbance was determined at 540nm in a spectrophotometer with results expressed in μmoles of NO_2^- g MF^{-1} h^{-1}.

3. Results

As the sodium chloride (NaCl) concentrations increased, and without the application of 24-epibrassinolide (EBL), the plant height of the cowpea seedlings decreased (Figure 1). With the application of EBL, we could not observe any mitigation of the salt stress effects on plant height. In fact, both EBL applications presented a negative effect on this variable, decreasing plant height in plants without NaCl and under moderate stress (50 mM NaCl), while in plants under severe stress (100 mM NaCl), EBL applications did not affect the plant height (Figure 1).

The number of leaves decreased under saline conditions without EBL. When applied, the hormone 24-epibrassinolide in both the 3 μM and 6 μM concentrations increased the number of leaves only under severe stress (100 mM NaCl). However, in the control (0 mM NaCl) and moderate stress (50mM NaCl treatment), the application of both EBL treatments did not produce any statistically significant effect (Figure 2). The application of EBL mitigates the effect of salt stress only in the highest concentration of salt, and

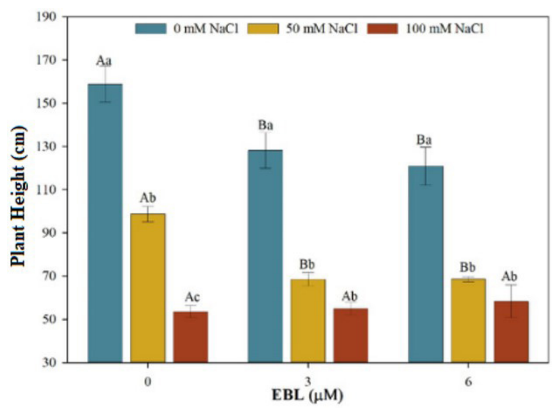

Figure 1. Effect of 24-epibrasinolide in the height of cowpea plants under salt stress. Capital letters indicate statistical differences between EBL treatments ($p < 0.05$) based on upon a Tukey's test; small letters indicate statistical differences between salt treatments ($p < 0.05$) based on upon a Tukey's test.

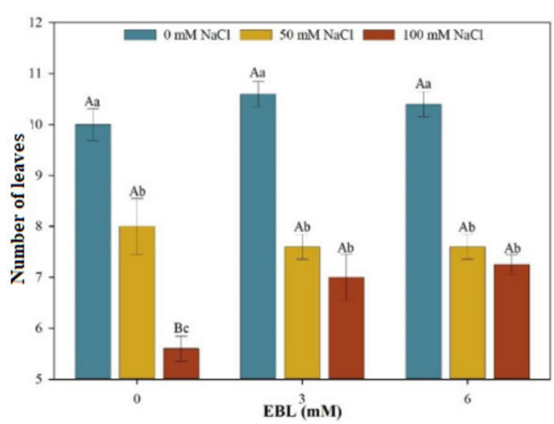

Figure 2. Effect of 24-epibrasinolide in the number of leaves of cowpea plants under salt stress. Capital letters indicate statistical differences between EBL treatments ($p < 0.05$) based on upon a Tukey's test; small letters indicate statistical differences between salt treatments ($p < 0.05$) based on upon a Tukey's test.

the increase in EBL concentrations from 3 to 6 μM did not increased the stress mitigation effect of this hormone in regard to this variable.

For the stem diameter, the stress conditions caused by the application of different concentrations of NaCl, without the application of EBL, decreased the cowpea growth not only in height (Figure 1), but also in diameter (Figure 3). Under both EBL treatments, the plants did not present the normal decrease in diameter with the increase of salt concentration, as the diameter did not differ between salt treatments. However, the increase in EBL concentrations from 3 to 6 μM had a negative effect in the stem diameter in every salt treatment, as even the non-stressed plant under the 6 μM treatment reached similar growth levels to the plants under severe stress without EBL (Figure 3).

The electrolyte leakage was negatively influenced by the increase of NaCl concentrations, as it increased in treatments with or without EBL applications (Figure 4). The application of EBL, especially in the 3 μM treatment, provided a decrease in the electrolyte leakage and indicated a positive effect of EBL on membrane integrity (Figure 4). The increase of EBL concentration from 3 to 6 μM EBL was not enough to reduce the electrolyte leakage in any salt stress levels.

The relative water content of cowpea plants for the 50mM NaCl treatment increased, but it did not statistically differ from the other treatments with 100mM NaCl and without EBL (Figure 5). When EBL was applied in the concentrations of 3 μM and 6 μM, the relative water content of the plants under stress (50mM NaCl e 100mM NaCl) decreased, but no statistical difference appeared. However, the 3 μM EBL treatment was the one who better expressed the increase in relative water content (Figure 5).

The enzymatic functionality of the nitrate reductase (NR) in the leaves was altered by the different salt stress treatments without EBL application, reaching values close to zero under severe stress (Figure 6). However, with EBL applications, the NR activity increased under stress

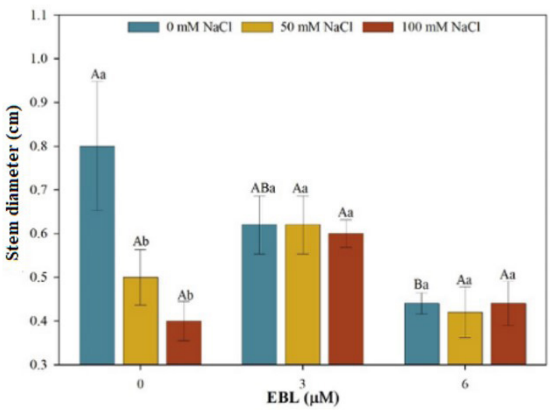

Figure 3. Effect of 24-epibrasinolide in the stem diameter of cowpea plants under salt stress. Capital letters indicate statistical differences between EBL treatments ($p < 0.05$) based on upon a Tukey's test; small letters indicate statistical differences between salt treatments ($p < 0.05$) based on upon a Tukey's test.

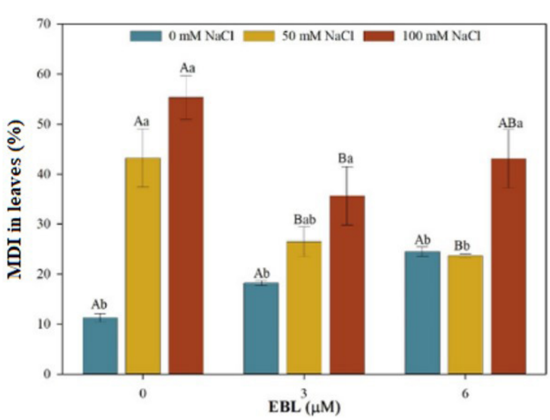

Figure 4. Effect of 24-epibrasinolide in the electrolyte leakage of cowpea plants under salt stress. Capital letters indicate statistical differences between EBL treatments ($p < 0.05$) based on upon a Tukey's test; small letters indicate statistical differences between salt treatments ($p < 0.05$) based on upon a Tukey's test.

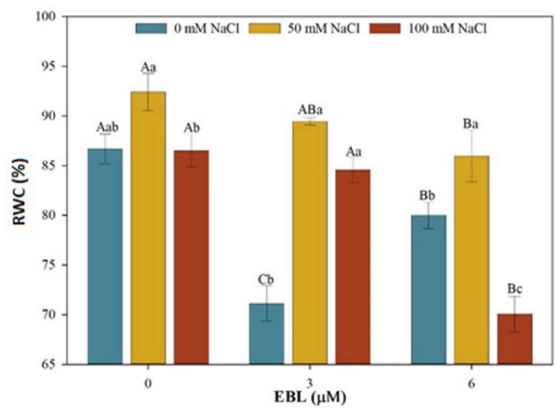

Figure 5. Effect of 24-epibrasinolide in the relative water content of cowpea plants under salt stress. Capital letters indicate statistical differences between EBL treatments ($p < 0.05$) based on upon a Tukey's test; small letters indicate statistical differences between salt treatments ($p < 0.05$) based on upon a Tukey's test.

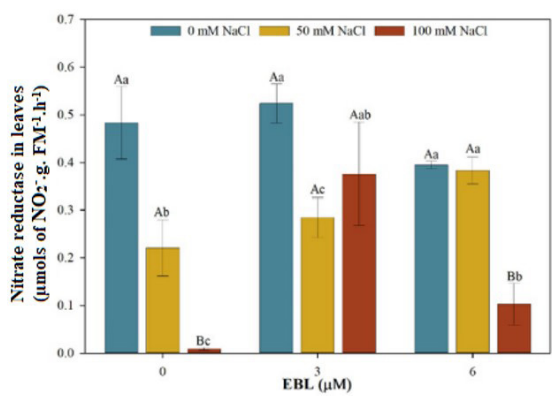

Figure 6. Effect of 24-epibrasinolide in the activity of the enzyme nitrate reductase of cowpea leaves under salt stress. Capital letters indicate statistical differences between EBL treatments ($p < 0.05$) based on upon a Tukey's test; small letters indicate statistical differences between salt treatments ($p < 0.05$) based on upon a Tukey's test.

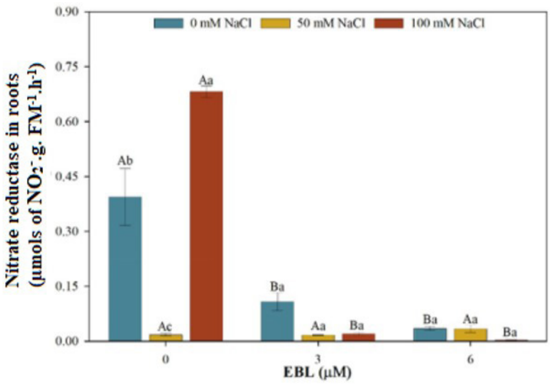

Figure 7. Effect of 24-epibrasinolide in the activity of the enzyme nitrate reductase of cowpea roots under salt stress. Capital letters indicate statistical differences between EBL treatments ($p < 0.05$) based on upon a Tukey's test; small letters indicate statistical differences between salt treatments ($p < 0.05$) based on upon a Tukey's test.

conditions. The highest values were for NR activity under moderate stress were attained with the application of 6 μM of EBL, while under severe stress, the application of 3 μM of EBL demonstrated better results (Figure 6).

The NR activity in the root system without the application of EBL decreased with moderate stress, while increasing with severe stress (Figure 7). With the application of EBL in both concentrations there was a significant decrease in the activity of this enzyme, revealing the inefficiency of the hormone regarding the NR in the roots (Figure 7).

4. Discussion

4.1. Growth variables

The salinity decreases the plant growth through the decrease in cellular expansion due to low water absorption, followed by the toxic of the concentrated Na^+ ions in the protoplasm (Ferreira and Borguetti, 2004). This toxic effect compromises the biomass production of the shoot, influencing the number of leaves and plant height (Figueredo et al., 2018). Working with red dragon fruit, Oliveira et al. (2024) also observed that salinity compromises the shoot and root systems.

Despite brassinosteroids role in the regulation of several key genes in physiological and plant growth processes (Anwar et al., 2018), it was not enough to increase the plant height under stress conditions. However, for phytohormone efficiency, it is necessary to study the method of application, specie, climate, development stage and other factors (Jun et al., 2018).

The decrease in the number of leaves with increasing salt concentrations occurs due to the negative effect of salts in the osmotic potential, and as the osmotic potential became more negative, the smaller the number of leaves produced (Cruz et al., 2020). This effect is because these ions reduce the capability of water and nutrients absorption, from there, the plant tends to undergo constant stomatal closure and consequently less CO_2 fixation. In this situation, there is less production of photoassimilates and thus, reducing the development rate, resulting in fewer and smaller leaves (Esteves and Suzuki, 2008).

The effect of the epibrassinolide in this variable was positive, due to the probable action of this hormone in the activation of certain enzymes of the photosynthetic process, such as the ribulose-1,5-bisphosfate carboxilase/ oxygenase (RubisCO) and Phosphoenolpyruvate carboxylase (PEPcase) (Nolan et al., 2020).

4.2. Physiological variables

Physiologically speaking, the salinity decreases the turgor pressure within the cells, due to the reduction of water content in the tissues, causing a decline in cell wall expansion and, consequently, decreasing plant growth as observed in the stem diameter (Silva et al., 2017; Bezerra et al., 2019).

The plasmatic membrane is the main tissue affected by the absorption of Na^+ and Cl^- ions, and the elevated concentration of Na^+ can damage the plasmatic membranes and alter their selectivity (Pacheco et al., 2013). The excessive disorders in water quantity due to the sodium chloride ions on the plant membrane tissues allowed structural changes, probably the advance of the oxidative stress and protein denaturation (Heringer et al., 2013).

The application of EBL promoted the reduction of the integrity of those membranes, this biochemical response occurred due to the brassinosteroids membrane permeability regulation and its connection to membrane proteins. Increasing the enzymatic and metabolic activity to promote detoxification in stresses plants (Shahzad et al., 2018)

The reduction of the relative water content in most crops is a recurrent action when there is the increase of soil salinity (Ferraz et al., 2015), negatively affecting the water absorption by the roots (Pedrotti et al., 2015). One of the explanations for the decrease in water content can be the accumulation of Na^+ and Cl^- on leaf tissues and the decrease of cellular osmotic potential (Silva Júnior et al., 2016; Souza et al., 2019).

The hormone epibrassinolide maintained high water content in the plant tissues, mainly on the 3mM dosage of EBL. This is justified by the fact that this hormone is efficient increasing the resistance to saline conditions (Vardhini, 2012). Possibly, there was the deposition on the leaf cell walls, forming a double protector layer, avoiding water losses under these conditions, due to the lower rate of plant evapotranspiration (Kaya et al., 2006).

4.3. Enzymatic variables

The nitrate reductase is a key enzyme in the regulation of nitrogen assimilation in plants, in this study we can observe that the enzymatic activity was higher in the leaves rather than on the roots. One probable reason for the increase in the activity of this enzyme in this specific organ is that there was a higher absorption on NO_3^-, since this ion induces the activity of NR though the modulation of its gene expression, increase its transcription (Ehlting et al., 2007). On the other hand, the reduction of NR activity in the root system, can be attributed to the inhibition of NO_3^- absorption by Cl^-, reducing the substrate concentrations on the enzyme active site (Nathawat et al., 2005). For Ashraf et al. (2018), they related that the salinity affects the enzymatic activity of the enzymes from the nitrogen metabolism.

According to Chaves et al. (2002), some metabolical changes, such as the reduction of the activity of the enzyme NR occurs as a result of the osmotic stress caused by the salinity, and these changes contribute to maintaining the osmotic pressure within the photosynthetic cells by increasing the concentration of nitrate and reducing the flow of carbohydrate.

The non-significant involvement of the hormone epibrassinolide on the enzymatic activities of the NR, mainly in the root system, is probably due to the low correlation of increased gene expression (Divi et al., 2016).

5. Conclusion

The use of brassinosteroids as a promoter of tolerance to saline condition in cowpea seedlings was positive, promoting attenuation of saline effects, mainly in shoots.

The concentration of 3µM of EBL provided the most satisfactory effect in tolerating the deleterious effects of the saline condition. The same cannot be concluded for the concentration of 6µM of EBL that did not promote tolerance to some variables.

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