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Revista Facultad Nacional de Agronomía

Mitigation of salt stress in cowpea cultivars by brassinosteroids: physiological and antioxidant responses

Mitigación del estrés salino en cultivares de caupí mediante brasinoesteroides: respuestas fisiológicas y antioxidantes

https://doi.org/10.15446/rfnam.v79.118824

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ABSTRACT

Cowpea (*Vigna unguiculata* (L.) Walp.) is a crucial crop for food security in tropical regions, but its productivity is severely limited by soil salinity. Brassinosteroids (BR) have emerged as promising natural regulators that can enhance plant resilience to abiotic stresses. This study aimed to evaluate the mitigating effect of BR on salt stress in two cowpea cultivars with contrasting tolerance (BRS Tapaihum, tolerant; Manteiguinha, sensitive) by analyzing physiological and biochemical parameters. A completely randomized design was employed in a 2x3x3 factorial scheme, combining two cultivars, three NaCl concentrations (0, 75, and 150 mM), and three BR concentrations (0, 0.2, and 0.4 μM). Key analyses included electrolyte leakage, leaf Na^+ and K^+ content, and the activity of antioxidant enzymes, alongside lipid peroxidation (MDA) assessment. Salinity stress (150 mM NaCl) significantly increased electrolyte leakage and sodium accumulation while decreasing potassium content. The activity of antioxidant enzymes was elevated under salt stress. Foliar application of BR significantly mitigated these effects by reducing Na^+ accumulation, enhancing K^+ retention, improving the K^+/Na^+ ratio, and modulating antioxidant enzyme activity, which consequently decreased oxidative membrane damage (MDA). The tolerant cultivar, BRS Tapaihum, consistently exhibited a superior response to BR application compared to the sensitive Manteiguinha. These results demonstrate that BR application effectively enhances salt stress tolerance in cowpea by improving ionic homeostasis and reinforcing the antioxidant system. This suggests its strong potential as a biostimulant for sustainable cultivation in salinity-affected areas.

Keywords:
Antioxidant enzymes
Ionic homeostasis
Lipid peroxidation
Plant biostimulant
Vigna unguiculata

CITATION: Lobo LFF, Nogueira GA dos S, Teixeira de Oliveira J, Silva PA and Ferreira de Oliveira Neto C (2026) Mitigation of salt stress in cowpea cultivars by brassinosteroids: physiological and antioxidant responses. Revista Facultad Nacional de Agronomía Medellín 79: e118824. doi: https://doi.org/10.15446/rfnam.v79.118824

RESUMEN

El caupí (*Vigna unguiculata* (L.) Walp.) es un cultivo crucial para la seguridad alimentaria en las regiones tropicales, pero su productividad está severamente limitada por la salinidad del suelo. Los brasinoesteroides (BR) han emergido como prometedores reguladores naturales que pueden mejorar la resiliencia de las plantas a los estreses abióticos. Este estudio tuvo como objetivo evaluar el efecto mitigador de BR sobre el estrés salino en dos cultivares de caupí con tolerancia contrastante (BRS Tapaihum, tolerante; Manteiguinha, sensible) mediante el análisis de parámetros fisiológicos y bioquímicos. Se empleó un diseño completamente aleatorizado en un esquema factorial 2x3x3, combinando dos cultivares, tres concentraciones de NaCl (0, 75 y 150 mM) y tres concentraciones de BR (0, 0,2 y 0,4 μM). Los análisis clave incluyeron la fuga de electrolitos, el contenido de Na^+ y K^+ en las hojas y la actividad de las enzimas antioxidantes, junto con la evaluación de la peroxidación lipídica (MDA). El estrés salino (150 mM de NaCl) incrementó significativamente la pérdida de electrolitos y la acumulación de sodio, a la vez que disminuyó el contenido de potasio. La actividad de las enzimas antioxidantes se elevó bajo estrés salino. La aplicación foliar de BR mitigó significativamente estos efectos al reducir la acumulación de Na^+, mejorar la retención de K^+, mejorar la relación K^+/Na^+ y modular la actividad de las enzimas antioxidantes, lo que, en consecuencia, disminuyó el daño oxidativo a la membrana (DAM). El cultivar tolerante, BRS Tapaihum, mostró consistentemente una respuesta superior a la aplicación de BR en comparación con el cultivar sensible Manteiguinha. Estos resultados demuestran que la aplicación de BR mejora eficazmente la tolerancia al estrés salino en el caupí al mejorar la homeostasis iónica y reforzar el sistema antioxidante. Esto sugiere su gran potencial como bioestimulante para el cultivo sostenible en zonas afectadas por la salinidad.

Palabras clave:
Enzimas antioxidantes
Homeostasis iónica
Peroxidación lipídica
Bioestimulante vegetal
Vigna unguiculata

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Received: May 26, 2025; Accepted: September 21, 2025

Rev. Fac. Nac. Agron. Medellín 79: e118824. 2026. ISSN 0304-2847 / e-ISSN 2248-7026

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most important and strategic food sources for tropical and subtropical regions of the world. Cabreira et al. (2024), studying the economic feasibility of central pivot irrigation with cowpea cultivation in sandy soils, concluded that cowpea is a crop that provides high productivity and excellent profits to rural producers.

Cowpea beans contain a high proportion of protein (19 to 35%) and carbohydrates (63%), with low fat content (1.5%) (Alvarez et al. 2021). Harvesting plays an important role in promoting global food security due to its nutritional characteristics and because it is considered undemanding in terms of production, making it possible to grow under low-consumption production systems (Horn and Shimelis 2020)

Plant tolerance and adaptation to salinity is attributed to a complex pathway of biochemical signals that include phospholipids, plant hormones, calcium ions (Ca^{2+}), osmotic stress recognition protein sensors, signaling transducers, gene transcription factors and stress response metabolites which promote the reduction of osmotic stress, by water retention and/or acquisition, and by the ability to accumulate and exclude sodium (Na^+) or chloride (Cl^-) ions preserving ionic homeostasis and, if necessary, the onset of enzymatic activity (Vásquez et al. 2019).

Phytohormones that guarantee different tolerance levels to the lethal concentrations through biochemical strategies, such as compartmentalization and exclusion of solutes at the cellular level, control of ion intake, osmolith synthesis, alteration of photosynthetic pathways, modification of membrane structures, and induction or inhibition of other antioxidant enzymes and other phytohormones (Oliveira et al. 2017).

Brassinosteroids are a class of polyhydroxysteroid phytohormones that play a crucial role in several plant metabolic pathways and increase plant tolerance to stress (El-Banna et al. 2022). There is convincing evidence to state that BR plays a prominent role in controlling the balance between normal growth and resistance to environmental aggression, due to its ability to regulate the antioxidant defense system, acting via "crosstalk" with abscisic acid (ABA) or independently (Planas-Riverola et al. 2019). Ohashi et al. (2020) concluded that the relative water content and electrolyte leakage were directly affected by the application of brassinosteroid, as well as the sodium and potassium contents and the Na^+/K^+ ratio in the bean area part. For these authors, the use of hormones was efficient in activating the antioxidant system of beans.

Therefore, based on the recognized role of brassinosteroids in modulating plant stress responses, it was hypothesized that the application of 24-Epibrassinolide (24-EBL) would mitigate the deleterious effects of salt stress in cowpea plants by enhancing antioxidant enzyme activity and improving ion homeostasis, particularly in a genotype-dependent manner.

Thus, this study aimed to evaluate the mitigating effect of 24-Epibrassinolide on salt stress in two cowpea cultivars (BRS Tapaihum and Manteiguinha) by analyzing physiological and biochemical parameters, including electrolyte leakage, Na^+ and K^+ content, and the activity of key antioxidant enzymes.

MATERIAL AND METHODS

Plant material and experiment conditions

The experiment was carried out in the greenhouse of the Laboratory of Plant Physiology, Institute of Agrarian Sciences of the Federal Rural University of the Amazon, Belém - PA, from December 17, 2018, to February 7, 2019. Biochemical and physiological analyses were carried out at the Laboratory of Biodiversity Studies in Higher Plants, established in the same site.

For the performance of this experiment, the following treatments were used: 0 - control; 0.2 μM BR; 0.4 μM BR; 75.0 mM NaCl; 150.0 mM NaCl; 0.2 μM BR + 75.0 mM NaCl; 0.2 μM BR + 150.0 mM NaCl; 0.4 μM BR + 75.0 mM NaCl; 0.4 μM BR + 150.0 mM NaCl, using two cultivars of *Vigna unguiculata* (L.) WALP., BRS Tapaihum, and Manteiguinha, totaling 18 treatments, with four replications and 72 experimental units.

The cultivars of *Vigna unguiculata* (L.) WALP., BRS Tapaihum and Manteiguinha were classified as tolerant and sensitive to saline stress, from Embrapa's germplasm bank. Uniform and healthy seeds were sown in disposable cups (300 mL) filled with washed and sterilized sand. Five seeds were planted per cup at a depth of 1.5 cm, and the substrate was kept moist with distilled water. Germination occurred on the fourth day after sowing (DAS).

In the 6th DAS, the transplant was performed for pots with a capacity of 500 mL containing washed and sterilized sand, leaving only two seedlings per pot, which contained nutrient solution, with 1/4 of the ionic strength. The solution consisted of: Macronutrients (mM): KNO_3: 5.0 mM, $Ca(NO_3)_2$ $4H_2O$: 5.0 mM, $MgSO_4$ $7H_2O$: 2.0 mM, KH_2PO_4: 1.0 mM. Micronutrients (µM): H_3BO_3: 46.0 µM, $MnCl_2$ $4H_2O$: 9.0 µM, $ZnSO_4$ $7H_2O$: 0.8 µM, $CuSO_4$ $5H_2O$: 0.3 µM, Na_2MoO_4 $2H_2O$: 0.1 µM, Fe-EDTA: 50.0 µM. The solution was maintained at a pH of 5.5±0.5. Initially, the seedlings were acclimated to the solution at 1/4 ionic strength, increasing it to 1/2 ionic strength after three days.

The nutrient solution was replaced every three days, and the application in the vessels was carried out daily at 08:00 and 17:00 h. Using a pH meter, the pH was maintained at 5.5±0.5 with 1 N NaOH or HCl solutions, as needed. After three days, the nutrient solution was modified to 1/2 of the ionic strength of the original concentration. A total of 36 vessels were used, each corresponding to an experimental unit.

The 24-epibrassinolide (24-BR) was dissolved in ethanol (1 mg mL^{-1}), diluted in a nutrient solution with 1/2 of the ionic strength. In the 10^{th} DAS period, in which the plants developed the second pair of leaves, treatment with BR was started at a concentration of 0, 0.2, and 0.4 µM, remaining for 7 days for acclimatization. The application occurred at the base of the stem insertion - root following a pre-fixed chronogrâma. After the acclimatization period, the plants were submitted to saline treatment at a concentration of 0, 75, and 150 mM NaCl. NaCl and BR concentrations were added to vessels containing nutrient solutions, and each concentration corresponded to one treatment, with four replications. The plants were kept under BR and saline treatment for 7 days.

Collection of plants

The plants were collected at the 24th DAS at 04:30 h. For the determination of relative water content (RWC) *in vivo,* completely expanded primary leaves of each of the repetitions were initially selected. Part of the material was reserved for the determination of electrolyte leakage (VE). Subsequently, the plants were separated into stem and leaf, wrapped in aluminum foil, and stored in a freezer at –80 °C for oxidative stress analysis. For the determination of total soluble proteins, the material was placed in a forced-air oven at 65 °C for 48 h until a constant mass was achieved. After drying, the leaf dry mass was recorded, and the material was ground in a mill until a fine powder was obtained.

Variables analyzed

The extracts for the determination of lipid peroxidation and the activity of antioxidant enzymes were obtained from the macerated liquid nitrogen of 1 g of fresh leaf tissues with 4.0 mL of Tris-HCl buffer at 0.05 M, pH=8.0, containing EDTA at 0.1 mM. The homogenized was centrifuged at 12,000 g for 15 min, at 4 °C, and the supernatant was collected and stored at -20 °C until analysis.

The activities of antioxidant enzymes were determined using specific methodologies: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX). Lipid peroxidation was estimated by quantifying malondialdehyde (MDA) using the thiobarbituric acid (TBA) method. All these analyses are described in Halliwell and Gutteridge (2007).

In addition, physiological parameters related to abiotic stress were evaluated: relative water content (RWC), sodium (Na^+) and potassium (K^+) content, and K^+/Na^+ ratio using the same technology employed by Zhu et al. (2025). Electrolyte leakage (EL) and total soluble protein content, determined by Li et al. (2021).

Experimental design and data analysis

The experimental design used was completely randomized (CRD) in a 2x3x3 factorial scheme, being two cowpea cultivars (BRS Tapaihum and Manteiguinha), three concentrations of BR (0, 0.2, and 0.4 µM BR), and three concentrations of NaCl (0, 75, and 150 mM). The experimental data were submitted to the Shapiro-Wilks test (P<0.01) to verify residual normality and homoscedasticity, using the statistical software RBio (Bhering and Teodoro 2021). After the assumptions, the experimental data were submitted to variance analysis, and the means were compared by the Scott-Knott test at 5% probability, using the statistical software SISVAR.

RESULTS AND DISCUSSION

Relative Water Content - RWC

Figure 1 shows bean plants of the variety BRS Tapaihum submitted to treatments with 150 mM of NaCl and 0.4 μM of BR + 150 mM of NaCl presented the highest RWC values (70.5 and 79.5%) compared to the Manteiguinha variety (65 and 77.25%), corresponding to an increase of 7.80 and 2.83%, respectively.

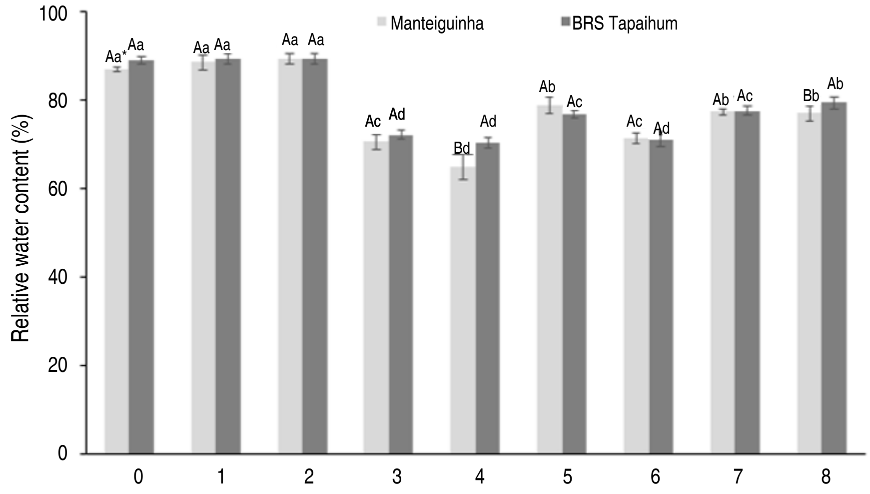

Figure 1. Relative water content (RWC) in cowpea cultivars as a function of application with brassinosteroids (0.2 and 0.4 μM) and NaCl (75 and 150 mM). Legend: (0) - 0 = Control; (1) - 0.2 μM BR; (2) - 0.4 μM of BR; (3) - 75 mM of NaCl; (4) - 150 mM of NaCl; (5) - 0.2 μM of BR + 75 mM of NaCl; (6) - 0.2 μM BR + 150 mM NaCl; (7) - 0.4 μM BR + 75 mM NaCl; (8) - 0.4 μM of BR + 150 mM of NaCl. *Capital letters compare cowpea cultivars, and lowercase letters compare BR and NaCl doses according to the Scott–Knott test at 5% probability.

This result may indicate that the variable RWC is one of the first characteristics of this variety of beans that is affected by the application of salt. According to Akrami and Arzani (2018) the increase in water content usually occurs when the plant is subjected to high salt concentrations. It can also be seen that the application of BR to this variable did not soften the effect of salt. Ohashi et al. (2020), working with BR e stress, also found that the application of BR increased the relative water content. These same authors also verified that plants that did not receive treatment with saline solution and without BR were the ones that showed a decrease in water content, indicating negative effects of NaCl on cowpea plants. Their results corroborate those found in this research.

When comparing the different treatments in the Manteiguinha variety, it is observed that the control treatments (87.25%), 0.2 μM of BR (88.75%), and 0.4 μM of BR (89.5%) presented the highest RWC values, while the plants submitted to treatment with 150 mM of NaCl (65%) presented the lowest RWC values. For the variety BRS Tapaihum it was found that the plants submitted to control treatments (89%), 0.2 μM of BR (89.5%) and 0.4 μM of BR (89.5%) also presented the highest RWC values, while the plants submitted to treatments 75 mM of NaCl (72.25%), 150 mM of NaCl (70.5%) and 0.2 μM OF BR + 150 mM of NaCl (71.25%) presented the lowest RWC values (Figure 1).

This study demonstrates that the Manteiguinha variety may be more tolerant to NaCl application. In addition, the application of BR to the evaluated varieties may have helped maintain relative water content, possibly because this hormone can regulate aquaporin activity in the plasma membrane, thereby increasing membrane permeability and promoting water influx. BR may also contribute to loosening of the cell wall, further facilitating water entry. Salt stress impairs water absorption because the water potential of the external solution decreases due to the accumulation of sodium and chloride ions (Andrade Júnior et al. 2011).

Electrolyte Leakage - EL

Figure 2 shows that the Manteiguinha variety bean plants submitted to treatments with 75 mM of NaCl (67.25%), 0.2 µM of BR + 75 mM of NaCl (56.5%) and 0.2 µM of BR + 150 mM of NaCl (57.5%) were the ones with the highest electrolyte leakage values compared to the BRS Tapaihum variety (75mM NaCl - 61.25%, 0.2 µM BR + 75 mM NaCl - 51%) and 0.2 µM of BR + 150 mM of NaCl - 48.75%, corresponding to an increase of 8.92, 9.73 and 15.22%, respectively. This result indicates that the Variety BRS Tapaihum may be more tolerant to the effect of salt with BR association than the Manteiguinha variety.

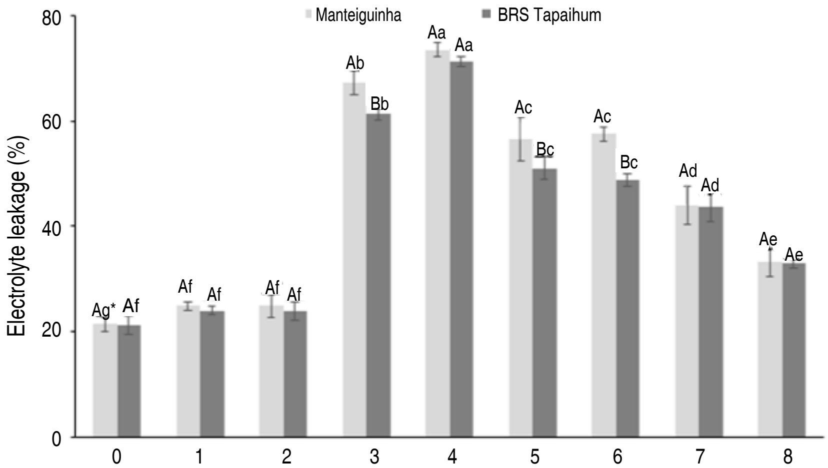

Figure 2. Electrolyte leakage (EL) in cowpea cultivars as a function of application with brassinosteroids (0.2 and 0.4 µM) and NaCl (75 and 150 mM). Legend: (0) - 0 = Control; (1) - 0.2 µM BR; (2) - 0.4 µM of BR; (3) - 75 mM of NaCl; (4) - 150 mM of NaCl; (5) - 0.2 µM of BR + 75 mM of NaCl; (6) - 0.2 µM BR + 150 mM NaCl; (7) - 0.4 µM BR + 75 mM NaCl; (8) - 0.4 µM of BR + 150 mM of NaCl. *Capital letters compare cowpea cultivars, and lowercase letters compare BR and NaCl doses according to the Scott–Knott test at 5% probability.

When comparing the different treatments in the Manteiguinha variety, it is observed that the plants submitted to the treatment of 150 mM of NaCl (73.5%) were the ones with the highest percentages of electrolyte leaks, while the plants submitted to the control treatment had the lowest percentages of electrolytes. For the BRS Tapaihum variety, it was found that the plants submitted to the treatment of 150 mM of NaCl (71.25%) also presented the highest percentages of electrolyte leakage, while plants submitted to control treatments (21.25%), 0.2 µM of BR (24%) and 0.4 µM of BR (24%) showed the highest percentages of electrolyte leakage. This result was possibly due to the fact that there was an increase in electrolyte leakage, which may have caused a decrease in plasma membrane integrity, causing greater damage due to increased NaCl. Cruz et al. (2019), studying salt stress in corn plants, also verified similar results, where electrolytic leakage in leaves grew as salt was added.

According to Yusuf et al. (2016), electrolyte leakage (EL) is an excellent indicator of loss of permeability of cell membranes and tissue damage, which are due to the ionic unbalance promoted by salt stress, as well as lipid peroxidation, where the increase in EL as a function of salinity can be explained, in parts, by the accumulation of Na^+ ions in the cell compartments, which at high concentrations becomes toxic and competes for membrane binding sites with Ca_2^+, contributing to its destabilization and the consequent leakage of cytoplasmic components.

The findings regarding the ameliorative role of 24-BR in sustaining ion homeostasis and antioxidant capacity are further supported by ultrastructural evidence reported in other species. Research on salt-affected grapevines demonstrated that brassinolide spraying effectively mitigated salinity-induced cellular damage, preserving chloroplast integrity, maintaining well-organized grana stacking, and reducing thylakoid swelling. These anatomical and subcellular improvements align with the sustained photosynthetic pigment concentrations and reduced membrane permeability observed in the present study, underscoring the fundamental role of BR in protecting cellular organelles from oxidative damage and maintaining physiological function under salinity stress (El-Banna et al. 2022).

Sodium concentration (Na^+)

For the sodium contents in the plant, Figure 3 shows, that the plants of variety Manteiguinha beans submitted to treatments with 0.2 µM of BR + 150 mM of NaCl (506.99 µmol g^{-1}) and 0.4 µM of BR + 150 mM of NaCl (429.05 µmol g^{-1}) were the ones with the highest leaf Na values compared to the variety BRS Tapaihum (0.2 µM of BR + 150 mM of NaCl - 473.67 µmol g^{-1} and 0.4 µM of BR + 150 mM of NaCl - 403.19 µmol g^{-1}), corresponding to an increase of 6.57 and 6.03%, respectively. However, plants submitted to treatments 75 mM of NaCl (541.87 µmol g^{-1}), 150 mM of NaCl (828.41 µmol g^{-1}) and 0.2 µM of BR + 75 mM of NaCl (350.92 µmol g^{-1}) d the variety BRS Tapaihum showed the highest sodium concentration soda compared to the variety (75 mM NaCl - 454.63 µmol g^{-1}, 150 mM NaCl - 782.24 µmol g^{-1} and 0.2 µM of BR + 75 mM NaCl - 317.48 µmol g^{-1}), corresponding to an increase of 16.10, 5.57 and 9.54%, respectively, indicating that for both varieties treatments with application of the hormone managed to soften the accumulation of Na^+ in plants.

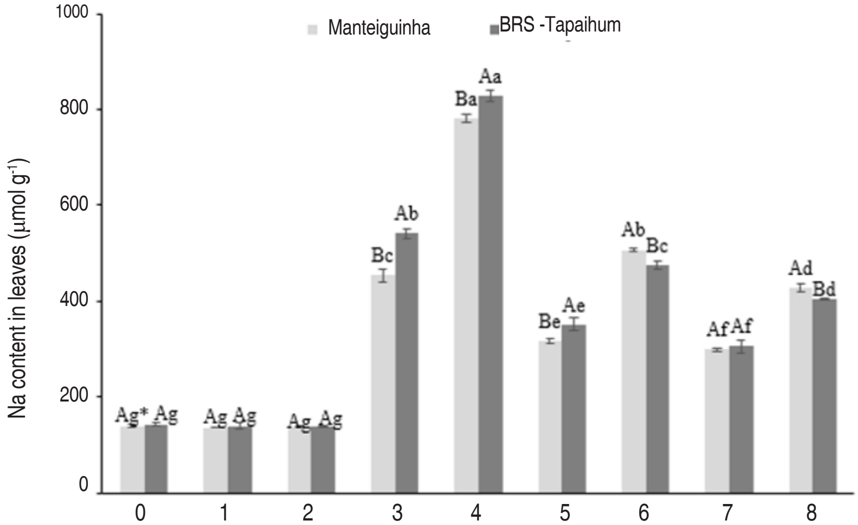

Figure 3. Sodium concentration (Na^+) µmol g^{-1} as a function of treatments with BRs (0.2 and 0.4 µM) and NaCl (75 and 150 mM). Legend: (0) - 0 = Control; (1) - 0.2 µM BR; (2) - 0.4 µM of BR; (3) - 75 mM of NaCl; (4) - 150 mM of NaCl; (5) - 0.2 µM of BR + 75 mM of NaCl; (6) - 0.2 µM BR + 150 mM NaCl; (7) - 0.4 µM BR + 75 mM NaCl; (8) - 0.4 µM of BR + 150 mM of NaCl. *Capital letters compare cowpea cultivars, and lowercase letters compare BR and NaCl doses according to the Scott–Knott test at 5% probability.

These results corroborate the findings of Ohashi et al. (2020), who reported that the application of BR significantly reduced sodium concentration compared with treatments that did not receive brassinosteroids.

For the Manteiguinha variety, plants subjected to the 150 mM NaCl treatment (782.23 µmol g^{-1}) showed the highest leaf Na levels, whereas the lowest values were observed in the plants under the control (140.21 µmol g^{-1}), 0.2 µM BR (136.82 µmol g^{-1}), and 0.4 µM BR (138.79 µmol g^{-1}) treatments.

For the BRS Tapaihum variety, the highest Na levels were observed in plants subjected to the 150 mM NaCl treatment (828.41 µmol g^{-1}), whereas the lowest Na levels were recorded in the plants under the control (142.97 µmol g^{-1}), 0.2 µM BR (140.21 µmol g^{-1}), and 0.4 µM BR (138.72 µmol g^{-1}) treatments. This result may indicate that the hormone was able to mitigate the harmful effect of salt on bean plants, as it reduced the levels of Na in plants treated with BR. Ohashi et al. (2020) also found that the increase in salt content favored a significant accumulation of sodium in cowpea leaves (BRS Guariba and BR3 Tracuateua).

Potassium concentration (K^+)

In relation to the leaf K concentration, Figure 4 shows that the variety kept in plants submitted to treatments

0.4 µM of BR (1903.54 µmol g^{-1}) and 75 mM of NaCl (965.04 µmol g^{-1}) were the ones with the highest levels of k when compared to the variety BRS Tapaihum (0.4 µM BR - 1763.11 µmol g^{-1} and 75 mM NaCl - 795.40 µmol g^{-1}), corresponding to an increase of 7.38 and 17.58%, respectively. The plants submitted to treatments with BR showed higher K concentration in both varieties, indicating that the hormone softened the negative effect of salt.

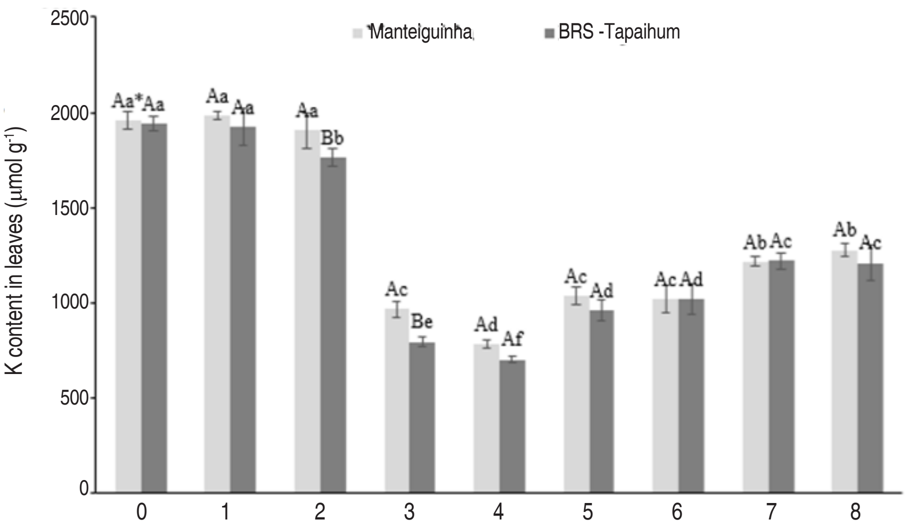

Figure 4. Potassium concentration (K^+) µM g^{-1} as a function of treatments with BR (0.2 and 0.4 µM) and NaCl (75 and 150 mM). Legend: (0) - 0 = Control; (1) - 0.2 µM BR; (2) - 0.4 µM of BR; (3) - 75 mM of NaCl; (4) - 150 mM of NaCl; (5) - 0.2 µM of BR + 75 mM of NaCl; (6) - 0.2 µM BR + 150 mM NaCl; (7) - 0.4 µM BR + 75 mM NaCl; (8) - 0.4 µM of BR + 150 mM of NaCl. *Capital letters compare cowpea cultivars, and lowercase letters compare BR and NaCl doses according to the Scott–Knott test at 5% probability.

When comparing the K concentration between the treatments of the Manteiguinha variety, the highest Levels of K in plants submitted to control treatments (1958.84 µmol g^{-1}), 0.2 µM of BR (1982.69 µmol g^{-1}), and 0.4 µM of BR (1903.54 µmol g^{-1}), and lower in plants submitted to treatment 150 mM of NaCl (782.54 µmol g^{-1}) are observed. For the variety BRS Tapaihum, the highest levels of K were observed in plants submitted to control treatments (1939.77 µmol g^{-1}) and 0.2 µM of BR (1926.40 µmol g^{-1}), and the lowest in plants submitted to 150 mM of NaCl (702.17 µmol g^{-1}). This increase in potassium concentration in cowpea leaves may have occurred due to the application of BR, and this has favored the decrease of the nano index in the membrane, providing an increase in the selectivity of the membrane. Ohashi et al. (2020) found that potassium (K^+) contents were strongly affected by saline treatment.

Potassium/ Sodium Ratio (K^+/Na^+)

For the relationship K^+/Na^+ it is observed in Figure 5 that the highest values of this relationship were found in plants of the Manteiguinha variety submitted to control treatments (13.98) and 0.2 µM of BR (14.49) when compared to the variety BRS Tapaihum (control - 12.33 and 0.2 µM of BR - 13.74), corresponding to an increase of 11.80 and 5.18%, respectively, indicating that the Manteiguinha variety accumulated more sodium and potassium in the leaves due to the presence of the hormone.

When co-commencing the different treatments for the Manteiguinha variety, the highest K^+/Na^+ ratio is observed for plants submitted to control treatments (13.98), 0.2 µM of BR (14.49), and 0.4 µM of BR (13.72), and lower in those submitted to treatment 150 mM of NaCl (1.00). For the variety BRS Tapaihumit was observed that the highest ratio K^+/Na^+ presented the same behavior as the Manteiguinha variety, and the lowest values of the K^+/Na^+ ratio were found in the treatments 75 mM of NaCl (1.47) and 150 mM of NaCl (0.85). The reduction in sodium concentrations from the application of 24-EBL was possibly the result of the greater accumulation or synthesis of compatible solutes in the plant and the possible dilution effect of toxic salts, due to a probable increase in the water content in the leaf RWC promoted by the hormone. In addition, the storage of sodium ions (Na^+) in the organs of the plant, especially in the leaves, can cause the stomata to close, which will hinder transpiration, the flow of CO_2 into the atmosphere, and affect photosynthesis. According to Abdelgawad et al. (2016), the lower assimilation of CO_2 and the reduction of growth and reduction of fresh mass accumulation occur due to the storage of Na^+ and Cl^- in the leaf organ, which causes the closure of stomata, or damage may occur in the photosynthesis process.

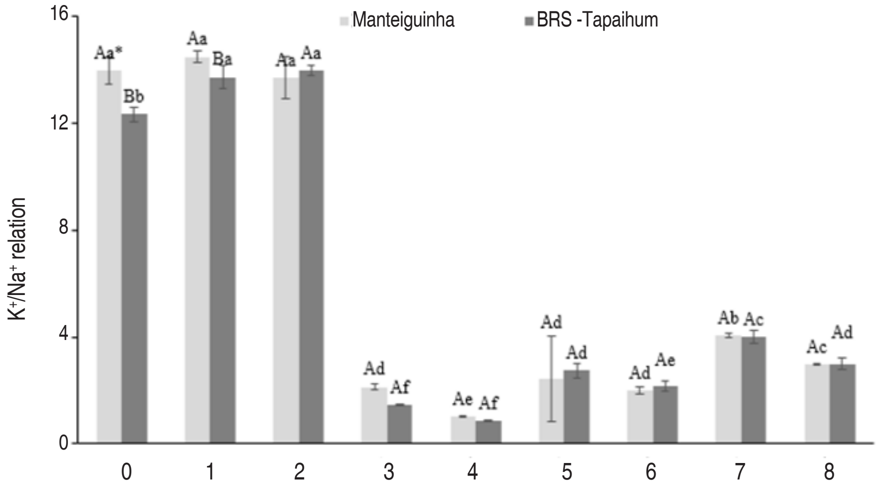

Figure 5. K^+/ Na^+ ratio as a function of treatments with BR (0.2 and 0.4 μM) and NaCl (75 and 150 mM). Legend: (0) - 0 = Control; (1) - 0.2 μM BR; (2) - 0.4 μM of BR; (3) - 75 mM of NaCl; (4) - 150 mM of NaCl; (5) - 0.2 μM of BR + 75 mM of NaCl; (6) - 0.2 μM BR + 150 mM NaCl; (7) - 0.4 μM BR + 75 mM NaCl; (8) - 0.4 μM of BR + 150 mM of NaCl. *Capital letters compare cowpea cultivars, and lowercase letters compare BR and NaCl doses according to the Scott–Knott test at 5% probability.

The absorption and accumulation of Na^+ in plants under saline stress inhibit the intake of K^+ and Cl^-, especially inhibiting the intake of $NO_{3,}$ disturbing the ionic balance (ALP et al. 2017). Also, in shoots of *Phaseolus vulgaris* was observed the accumulation of Na^+, associated with marked decrease in K^+ content exposed to salt stress, being due to the competition between the absorption of these ions in the medium, because the Na^+ interferes with the K^+ by the selective channels in plasma membranes of the roots and thus reduces the availability of many ions (Nafie et al. 2015).

Thus, numerous evidences indicate the participation of high and low affinity transporters for K^+ in the transport of Na^+ into the cell, when Na^+ is in high concentrations in the soil, which demonstrates that the high K^+/ Na^+ ratio presents a direct proportion with salt resistance (ALP et al. 2017).

Total Soluble Protein concentration

For the protein concentration, it is observed in Figure 6A a that the variety BRS Tapaihum presented the highest protein content sums in all treatments when compared to the Manteiguinha variety, corresponding to an increase of 17.09% in control,15.37% in the 0.2 μM of BR, 16.04% in the 0.4 μM of BR, 26.78% in the 75 mM of NaCl, 40.68% in the 150 mM of NaCl, 10.36% in 0.2 μM of BR + 75 mM of NaCl, 28.82% in 0.2 μM of BR + 150 mM of NaCl and 13.46% in 0.4 μM of BR + 75 mM of NaCl. Only for treatment with 0.4 μM of BR + 150 mM of NaCl (4.21 mg g^{-1}), the BRS Tapaihum variety presented a lower value when compared to the Manteiguinha variety (0.4 μM of BR + 150 mM of NaCl - 4.91 mg g^{-1}). This demonstrates that the BRS Tapaihum variety, even in the presence of salt, was able to maintain protein levels in plants. The action of BR may have positively influenced this variety.

When checking the effect of the treatments for the Manteiguinha variety, it is observed that the plants submitted to control treatments (5.24 mg g^{-1}), 0.2 μM of BR (5.45 mg g^{-1}) and 0.4 μM of BR (5.39 mg g^{-1}) were the ones with the highest protein concentration, while the lowest levels were found in plants submitted to 150 mM of NaCl (2.10 mg g^{-1}). This result indicates that the doses of the hormone applied in this study may have favored protein production and consequently managed to mitigate, even if the negative effects of salt application are mild.

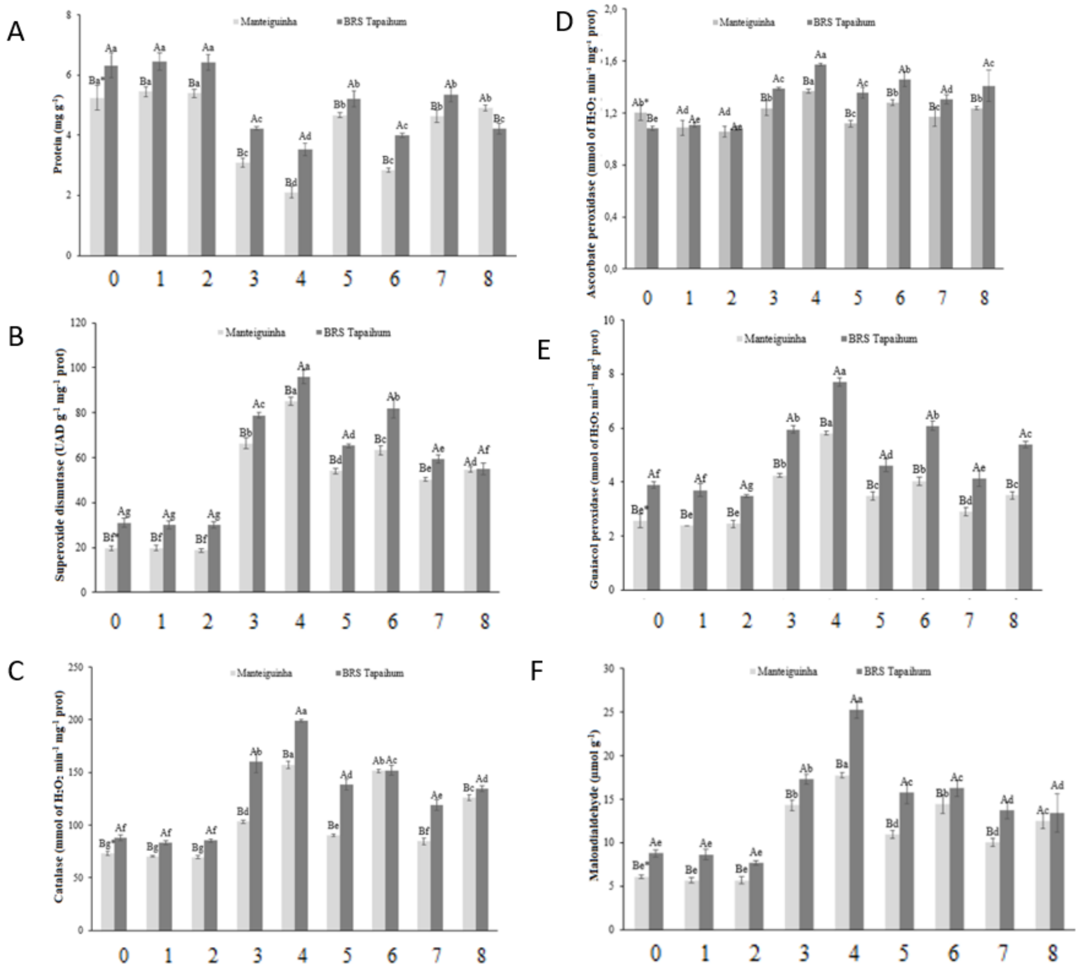

Figure 6. Cultivars as a function of the application of BR (0.2 and 0.4 μM) and NaCl (75 and 150 mM), **A**) protein, **B**) Concentration of the enzyme SOD (UA g^{-1} mg^{-1} prot.), **C**) Concentration of the enzyme catalase (mmol of H_2O_2 min^{-1} mg^{-1} prot.), **D**) Concentration of the enzyme APX (mmol of H_2O_2 $min^{-1}mg^{-1}$ prot.), **E**) Concentration of the GPX enzyme (mmol of H_2O_2 $min^{-1}mg^{-1}$ prot.) and **F**) Malondialdehyde content (μmol g^{-1} of MF). Legend: (0) - 0 = Control; (1) - 0.2 μM BR; (2) - 0.4 μM of BR; (3) - 75 mM of NaCl; (4) - 150 mM of NaCl; (5) - 0.2 μM of BR + 75 mM of NaCl; (6) - 0.2 μM BR + 150 mM NaCl; (7) - 0.4 μM BR + 75 mM NaCl; (8) - 0.4 μM of BR + 150 mM of NaCl. *Capital letters compare cowpea cultivars, and lowercase letters compare BR and NaCl doses according to the Scott–Knott test at 5% probability.

The action of BR on prosthetic metabolism is presented in cell growth and division; vascular and root development; reproduction, and development of stomata, demonstrating the importance of its action (Wang and He 2017).

Superoxide Dismutase - SOD

Figure 6B shows that the BRS Tapaihum variety showed the highest values of superoxide dismutosis in all treatments, corresponding to an increase of 36.56% in control, 34.68% in 0.2 µM of BR, 37.83% in 0.4 µM of BR, 15.92% in 75 mM of NaCl, 11.23% in 150 mM of NaCl, 17,23% at 0.2 µM of BR + 75 mM of NaCl, 22.93% in 0.2 µM of BR + 150 mM of NaCl and 15.09% in 0.4 µM of BR + 75 mM of NaCl in relation to the Manteiguinha variety. For the treatment with 0.4 µM of BR + 150 mM of NaCl (54.82 g mg^{-1}), the BRS Tapaihum variety presented a lower value in relation to the Manteiguinha variety (0.4 µM of BR + 150 mM of NaCl - 54.73 g mg^{-1}). It is noticed that the Manteiguinha variety may be more resistant to saline stress than the BRS

Tapaihum variety, and perhaps in this variety, the BR hormone managed to keep the plant defense system more active. In addition, SOD is one of the most important useful enzymes against oxidative stress of the plant defense system and are ubiquitous throughout plant anatomy (Moharramnejad and Valizadeh 2014).

When observing the effect of the treatments on the Manteiguinha variety, Figure 6B shows that the highest values of superoxide dismutase were found in plants submitted to treatment with 150 mM NaCl (85.21 g mg^{-1}) and the lowest in plants submitted to treatments control (19.66 g mg^{-1}), 0.2 µM BR (19.68 g mg^{-1}) and 0.4 µM BR (18.64 g mg^{-1}). While for the variety BRS Tapaihum, the same behavior was observed. The increase in SOD with the application of saline stress may have happened because this enzyme was used directly for the formation of several reactive oxygen species in plants, besides being directly involved in the plant defense system. According to Ohashi et al. (2020), the level of H_2O_2 rises due to the activity of SOD, favoring the action of neutralizing enzymes such as CAT and peroxidases, APX, and GPX. The activity of these enzymes favors the prevention of severe damage caused to cell membranes.

Catalase - CAT

Figure 6C shows that the BRS Tapaihum variety presented the highest catalase values in all treatments, except for treatment with 0.2 µM BR + 150 mM NaCl (152.02 mmol H_2O_2 min mg^{-1}) compared to the Manteiguinha variety (0.2 µM BR + 150 mM NaCl - 151.50 mmol H_2O_2 min mg^{-1}), corresponding to a 16.78% increase in control, 15.74% in 0.2 µM of BR, 17.27% in 0.4 µM of BR, 35.54% in 75 mM of NaCl, 20.82% in 150 mM of NaCl, 34.89% in 0.2 µ BrM + 75 mM NaCl, 28.87% in 0.4 µM of BR + 75 mM of NaCl and 6.67% in 0.4 µM of BR + 150 mM of NaCl. The results indicate that the Manteiguinha variety was more resistant to catalase variation than BRS Tapaihum when exposed to the hormone.

When observing the effect of the treatments on the Manteiguinha variety, Figure 6C shows that the highest conceptions of the enzyme catalase were found in plants submitted to treatment with 150 mM of NaCl (157.53 mmol of H_2O_2 min mg^{-1}) and the smallest in plants submitted to control treatments (73.25 m O_2 min mg^{-1}), 0.2 µM BR (70.32 mmol H_2O_2 min mg^{-1}) and 0.4 µM BR (69.61 mmol H_2O_2 min mg^{-1}). The BRS Tapaihum variety presented the same behavior. This increase in catalase in plants under salt stress may have occurred because lipid peroxidation levels remained when salt was applied to the solution.

The reduction in catalase activity in plants exposed to the hormone was also reported by Ohashi et al. (2020) in cowpea cultivars. In the present study, plants subjected to combined brassinosteroid and salt treatments did not exhibit high catalase levels, likely because the hormone attenuated the effects of sodium and chlorine on cellular metabolism.

Ascorbate peroxidase - APX

Figure 6D shows the design of the enzyme APX, in which the Manteiguinha variety presented values higher than the BRS Tapaihum variety only in plants submitted to control treatment (Manteiguinha - 1.20 and BRS Tapaihum - 1.09), with an increase of 10%. While the BRS Tapaihum variety showed the highest concentrations of the APX enzyme in the other treatments compared to the Manteiguinha variety, corresponding to an increase of 1.80% in the 0.2 µM of BR, 1.85% in the 0.4 µM of BR, 10.79% in the 75 mM of NaCl, 12.74% in 150 mM of NaCl, 17.65% in the 0.2 µM of BR + 75 mM NaCl, 12.33% at 0.2 µM of BR + 150 mM of NaCl, 10% in 0.4 µM of BR + 75 mM of NaCl and 12.06% in 0.4 µM of BR + 150 mM of NaCl. With this result, it can be inferred that the variety BRS Tapaihum is more sensitive to the harmful effects of salt than the Manteiguinha variety.

When observing the effect of the treatments on the Manteiguinha variety, it was verified that the plants submitted to 150 mM of NaCl (1.37 mmol of H_2O_2 min mg^{-1}) presented the highest values of the Enzyme APX and lower in plants submitted to 0.2 µM of BR (1.09 mmol of H_2O_2 min mg^{-1}) and 0.4 µM of BR (1.06 mmol of H_2O_2 min mg^{-1}). The BRS Tapaihum variety showed higher values of the APX enzyme in plants submitted to 150 mM of NaCl (1.57 mmol of H_2O_2 min mg^{-1}) and lower concentrations in plants submitted to control treatments (1.08 mmol H_2O_2 min mg^{-1}), 0.2 μM BR (1.11 mmol H_2O_2 min mg^{-1}), and 0.4 μM BR (1.08 mmol H_2O_2 min mg^{-1}). According to Ohashi et al. (2020), this increase occurs because this enzyme turns H_2O_2 into water. These same authors also verified that in plants that were not submitted to saline stress, there was an increase in APX levels.

Guaiacol Peroxidase - GPX

For the concentration of the GPX enzyme it is observed (Figure 6E) that the variety BRS Tapaihum presented the highest concentrations of the GPX enzyme in all treatments when compared to the Manteiguinha variety, corresponding to an increase of 33.93% in control, 35.68% in 0.2 μM of BR, 29.80% in 0.4 μM of BR, 28.57% in the 75 mM of NaCl, 24.64% in the 150 mM of NaCl, 24.51% in the 0.2 μM of BR + 75 mM of NaCl, 33.55% in the 0.2 μM of BR + 150 mM of NaCl, 29.37% in 0.4 μM of BR + 75 mM of NaCl and 35.19% in 0.4 μM of BR + 150 mM of NaCl. This result may indicate that the BRS Tapaihum variety may be more resistant to the harmful effects of the sea.

According to Hasanuzzaman et al. (2017), GPX, as well as APX, is responsible for the elimination of H_2O_2, and the increase in its activity has been related to plant tolerance to salinity, being observed in the present study, in which the increase in GPX activity was significantly higher for BRS Tapaihum (tolerant) compared to Manteiguinha (sensitive).

When checking the effect of the treatments for the Manteiguinha variety, it is observed that the plants submitted to 150 mM NaCl treatments (5.81 mmol of H_2O_2 min mg^{-1}) were the ones with the highest concentrations of the GPXenzyme, while the lowest concentrations were found in plants submitted to control treatments (2.57 mmol of H_2O_2 min mg^{-1}), 0.2 μM BR (2.38 mmol H_2O_2 min mg^{-1}) and 0.4 μM BR (2.45 mmol H_2O_2 min mg^{-1}). The same behavior was observed for the variety BRS Tapaihum in relation to the highest concentrations of the GPX enzyme, but this variety presented the lowest concentrations in plants submitted to 0.4 μM of BR (3.49 mmol of H_2O_2 min mg^{-1}). The increase in GPX enzyme concentration in plants submitted to salinity may have occurred due to the cell wall and vacuole expelling hydrogen peroxide. Ohashi et al. (2020) also found that plants that did not receive salt and hormone application and those treated only with brassinosteroid had lower concentrations of GPX, due to not manifesting symptoms of salt stress.

Peroxidation of Membrane Lipids

For the malondialdehyde content (Figure 6F) that the variety BRS Tapaihum presented the highest values in all treatments when compared to the Manteiguinha variety, corresponding to an increase of 30.44% control, 34.45% in 0.2 μM of BR, 26.04% in 0.4 μM of BR, 17.26% in 75 mM of NaCl, 29.61% in 150 mM of NaCl, 30.30% in 0.2 μM of BR + 75 mM of NaCl, 11.09% in 0.2 μM of BR + 150 mM of NaCl and 26.78% in 0.4 μM of BR + 75 mM of NaCl, respectively, except for plants submitted to treatment 0.4 μM of BR + 150 mM of NaCl that did not differ between varieties (BRS Tapaihum: 13.45 μmol g^{-1}, Manteiguinha: 12.46 μmol g^{-1}). The cultivar Manteiguinha responded better to saline stress when associated with the application of BRs compared to BRS Tapaihum, showing to be more resistant.

When verifying the effect of the treatments for the Manteiguinha variety, it is observed that the plants submitted to the treatments 150 mM of NaCl (17.76 μmol g^{-1}) were the ones with the highest levels of malondialdehyde, while the lowest concentrations were found in plants submitted to control treatments (6.10 μmol g^{-1}), 0.2 μM of BR (5.67 μmol g^{-1}) and 0.4 μM of BR (5.68 μmol g^{-1}). The same behavior was observed for the variety BRS Tapaihum. The addition of BR influenced the malondialdehyde contents, attenuating the effect of salt stress, indicating that the use of this hormone may have hindered the oxidative deterioration of lipids, which caused the decrease in MDA concentrations, since this is due to the incorporation of molecular oxygen into the fatty acids of the cell membrane.

These results corroborate those of Ohashi et al. (2020), which verified increases in MDA content and reductions in bean plants submitted to the association of NaCl and BR. The production of MDA, due to the peroxidation of lipids from cell membranes, has been used as an indication of oxidative damage in plant tissues, as well as plant sensitivity to saline stress (Bali and Sidhu 2019).

It is possible that the increases in the activity of SOD, CAT, APX and GPX enzymes, promoted by the action of BR on leaves of plants under salt stress, observed during the experimental period, are responsible for reducing oxidative damage generated by salt stress; but as resistance and this type of stress is a quantitative character that involves several metabolic networks, with strong interaction with the environment, probably the and made of 24-BR in tolerance to saline stress in cowpea cultivars, was part of a cascade of biochemical reactions of perception and expression of genes linked to stress modulating factors (presence of ions, oslotic potential, changes in turbidity pressure, etc.), which leads to the need for complementary research.

CONCLUSION

This study demonstrated that foliar application of 24-Epibrassinolide (24-BR) effectively mitigated the deleterious effects of salt stress in two cowpea cultivars, with the tolerant genotype (BRS Tapaihum) showing a superior physiological and biochemical response compared to the sensitive one (Manteiguinha). The application of 24-BR significantly alleviated ionic imbalance by reducing Na^+ accumulation and enhancing K^+ retention in leaves, resulting in a more favorable K^+/Na^+ ratio. Furthermore, 24-BR reinforced the plant's antioxidant defense system, modulating the activity of key enzymes such as SOD, CAT, APX, and GPX, which consequently reduced oxidative damage, as evidenced by lower lipid peroxidation (MDA) and electrolyte leakage.

These findings highlight the practical potential of using 24-BR as a natural and sustainable strategy to enhance salt tolerance in cowpea, particularly in semi-arid regions or areas affected by secondary salinization. From a scientific perspective, the results underscore the role of brassinosteroids in modulating complex stress-response mechanisms, including ion homeostasis and oxidative protection.

For future research, field trials are recommended to validate the efficacy of 24-BR under real agricultural conditions. Additionally, molecular studies should be conducted to elucidate the genetic and regulatory mechanisms underlying the differential responses between cultivars and the specific pathways activated by 24-BR.

CONFLICT OF INTERESTS

The authors declare no known financial or personal conflicts of interest that could have influenced the work reported in this manuscript.

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