Physiological Responses of Cowpea (*Vigna unguiculata*) Under Irrigation With Saline Water and Biostimulant Treatment

Leonardo Vieira de Sousa¹, Rayanne Maria Paula Ribeiro², Manoel Galdino dos Santos², Fernando Sarmento de Oliveira², Hugo Ferreira², Francisco Rafael Rodrigues Gerônimo², Antônio Genilson Rodrigues Araújo², Toshik Iarley da Silva³ & Aurélio Paes Barros Júnior²

¹ Department of Plant Science and Environmental Sciences, Federal University of Paraíba, Areia, Paraíba, Brazil

² Department of Agronomic and Forest Sciences, Federal Rural Semi-Arid University, Mossoró, Rio Grande do Norte, Brazil

³ Department of Plant Science, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil

Correspondence: Toshik Iarley da Silva, Department of Plant Science, Postgraduate Program in Plant Science, Federal University of Viçosa, Campus Universitário, CEP: 36570-900, Viçosa, Minas Gerais, Brazil. E-mail: iarley.toshik@gmail.com

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Abstract

Cowpea (Vigna unguiculata) is one of the world's main crops, and it is a fundamental source of protein for semiarid regions population. In these regions, the use of high salts concentration water in irrigation systems is one of the major factors that contributes to reduced cowpea yield. One way to alleviate the negative effects of salinity is through the biostimulants application, which is a product that has beneficial substances to the plants metabolism. The aim of this study was to evaluate the application of biostimulant in cowpea cultivars under irrigation with saline water. The study was carried out in the Agrarian Sciences Center, of the Department of Agronomic and Forest Sciences of the Federal Rural University of the Semi-Arid, in the city of Mossoró, RN. The experimental design was completely randomized, with four replications. The treatments were arranged in 5 $\times 2 \times 2$ factorial scheme, with five doses of biostimulant (0, 15, 30, 45 and 60 mL L⁻¹), two electrical conductivities of the irrigation water (0.5 and 5.0 dS m⁻¹), and two cowpea cultivars (IPA-206 and BRS Guariba). The evaluated characteristics were: chlorophyll content index, stomatal conductance, net photosynthesis, internal CO₂ concentration, transpiration rate, shoot height, stem diameter and shoot dry mass. The biostimulant application was not efficient in attenuating the salinity stress effect on the development of cowpea cultivars. The higher biostimulant concentrations along with the use of saline water increased the negative effects of salinity on the cowpea plants physiology. There was no difference between the cultivars regarding the tolerance to saline stress and the application of biostimulant.

Keywords: Ascophyllum nodosum, salinity stress, gas exchange

1. Introduction

The cowpea (*Vigna unguiculata* (L.) Walp.) is one of the main sources of vegetable protein in tropical and subtropical regions of the world (Santos et al., 2014). In this scenario, Brazil is classified as the world's third largest producer, with estimated production of 749.4 thousand tons for the 2017/2018 harvest (CONAB, 2018). In the Brazilian national ranking, the Northeast region is the largest producer, with approximately 51% of the national production, produced in an area of 1,134.3 hectares. Despite the high productive potential, the average yield of cowpea in the Northeast is still low, with only 336 kg ha⁻¹ (CONAB, 2018).

Several factors (low rainfall, salinity of soil and of the irrigation water, high temperature, among others) are responsible for the low cowpea productivity in the Northeast region, the use of water with high salinity levels stands out (Silva et al., 2013). Although cowpea is classified as a moderately salt tolerant crop, with a salinity threshold of 3.3 dS m^{-1} (Ayers & Westcot, 1999). Researchers have observed that the use of saline water in irrigation directly impairs its development, affecting plant height, stem diameter and dry matter production, in addition to interfere in physiological characteristics, such as stomatal conductance, transpiration and net photosynthetic rate (Prazeres et al., 2015; Aquino et al., 2017).

The development of irrigation management strategies, especially those aimed at mitigating the effects of saline stress on plants (Silva et al., 2013; Oliveira et al., 2015), is one way to increase the cowpea productivity in regions with salinity problems. Among these strategies, the application of natural or synthetic substances that alleviate the salinity stress on plants may be an important alternative (Oliveira et al., 2017). Several studies have been conducted aiming the development of techniques that might alleviate the negative effects of irrigation with saline water, the use of biostimulants is among the techniques studied (Oliveira et al., 2013, 2017). Biostimulants are products that can assist plants in overcoming abiotic stresses, mainly due to their roles as hormonal and nutritional stimulants (Oliveira et al., 2016).

Many commercial products based on the seaweed extract *Ascophyllum nodosum* have been used as a biostimulant, which is an alternative source of nutrients to plants that leaves no residues or pollutants in the environment. The Acadian[®] (Acadian Seaplants liquid, Canada) stands out as one of the most used biostimulants in agriculture (Hurtado & Critchley, 2018). Acadian is the trade name for the seaweed *A. nudosum* L. This is commonly used in agriculture as a growth stimulant, which contributes to the quality of different crops, in addition to increasing the photosynthetic rate. This is rich in many growth regulators such as auxins, gibberellins, cytokinins, macro and micronutrients such as Ca, K and Mo. This substances are beneficial to the plant metabolism, which gives it the biostimulating effect (Acadian Agritech, 2009).

Several studies have already proven the beneficial effects of biostimulants applications (Oliveira et al., 2015, 2017). However, in most of these researches the biostimulant used was the Stimulate[®] (plant growth regulator of the chemical group composed of the hormones cytokinin, gibberellin and indolalkanoic acid). There is little information in the literature about the biostimulant Acadian[®] being used in cowpea, especially under salinity stress conditions.

Due to the above mentioned considerations, the hypothesis was raised that the biostimulant application can reduce the effects of salinity stress on cowpea. The objective of this work was to evaluate the effects of biostimulant application on the physiological responses of cowpea cultivars (*Vigna unguiculata* (L.) Walp.) irrigated with saline water.

2. Material and Methods

The study was carried out from April to June 2016 in a greenhouse in the Didactic Vegetable Garden of the Agrarian Sciences Center of the Department of Agronomic and Forest Sciences of the Federal Rural University of the Semi-Arid (UFERSA), in Mossoró, RN, Brazil (5°11'31" S; 37°20'40" W; altitude 18 m).

A mixture of soil and commercial substrate (Plantmax[®]) in a 3:6 ratio, was the material used as substrate. The soil used is classified as Eutrophic Haplic Planosol (EMBRAPA, 2013), collected in the 0-20 cm depth, and its physico-chemical characteristics are presented in Table 1.

Chemical	characteristi	CS							
pН	OM	Р	K^+	Na ⁺	Ca ²⁺	Mg ²⁺	Al ³⁺	H^+	CEC
(H ₂ O)	%	mg dm ⁻³				cmolc dm ⁻³			
6.5	1.0	64.4	7.2	3.2	2.5	1.8	0.0	0.0	4.5
Physical	characteristic	S							
Granulometric fraction (g kg ⁻¹)						Taytypal alog			
Sand		Silt		Clay		- Textural class			
820.2		120.4		59.4		Sandy fr	anc		

Table 1. Physical and chemical characteristics of the substrate used in the study

Note: OM = Organic matter; CEC = Cation exchange capacity.

The experimental design was completely randomized, with the treatments arranged in a $5 \times 2 \times 2$ factorial scheme. The first factor was two electrical conductivities of the irrigation water (ECw) (0.5 and 5.0 dS m⁻¹); the second two cowpea cultivars (IPA-206 and BRS Guariba); and the third five biostimulant doses applied via leaves (0, 15, 30, 45, 60 mL of Acadian[®] per liter of water) with four replicates. Each experimental unit was represented by a 1.6 dm⁻³ volumetric capacity plastic bag containing one plant. Five cowpea seeds were sown in each plastic bag, at a depth of two centimeters. Seven days after sowing, the thinning was performed, leaving in each bag the most vigorous plant. After that, the treatments with saline water irrigation started.

For the lowest ECw (0.5 dS m⁻¹), water from a deep well located at the UFERSA Central Campus was used. To prepare the water with the highest ECw (5.0 dS m⁻¹), a mixture of the following salts: NaCl, CaCl₂·2H₂O and MgCl₂·6H₂O was added to the 0.5 dS m⁻¹ water, in a 7:2:1 ratio (Rhoades et al., 2000). The biostimulant used was the seaweed extract of the species *Ascophyllum nodosum* (Acadian[®]), composed of: N—8.12; P—6.82; K—12.00; Ca—1.60; Mg—2.03; S—8.16 g kg⁻¹; B—5.74; Cu—13.60; Fe—11.5; Mn—0.04; Zn—24.40 and Na—20000 mg kg⁻¹; potassium hydroxide, with 61.48 g L⁻¹ of water-soluble K₂O; 69.60 g L⁻¹ of total organic carbon; and a density of 1.16 g dm⁻³ (Silva et al., 2016). Two applications of biostimulant were carried out at 7 and 25 days after sowing. The applications were performed in the morning, starting at 8:00 am. The whole aerial part of the plant was sprayed until runoff, using a 5 L capacity hand sprayer, applying a water volume equivalent to 300 L ha⁻¹ (Abrantes et al., 2011). For the treatments that did not receive the biostimulant dose (0 mL of Acadian[®]), the plants were sprayed only with water, applying the same volume of the other treatments.

The following variables were evaluated at 40 days after sowing: chlorophyll content index (CCI), stomatal conductance (gs; mol H₂O m⁻² s⁻¹), net photosynthesis (A; µmol CO₂ m⁻² s⁻¹), internal CO₂ concentration (Ci; µmol CO₂ m⁻² s⁻¹) and transpiration rate (E; mmol H₂O m⁻² s⁻¹). The gs, A, Ci and E measurements were performed using an infrared gas analyzer (IRGA, portable model LI-6400, li-color, Lincoln, Nebraska, USA), and the readings were performed between 08:00 and 10:00 am. The CO₂ contents were set at 400 µmol m⁻² s⁻¹ and the luminous intensity at 1500 µmol photons m⁻² s⁻¹. Young newly expanded, undamaged and well lit leaves (when the light intensity was greater than 1000 µmol of photons m⁻² s⁻¹) were evaluated. The CCI was determined using a portable chlorophyll-meter (model CCM-200, Opti-Science), and two readings were performed per plant, always in the third and fourth leaf of each plant, counting from the apex.

At 45 days after sowing, the plants were harvested. The following variables were evaluated: shoot height (SH), measured with a ruler graduated in cm; stem diameter (SD), determined at 3 cm from the ground, using a digital caliper; and shoot dry mass (SDM), where the aerial plant part (leaves + stem) was packed in paper bags and placed in a forced air oven at a temperature of 65 ± 1 °C until reaching a constant mass. After that, the dried shoot was weighed in analytical balance to obtain its dry mass.

The data obtained were submitted to analysis of variance by the F test ($p \le 0.05$), and the results were analyzed using the SISVAR software (Ferreira, 2011). The effect of the salinity and cultivars factors were analyzed using the Tukey's test ($p \le 0.05$), while the biostimulant effect was evaluated by regression analysis.

3. Results and Discussions

There were triple interactions (cultivar \times salinity \times biostimulant) for the internal CO₂ concentration, stomatal conductance, net photosynthesis, chlorophyll content index and shoot dry mass. For the shoot height, there was a double interaction between cultivar and biostimulant and between salinity and biostimulant. For the transpiration rate, there was an isolated effect of the cultivar and interaction between salinity and biostimulant. For the stem diameter, there was an isolated effect of the factors (Table 2).

Table 2. Analysis of variance summary for internal CO_2 concentration (Ci), stomatal conductance (gs), net photosynthesis (A), chlorophyll content index (CCI), shoot dry mass (SDM), shoot height (SH), transpiration rate (E), and stem diameter (SD) in cowpea (*Vigna unguiculata* (L.) Walp.) cultivars as a function of biostimulant doses and salinity of the irrigation water

SV	DF	Mean Squares							
		Ci	Gs	A	CCI	SDM	SH	Ε	SD
Blocks	3	0.84 ^{ns}	1.31 ^{ns}	0.63 ^{ns}	0.68 ^{ns}	3.22 ^{ns}	3.29 ^{ns}	1.80 ^{ns}	1.33 ^{ns}
Cultivar (C)	1	0.00^{ns}	0.11 ^{ns}	19.04**	88.79**	8.34**	0.00^{ns}	103.64**	4.56**
Salinity (S)	1	127.17**	1407.88**	1628.79**	164.38**	629.18**	140.71**	1393.46**	139.53 ^{ns}
Biostimulant (B)	4	22.18**	27.90^{**}	127.90**	16.68**	16.85**	12.21**	48.10**	12.51**
Interaction $\mathbf{C} \times \mathbf{S}$	1	0.001^{ns}	13.11**	36.11**	431.42**	0.03 ^{ns}	0.09 ^{ns}	1.79 ^{ns}	0.32 ^{ns}
Interaction $C \times B$	4	5.43**	18.32**	9.49**	31.49**	0.58 ^{ns}	4.77**	0.59 ^{ns}	0.99 ^{ns}
Interaction $S \times B$	4	9.98**	40.40^{**}	36.42**	83.93**	0.98 ^{ns}	13.96**	6.42**	1.19 ^{ns}
Interaction $C \times S \times B$	4	13.30**	32.51**	10.18**	10.86**	5.74**	1.28 ^{ns}	1.57 ^{ns}	1.16 ^{ns}
CV (%)		9.22	14.67	8.03	13.41	9.05	6.23	12.89	7.62

Note. SV = Source of variation; DF = Degree of freedom; CV = Coefficient of variation; (**) Significant at 1% probability by F-test; (^{ns}) not significant.

In general, cowpea cultivars irrigated with the highest ECw level (5.0 dS m⁻¹) presented lower values of internal CO_2 concentration (Ci), stomatal conductance (gs) and net photosynthesis (A), in relation to those that were irrigated with water of lower ECw (0.5 dS m⁻¹). The reduction of these variables is possibly a reflection of the ionic and osmotic effects caused under salinity stress conditions. This first effect (ionic) results from reduced soil water potential, while the second one (osmotic) is caused by the ions accumulation in plant tissues (Munns & Tester, 2008). Salinity stress can lead to stomatal closure and, as a result, reduce stomatal conductance. This results in lower intercellular CO_2 availability in the leaves and carbon fixation inhibition, while reducing transpirational water loss, resulting in lower photosynthetic rates (Praxedes et al., 2010; Huang et al., 2015). In the literature, several researchers have already reported the negative effects of salinity on cowpea gas exchanges (Neves et al., 2009; Souza et al., 2011; Silva et al., 2013; Prazeres et al., 2015).

In relation to *Ci*, in the plants of cultivar IPA-206 (Figure 1A) submitted to ECw of 0.5 dS m⁻¹, there was a positive linear effect as the biostimulant concentrations increased and, according to the regression equation, plants treated with 60 mL L⁻¹ of Acadian[®] provided the highest values, with 263.17 μ mol CO₂ m⁻² s⁻¹, representing a 15.9% increase compared to plants that did not receive biostimulant doses. In the ECw of 5.0 dS m⁻¹, increased biostimulant concentrations provided a quadratic effect, with a lower value (142.74 μ mol CO₂ m⁻² s⁻¹) recorded in the absence of the biostimulant, and a higher (236.55 μ mol CO₂ m⁻² s⁻¹) at the concentration of 33.4 mL L⁻¹.

The increase of the biostimulant doses at the two levels of salinity promoted a quadratic effect on the *Ci* values in the cultivar BRS Guariba (Figure 1B) and, according to regression equations, the lowest values were verified in the absence of biostimulant (0 mL of Acadian[®]). At the ECw of 0.5 dS m⁻¹, the highest value was 251.67 μ mol CO₂ m⁻² s⁻¹, obtained in the biostimulant dose of 33.7 mL L⁻¹, whereas, at the ECw of 5.0 dS m⁻¹, the highest value was 271.59 μ mol CO₂ m⁻² s⁻¹, recorded at the dose of 51.22 mL L⁻¹.

For the cultivar IPA-206 (Figure 1C), at the ECw of 0.5 dS m⁻¹, there was a quadratic response in relation to the biostimulant concentrations to variable *gs*, with maximum value (0.3586 mol H₂O m⁻² s⁻¹) in the treatment with 8.9 mL L⁻¹, decreasing (0.1144 mol H₂O m⁻² s⁻¹) in concentration with lower value in 60 mL L⁻¹. On the other hand, at the ECw of 5.0 dS m⁻¹, a higher value (0.0699 mol H₂O m⁻² s⁻¹) was observed when 45.7 mL L⁻¹ was applied, and the lowest value (0.0276 mol H₂O m⁻² s⁻¹) was obtained in the absence of the biostimulant. The obtained results differ from those found by Anjos et al. (2015), who assessed different doses of Stimulate[®], Booster[®] and Biozyme TF[®] on common bean (*Phaseolus vulgaris* L.) plants and did not observe any significant results for *gs*.

Regarding the cultivar BRS Guariba (Figure 1D), there was small oscillations in the *gs* values at the two ECw levels with the increase of biostimulant concentrations. The highest *gs* values were obtained in the biostimulant concentrations of 34.3 and 22.3 mL L⁻¹, at both ECw levels, whereas the lowest *gs* values were obtained in the concentrations of 60 and 0.2 mL L⁻¹, at the salinity levels of 0.5 and 5.0 dS m⁻¹, respectively. This may have happened due to the fact that in high concentrations of salts (which hinders or reduces the absorption of water) the plants tend to close their stomata and, consequently, reduce their stomatal opening. Similarly to *gs*, at the ECw of 0.5 dS m⁻¹, *A* in the cultivar IPA-206 had a quadratic response to the effect of the biostimulant concentration on, a reduction was observed, reaching the lowest value (10.94 µmol CO₂ m⁻² s⁻¹) in the dose of 60 mL L⁻¹. At the ECw of 5.0 dS m⁻¹, increasing biostimulant concentrations resulted in a linear decrease response in *A*, and according to the regression equation, the lowest *A* value (4.69 µmol CO₂ m⁻² s⁻¹) was found in the highest biostimulant concentration (60 mL L⁻¹).

Regarding the A in BRS Guariba cultivar (Figure 1F), there was a linear decreasing response to the biostimulant concentrations at the two ECw levels. The highest A values were found in the absence of biostimulant and the lowest values in the dose of 60 mL L⁻¹. The results found in the present study are similar to those of Prazeres et al. (2015), who verified a linear reduction in the cowpea net photosynthesis as the salinity of the irrigation water increased.



Figure 1. Internal CO₂ concentration (*Ci*), stomatal conductance (*gs*) and net photosynthesis (*A*) in the cowpea (*Vigna unguiculata* (L.) Walp.) cultivars IPA-206 (A, C and E) and BRS Guariba (B, D and F) as a function of biostimulant doses and salinity of the irrigation water

For the chlorophyll content index (CCI), at the ECw of 0.5 dS m⁻¹, there were increasing linear responses as the biostimulant concentrations increased, in both cultivars (IPA-206 and BRS Guariba) (Figures 2A and 2B). It presented the highest values (41.61 and 18.71) in the biostimulant concentration of 60 mL L⁻¹, which represents an increase of 102.68 and 78.02% for cultivars IPA-206 and BRS Guariba respectively, compared to plants that did not receive the biostimulant.

At the ECw of 5.0 dS m⁻¹, the biostimulant application provided different responses in the CCI (Figures 2A and 2B). For the two assessed cultivars there were quadratic responses with increasing biostimulant concentrations. The highest values were found in the concentrations of 9.41 and 25.19 mL L⁻¹ for the cultivars IPA-206 and BRS Guariba, respectively. In the presence of high salinity (5 dS m⁻¹), high doses of biofertilizer had a negative effect above 30 mL L⁻¹. This may be linked to the fact that in high concentrations, hormones can cause harmful effects rather than increase the activity of the photosynthetic apparatus.

The reduced CCI at the highest salinity level may be a result of salinity stress, which causes the destruction of the chlorophyll molecule and promotes instability of the pigment-protein complex (Jaleel et al., 2008). Another possible explanation for the reduction of CCI is the interference of saline ions in the *de novo* synthesis of new proteins, structural components of chlorophyll, rather than the degradation of chlorophyll (Jaleel et al., 2007). The salinity stress can also reduce the number of chloroplasts and lead to the decomposition of thylakoid membranes, resulting in increased chlorophyllase enzyme activity and, consequently, decreased chlorophyll content (Santos, 2004).

Plants cultivated under the ECw of 5.0 dS m⁻¹ presented lower SDM compared to those submitted to the ECw of 0.5 dS m⁻¹. For the cultivar IPA-206 (Figure 2C), at the ECw of 0.5 dS m⁻¹, a linear and positive response was observed in the SDM with the increase of the biostimulant concentration and, according to the regression equation, plants treated with 60 mL L⁻¹ provided the highest values (2.74 g plant⁻¹). At the salinity level of 5.0 dS m⁻¹, there was a quadratic response with maximum (1.74 g plant⁻¹) and minimum (1.24 g plant⁻¹) SDM value obtained in the biostimulant concentration of 39.8 mL L⁻¹ and 60 mL L⁻¹, respectively. For the cultivar BRS Guariba (Figure 2D), at both ECw levels, quadratic responses to biostimulant concentrations were found, and, according to the regression equations, the biostimulant doses of 43.7 and 47.6 mL L⁻¹ resulted in higher SDM values, obtained in the salinity levels of 0.5 and 5.0 dS m⁻¹, respectively. The negative effect of saline irrigation water on cowpea dry biomass was also observed by other researchers (Prazeres et al., 2015; Aquino et al., 2017).



Figure 2. Chlorophyll content index (CCI) and shoot dry mass (SDM) in the cowpea (*Vigna unguiculata* (L.) Walp.) cultivars IPA-206 (A and C) and BRS Guariba (B and D) as a function of biostimulant doses and salinity of the irrigation water

The shoot height (SH) was positively affected by the interaction between cultivars and biostimulant doses. Quadratic responses were observed with increasing biostimulant concentrations. The highest values were 24.51 and 23.71 cm for the cultivars IPA-206 and BRS Guariba, respectively (Figure 3A). These results are different from those found by Abrantes et al. (2011), who studied the effect of the biostimulant Stimulate[®] in common bean (*Phaseolus vulgaris* L.) and did not verify influence on the plants shoot height.

The interaction between biostimulant doses and salinity also influenced the SH (Figure 3B). At the ECw of 0.5 dS m⁻¹, SH increased as the biostimulant doses increased up to the concentration of 42.4 mL L⁻¹, and the highest value (25.82 cm) represents a 17.5% raise in relation to the treatment without biostimulant. At the ECw of 5.0 dS m⁻¹, from the biostimulant concentration of 19.7 mL L⁻¹ on, the plants presented a reduction in the SH, when it was obtained the highest value, with 22.87 cm. When evaluating the same salinity levels and the application of the biostimulant Stimulate[®] on cowpea, Oliveira et al. (2013) did not verify any effect on the plants shoot height.

In a study by Aquino et al. (2017), plants shoot height was severely affected with increasing salinity levels. The effect of salinity on plant growth can be explained by the impairment of biochemical and physiological functionalities, which are linked to toxic, osmotic and nutritional effects of the salts accumulation in the plants root zone (Taiz et al., 2017).



Figure 3. Shoot height on the cowpea (*Vigna unguiculata* (L.) Walp.) cultivars IPA-206 and BRS Guariba (A) and salinities of the irrigation water (B), as a function of biostimulant doses

The transpiration rate (*E*) was linearly and negatively influenced as the biostimulant concentration increased and, according to the regression equation, the concentration of 60 mL L^{-1} resulted in lower values, with decreases of approximately 54 and 121% at the ECw of 0.5 and 5.0 dS m⁻¹, respectively (Figure 4).



Figure 4. Transpiration rate (*E*) in cowpea (*Vigna unguiculata* (L.) Walp.) as a function of biostimulant doses and salinity of the irrigation water

For the stem diameter (Figure 5), increases in the biostimulant concentration had a linear and positive effect. The highest value was 4.53 mm, recorded in the concentration of 60 mL L⁻¹, which corresponds to a 18.27% increase in relation to the treatment with absence of biostimulant. These results are different from those found by Santos et al. (2013), who verified no effect of the biostimulants BU-RG[®], BU-VG[®] and BU-EC[®] on corn (*Zea mays* L.) stalk diameter.



Figure 5. Stem diameter of cowpea (Vigna unguiculata (L.) Walp.) as a function of biostimulant doses

In general, it was verified that the highest biostimulant concentration (60 mL L^{-1}), under high salinity conditions, intensified the saline stress effects on cowpea plants, by reducing its physiological and growth characteristics. These reductions probably occurred because the high biostimulant concentration enhanced the osmotic effects of salinity, hindering water absorption by the plants, as well as the ionic effects, that cause the increase of ions inside the cells, leaf injuries or reduced essential elements absorption (Munns, 2005). On the other hand, under favorable environmental conditions, such as low salinity levels, the biostimulant application can improve the crops growth characteristics. These results coincide with those found by Oliveira et al. (2017), who verified that the use of the biostimulant Stimulate[®] favors the cowpea growth and biomass production, but it is only effective in the absence of salinity stress.

4. Conclusions

The biostimulant application was not efficient in attenuating the salinity stress effect on the development of cowpea (*Vigna unguiculata* (L.) Walp.) cultivars. The higher biostimulant concentrations along with the use of saline water increased the negative effects of salinity on the chlorophyll content index, net photosynthesis and transpiration rate of cowpea cultivars. There was no difference between the cowpea cultivars regarding salinity stress tolerance and exogenous biostimulant application, since the studied variables were significantly affected and at similar intensities, except for the chlorophyll content index.

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