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Bradyrhizobium Inoculation Plus Foliar Application of Salicylic Acid Mitigates Water Deficit Effects on Cowpea

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Abstract

To evaluate the interaction between foliar application of salicylic acid and *Bradyrhizobium* inoculation on the morphophysiology of cowpea under water stress conditions, four genotypes (BRS Rouxinol, BRS Marataoã, BRS Aracê and BR 17 Gurguéia) were subjected to five combinations of water availability: 100% replacement of crop evapotranspiration (control); 50% replacement of crop evapotranspiration (water stress); water stress + salicylic acid; water stress + *Bradyrhizobium* inoculation; and water stress + salicylic acid + *Bradyrhizobium* inoculation. The experiment was set up in a 4 × 5 factorial randomized block design, with four replicates and four plants per plot. Water stress negatively affected the leaf water potential, growth, proline contents and antioxidant activity of the cowpea genotypes, and BRS Marataoã was the most sensitive. Under water stress conditions, *Bradyrhizobium* inoculation was efficient for BRS Rouxinol, but was only efficient for BRS Marataoã, BRS Aracê and BR 17 Gurguéia when associated with foliar application of salicylic acid, maintaining their values of leaf water potential, growth, proline content and activities of superoxide dismutase, ascorbate peroxidase, and catalase similar to those of the control treatment.

Keywords *Vigna unguiculata* · Water stress · Rhizobia · Antioxidant activity · Water status

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is cropped in more than 10 million hectares, located mainly in tropical and subtropical regions of America, Asia and Africa, with global production of about 5.5 million Mg (FAO 2017). Brazil currently has a cropped area of approximately 1051 thousand hectares, with an expected yield of 1268.9 kg ha^{-1} for the 2017/2018 season (CONAB 2018). Cowpea is an essential food source for the North and Northeast regions of Brazil, mainly its Northeastern semi-arid region (Koblitz 2011). This crop is predominantly cultivated in arid and semi-arid regions, develops regularly within a temperature range from 21 to 30 ºC, and requires 300 mm of rainfall for satisfactory production without using irrigation (Barros et al. 2012). Despite the low water requirement compared to other crops, cowpea faces an adverse condition in the semi-arid environment, due to the high temperatures and irregular rainfalls during its cycle, which most often makes a satisfactory production unfeasible (Didonet and Vitória, 2006; Freire-Filho et al. 2011).

Water stress limits cowpea yield as it causes alterations in physiological conditions, increasing both leaf temperature and leaf temperature/environmental temperature ratio and reducing leaf water potential, stomatal conductance, and grain yield (Nascimento et al. 2011).

Lack of water in soil often causes oxidative stress in plants, precluding normal cell functioning, producing

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reactive oxygen species (ROS) and, at more advanced level, causing plant death (Qu et al. 2013). According to Sharma et al. (2012), the most common reactive oxygen species are singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and superoxide anion ($O_2^{\cdot -}$). Under these conditions, plants maintain their water potential by accumulating osmoprotectants and/or compatible solutes in their cells, regardless of the volume resulting from water loss (Taiz et al. 2015), besides structural transformations to enhance cell functioning under water stress (Lisar et al. 2012).

ROS accumulation leads to several consequences, for instance, oxidation of proteins, enzymatic inhibition, damage to DNA and RNA, inadequate functioning of photosynthesis, and leaf necrosis (Qu et al. 2013). In response to ROS production, plants have developed an antioxidant system for signaling and defense against water stress (Barbosa et al. 2014; Dutra et al. 2017). The more efficient the assimilation of inorganic nitrogen (N), the better all the biochemical reactions of the plant, as a result of sufficient production of proteins and enzymes. There may be positive effects on enzymes and proteins responsible for the formation and maintenance of plasma membranes, thus allowing better arrangements of their structures during the storage period and also in germination (Possenti and Villela 2010).

Biological N fixation (BNF) represents the most important form of fixing atmospheric N (N_2) into ammonium and the key point for the entry of N in the food webs (Boyd and Peters 2013). Since atmospheric gases also diffuse into the soil porous space, N_2 can be used by soil microorganisms due to the action of the enzymatic complex nitrogenase, which is able to break the triple bond of N_2 and reduce it to ammonia, readily available for plant nutrition (Hungria 2011). Diazotrophs establish beneficial relationships with plants. Bacteria use part of the host plant photoassimilates as their energy source to survive and, in turn, they fix nitrogen for the host plants. Among those relationships, the legume-rhizobia association is the most well studied and understood. In addition to fixing N, rhizobia benefit the plants by several other forms of plant growth promotion, including the increase of drought tolerance (Sassi-Aydi et al. 2012; Staudinger et al. 2016; Barbosa et al. 2018), especially by bacteria isolated from dryland soils.

Present in most plants, salicylic acid (SA) has numerous regulatory functions in plant metabolism (Hayat et al. 2010; Mazzuchelli et al. 2014; Kang et al. 2014). Moreover, SA accumulation is a significant component in translation signals of the main pathways for systemic acquired resistance (Song et al. 2004). In addition, SA modulates the plant-microbial association, regulating the plant rhizospheric microbiome and its colonization by specific *taxa* (Lebels et al. 2015), enabling beneficial alterations in plant-microbial relationships. However, the exact mechanism of action of SA is not well understood, mainly because it may differ from species to species and vary according to environmental conditions (Pál et al. 2014).

Some plant growth-promoting rhizobacteria are capable of producing high levels of SA (Masciarelli et al. 2014). Dutra et al. (2017) found that SA application improves the growth and activity of antioxidant enzymes in cowpea plants under water deficit. Despite the single beneficial effects of SA application and *Bradyrhizobium* on plants, there are no reports in the literature of the combined action between SA and *Bradyrhizobium*, attenuating the water deficit in cowpea plants.

The selection of mitigating agents which can be used in the induction of tolerance to abiotic stresses in cowpea, and their association, is important in the strategy of adaptation to climate changes. Although the SA and rhizobia beneficial effects on cowpea under low water stress conditions has already been reported, the synergistic effect of both strategies has not yet been assessed until now. Therefore, the present study aimed to evaluate the interaction between foliar SA application and *Bradyrhizobium* inoculation on the growth, physiological and biochemical parameters of cowpea under water deficit stress conditions.

Materials and Methods

Study Location, Treatments and Statistical Design

The study was carried out in a Forest Nursery, situated at 07° 12′ 43″ S, 35° 54′ 36″ W at an altitude of 521 m, at the Paraíba State University facilities (Campina Grande, PB, Brazil). The region has an Aw climate, according to the Köppen-Geiger classification.

Four cowpea genotypes (G1—BRS Rouxinol, G2—BRS Marataoã, G3—BRS Aracê and G4—BR 17 Gurguéia) were subjected to five treatments, combining two levels of water availability (with and without stress), monitored with an evaporimeter (JR-200, Soil Control Agrisearch Equipment), and the condition with water stress associated with salicylic acid application and inoculation of a *Bradyrhizobium* sp. strain: W100 = corresponding to 100% of water replacement according to crop evapotranspiration (Control); W50 = corresponding to 50% replacement of crop evapotranspiration (water stress); W50 + SA = water stress + salicylic acid; W50 + BR = water stress + *Bradyrhizobium*; W50 + SA + BR = water stress + salicylic acid + *Bradyrhizobium*. The combination resulted in a 4 × 5 factorial scheme, arranged in completely randomized blocks, with four replicates and experimental plot composed of four plants.

Experimental Setup and Analyses

Plants were grown in polyethylene pots with volumetric capacity for 20 L, filled with sandy clay loam soil material (Table 1), properly corrected with soil washing, with twice the pore volume, and subsequent application of calcium carbonate (A.R.) (Dinâmica®, Brazil), in the period from August to November 2018, distributed in 8 rows with 16 pots each, totaling 128 experimental plots. A 75 cm spacing was used between rows and pots. After increasing soil moisture to a level close to field capacity, six seeds were planted in each pot at average depth of 2 cm.

To prepare the inoculant, bacteria were grown in YM liquid medium (Vincent, 1970), under constant orbital stirring (120 rpm) for 5 days, when the broth had 10^8 colony forming units per mL. The rhizobia strain used was *Bradyrhizobium* sp. ESA 17. This strain was chosen due to its good agronomic performance previously evaluated by Marinho et al. (2017). Two milliliters of the bacterial broth were inoculated in each seed at sowing.

Irrigations were carried out manually until SA application at V5 stage (25 days after emergence), and then irrigation depths were differentiated using an evaporimeter installed at the experimental site (Fig. 1). Foliar salicylic acid (Vetec® A.R.; Brazil) application was carried out at 25 days after emergence, using a backpack sprayer. Plant analyses were performed at the V9 stage at 42 days after emergence (Fig. 1). The V5 stage occurs when plants have

Table 1 Chemical and physical attributes of the soil material used in the experiment

Chemical characteristics									
			$cmol_c\ kg^{-1}$ (%)						
pH (H_2O) (1:2.5)	OM dag kg^{-1}	P mg kg^{-1}	K^+	Na^+	Ca^{2+}	Mg^{2+}	$H^+ + Al^{3+}$	ESP	ECse dS m^{-1}
4.30	2.503	6.89	0.29	1.70	0.87	0.77	5.48	11.36	5.96
Physical characteristics									
				dag kg^{-1}				($kg\ dm^{-3}$)	
Granulometric fraction g kg^{-1}			Textural class	Water content (kPa)		AW	Total porosity $m^3\ m^{-3}$	Ds	Dp
Sand	Silt	Clay		33.42	1519.5				
659	101	240	SCL	15.20	7.50	7.70	0.48	1.38	2.63

OM organic matter: Walkley–Black wet digestion; Ca^{2+} and Mg^{2+} extracted with 1 M KCl at pH 7.0; Na^+ and K^+ extracted with 1 M NH_4OAc at pH 7.0; Al^{3+} and ($H^+ + Al^{3+}$) extracted with 0.5 M CaOAc at pH 7.0; *ECse* electrical conductivity of the soil saturation extract; *SCL* sandy clay loam; *AW* available water; *Ds* soil bulk density; *Dp* soil particle density

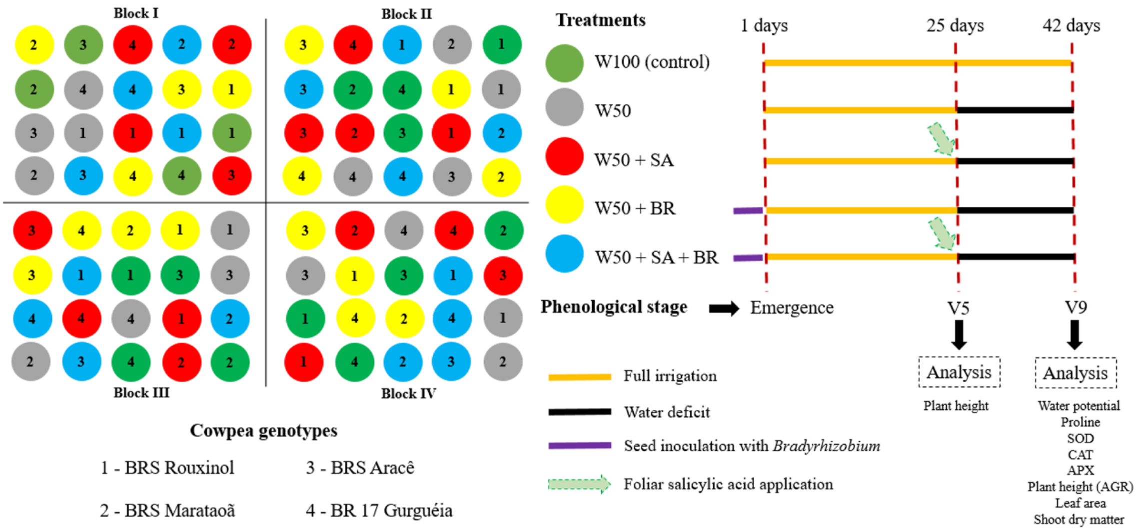

Fig. 1 Schematic representation of experimental design and treatments

the sixth node of the main branch with fully opened leaflets and emergence of the secondary branch primordium. The V9 stage occurs when the third leaf of the secondary branch has fully open leaflets. These stages of the vegetative period are critical for plant establishment in the field. At V5, there is an increasing demand of N, and V9 is the stage of transition between vegetative and reproductive period, a critical moment of high demand of water and nutrients.

Leaf Water Potential Analysis

Leaf water potential (Ψ_f) at V9 stage was determined using a Scholander pressure bomb (model 3035, Soil Moisture Equipment Corp). Initially, leaves (expanded and in good health conditions) were collected and placed in the pressure bomb chamber. Then, they were subjected to pressure up to exudation and, at this point, the applied pressure was measured (Scholander et al. 1965).

Growth Analysis

At V5 and V9 stages, plant height was evaluated in one plant per pot in both periods, one prior to SA application (V5 stage, 25 days after emergence) and one after SA application (V9 stage, 42 days after emergence).

Plant height (cm) was measured using a millimetric ruler from the base of the stem to the highest ramification of branches/leaves. Absolute growth rate (AGR) of plant height was calculated using the equation: (AGR = FH-IH/Δt), where FH corresponds to final height, IH corresponds to initial height, and Δt corresponds to time variation (Silva et al. 2000), expressed in centimeters per day.

In addition, one plant at V9 stage was collected from each treatment in each replicate and its leaf area was measured using a Li-Cor 3100 leaf area meter. Shoot (leaves and stems) dry matter was measured in the same plants collected for leaf area measurement. These plants were placed in Kraft paper bags, dried until constant weight in forced air circulation oven at 65 ºC, and weighed on analytical scale.

Biochemical Analyses

Quantification of Free Proline

Plants were collected at V9 stage and the same leaf isolated to measure water potential was used in the procedure. Free proline content in the tissues was quantified by the colorimetric method proposed by Bates et al. (1973) and modified by Bezerra Neto and Barreto (2011).

Briefly, 250 mg of fresh material was manually ground separately, with the aid of a mortar and a pistil, in 3% sulfosalicylic acid (NEON©, Brazil) (5 mL) and centrifuged at 2000 g for 10 min (Universal 320R, Hettich), and the supernatant was aspirated and used in the determinations. Color development was obtained by heating the threaded test tubes containing the mixture of extract + acid ninhydrin (composed by ninhydrin—NEON©, Brazil; phosphoric acid—NEON©, Brazil) + glacial acetic acid (Dinâmica®, Brazil), at 1:1:1 proportion (total volume of 3 mL), for one hour in water bath (Nova Instruments) regulated to 100 ºC.

After this period, the tubes were cooled in ice bath, with subsequent addition of 2 mL of toluene (NEON©, Brazil), vigorously hand shaken (AP 56, Phoenix) for 20 s and kept at rest for 10 min, in order to separate the phases. The supernatant was aspirated, placed in glass cuvette and transferred to a spectrophotometer (2000 UV, Nova Instruments), where absorbance was read at 520 nm wavelength, using pure toluene as blank.

Then, free proline concentration was quantified based on the standard curve of L-proline (VETEC©, Brazil) (0, 5, 10, 15, 20, 25, 50 mg L^{-1}) and expressed in µmol per gram of fresh matter (FM). Proline content was calculated using the average absorbance obtained in triplicate.

Activity of Antioxidant Enzymes

The activities of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) at V9 stage were quantified to verify the effect of the stressing agent on the antioxidant metabolism in cowpea plants. For enzymatic extraction, 200 mg of fresh material was manually ground separately, with the aid of a mortar and a pistil, in 2 mL of potassium phosphate buffer (final concentration of 50 mM and final pH of 7) (Monobasic – NEON©, Brazil and Dibasic – NEON©, Brazil) mixed with ascorbic acid (0.1 mM) (NEON©, Brazil), EDTA (0.1 mM) (NEON©, Brazil) and polyvinylpyrrolidone (5%) (VETEC©, Brazil). Then, the extracts were centrifuged at 20,000 g and 4 ºC temperature for 15 min (Universal 320R, Hettich). The supernatant was aspirated, placed in 1.5 mL centrifuge tubes and kept at -80° C until the analysis.

Catalase Activity

Catalase (CAT) activity was quantified according to Sudhakar et al. (2001). Briefly, the reaction was initiated by adding the enzymatic extract (50 µL) to the quartz cuvette containing reaction medium (2.95 mL) composed of potassium phosphate buffer (final concentration of 50 mM and final pH of 7) (Monobasic—NEON©, Brazil and Dibasic—NEON, Brazil) and mixed with hydrogen peroxide (VETEC©, Brazil) (20 mM). Then, after slight agitation, the mixture was taken to a spectrophotometer where the reduction of absorbance at 240 nm was monitored for 1.5 min, with readings taken every 15 s. The Lambert–Beer equation (A = ε.b.c), where A = decrease in absorbance (triplicate mean);

ε = molar extinction coefficient; b = optical path length; and c = concentration of the enzyme expressed as mol L^{-1}, was used. The molar extinction coefficient of hydrogen peroxide is 39.4 mol^{-1} cm^{-1}. Final CAT activity was expressed in μmol H_2O_2 g FM^{-1} min^{-1}.

Ascorbate Peroxidase Activity

Ascorbate peroxidase (APX) activity was determined based on the ascorbate consumption (Nakano and Asada 1981) by monitoring the reduction of absorbance at 290 nm. For this procedure, 100 μL of enzymatic extract were mixed to the reaction medium (2.7 mL) composed of potassium phosphate buffer (final concentration of 50 mM and pH of 6.0) (Monobasic – NEON©, Brazil and Dibasic—NEON©, Brazil) and mixed with ascorbic acid (0.8 mM) (NEON©, Brazil). The reaction was initiated by adding 200 μL of hydrogen peroxidase (2 mM) (VETEC©, Brazil) to the solution and immediately transferring the quartz cuvette to the spectrophotometer, where the reduction of absorbance was monitored for 1 min, with readings taken every 10 s. The Lambert–Beer equation was also used in the calculations, with the molar extinction coefficient of ascorbate of 2.8 mM^{-1} cm^{-1}. Final APX activity was expressed in ηmol ASC g FM^{-1} min^{-1}.

Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined based on the capacity to inhibit the photoreduction of nitro blue tetrazolium chloride (NBT) by the enzyme present in the extract (Beauchamp and Fridovich, 1971).

For this procedure, 40-μL aliquots of the enzymatic extract were added to test tubes, protected from light, containing reaction medium (1.5 mL) composed of potassium phosphate buffer (final concentration of 100 mM and pH of 7.8) (Monobasic—NEON©, Brazil and Dibasic – NEON©, Brazil) mixed with EDTA (0.1 mM) (NEON©, Brazil), methionine (13 mM) (Dinâmica®, Brazil) and nitro blue tetrazolium chloride (NBT) (750 mM) (Dinâmica®, Brazil). The reaction was initiated by adding riboflavin (final concentration of 7 μM) (VETEC©, Brazil) and immediately transferring the tubes to the sealed reaction chamber, with internal lighting (35 W) at room temperature, where they remained for 15 min.

Then, the readings were taken in spectrophotometer at 560 nm. Blanks were considered as the tubes with extract maintained in the dark (which represent 0% of NBT reduction) (dark blank) and the tubes without extract maintained under lighting (which represent 100% of NBT reduction) (light blank). One SOD unit was considered as the quantity of enzyme required to inhibit NBT photoreduction by 50% compared to the light blank, and its activity was expressed in U mg^{-1} protein min^{-1}. The reactions were performed in triplicate.

Statistical Analysis

The obtained data were evaluated by analysis of variance by F test $p < 0.05$, using the program SISVAR® version 5.6. After analysis of variance, means were compared in box-whisker plots, using Microsoft Excel.

Results

The genotype BRS Rouxinol and BRS Marataoã subjected to water deficit without rhizobia inoculation and SA application (W50) had the lowest water potential (Fig. 2a).

The genotype BRS Marataoã showed the highest contents of free proline and activity of the three antioxidant enzymes among all genotypes studied (Fig. 2b). It is remarkable that the contents of free proline and activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX) in the W50 treatment were higher than those in W100 (Figs. 2b, 3a, b). This genotype was the most affected by water stress. In the treatments SA and SA + BR, leaf water potential was higher than that in the W50 treatment (water stress) and similar to that of the control (W100) (Fig. 2a). In SA and SA + BR, proline contents were higher than in the control (W100) and lower than in W50 (Fig. 2b). In SA, the activities of SOD, APX and CAT were lower than in W50, and the activities of SOD and APX were similar to those observed in W100 (control) (Fig. 3a, b, c).

In the genotype BRS Aracê, leaf water potential increased with the application of BR and SA + BR under water deficit treatment (Fig. 2a). In the W50 + BR treatment, there were increments in proline content, APX and CAT activities concomitantly with reduction in SOD activity, compared to the treatments W100 and W50 (Figs. 2b, 3a, b, c). The treatment W50 + BR was also efficient for BRS Rouxinol, reducing its potential to levels similar to those found in the W100 treatment, as well as the free proline levels and activities of SOD, APX and CAT (Figs. 2, 3). On the other hand, in the treatment W50 + SA + BR, leaf water potential decreased but there were no significant alterations in free proline contents and SOD and APX activities compared to the W100 treatment, and no alterations in CAT activity compared to the W50 treatment (Figs. 2, 3). These alterations also occurred for BR 17 Gurguéia cultivar; in this treatment, leaf water potential decreased while free proline contents and SOD and APX activities stabilized compared to W100, but CAT activity increased (Figs. 2, 3). The positive responses of these treatments indicate that *Bradyrhizobium* inoculation mitigates the physiological and biochemical changes in cowpea

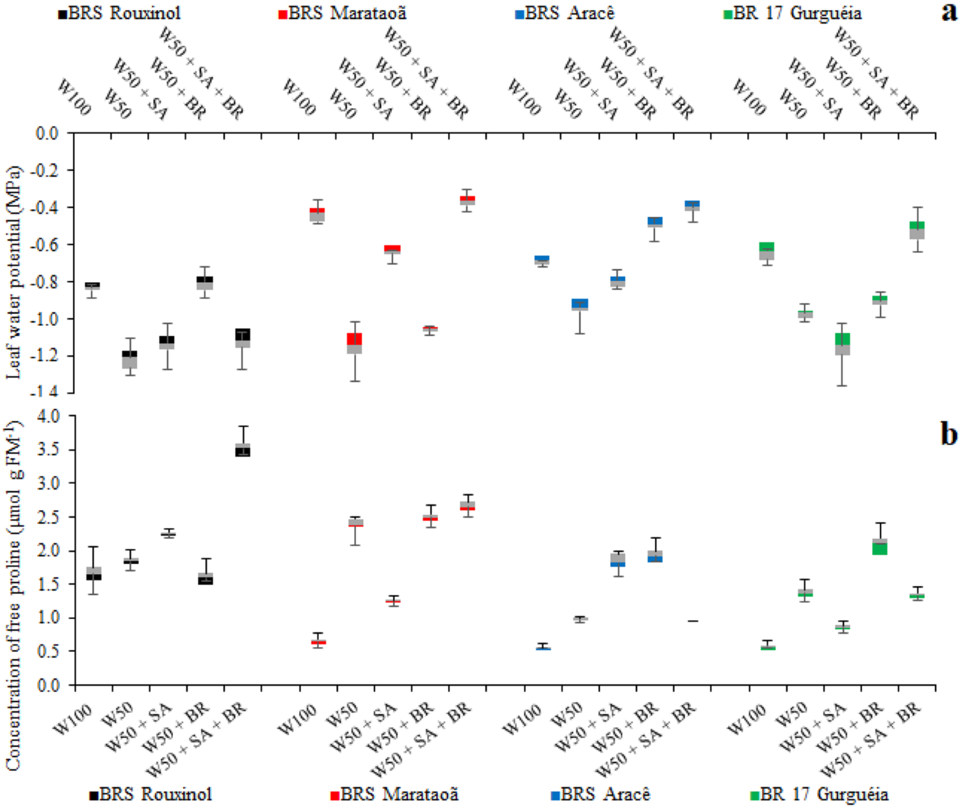

Fig. 2 Leaf water potential (**a**) and concentration of free proline (**b**) in leaves of cowpea genotypes at V9 stage subjected to different water availabilities, associated with salicylic acid application and *Bradyrhizobium* sp. ESA 17 inoculation. W100–100% replacement of crop evapotranspiration (control); W50–50% replacement of crop evapotranspiration (water stress); *SA* salicylic acid; *BR Bradyrhizobium* inoculation

caused by water stress, and the association SA + BR is more efficient for osmotic and redox homeostasis.

Under water deficit conditions, there was a reduction of leaf water potential in all genotypes. Under W50, in the absence of stress attenuators, leaf water potential was reduced by 31%, 62%, 25% and 43% in the genotypes BRS Rouxinol, BRS Marataoã, BRS Aracê and BR 17 Gurguéia, respectively, compared to the W100 treatment (Fig. 2a). According to the evaluation of leaf water potential in the genotypes under W50, BRS Aracê and BR 17 Gurguéia were the least affected by water deficit, showing higher level of natural tolerance to water deficit at the vegetative growth stage.

Sole application of SA was not effective in reducing water stress on the cowpea genotypes. Sole inoculation of *Bradyrhizobium* was efficient in mitigating water stress on the genotypes BRS Rouxinol and BRS Aracê, which had leaf water potentials similar to those found in the control treatment. However, the combination of SA application and *Bradyrhizobium* inoculation (W50 + SA + BR) was efficient to help cowpea overcome the drought effects on BRS Marataoã, BRS Aracê and BR 17 Gurguéia, maintaining their levels of leaf water potential close to those of the control.

In the analysis of absolute growth rate of plant height, there was no significant difference for any of the genotypes evaluated between water deficit (W50) and irrigated treatment (W100) (Fig. 4a). Only the absolute growth rates of BRS Aracê increased with the use of the water stress attenuators. This genotype showed the best intrinsic responses to water deficit regarding the reduction in leaf water potential and to the action of the drought stress attenuators (SA and BR), separately and combined. In this genotype, the applications of both SA + BR, combined, and of SA and BR, separately, increased the absolute growth rate (AGR) of height by 65%, 81% and 74%, respectively, compared to the treatment without application of attenuators (Fig. 4a).

Under water deficit conditions (W50), reductions in leaf area were observed only in the genotypes BRS Rouxinol and BRS Aracê, equal to 40% and 33%, respectively, compared to the W100 treatment. BRS Marataoã and BR 17 Gurguéia showed no significant differences between the irrigation depths (Fig. 4b).

With the inoculation of *Bradyrhizobium*, leaf area increased in all genotypes subjected to water deficit (W50), compared to the irrigated treatment, except for BRS Rouxinol, whose leaf area was similar to that found in W100 and 48.29% higher than that observed in W50. The presence of BR in the root environment, under W50 irrigation depth, increased leaf area by 39.25 and 51.26% in the genotypes BRS Marataoã and BR 17 Gurguéia, respectively, compared to the W100 irrigation depth (Fig. 4b). On the other hand, sole application of SA under water deficit conditions

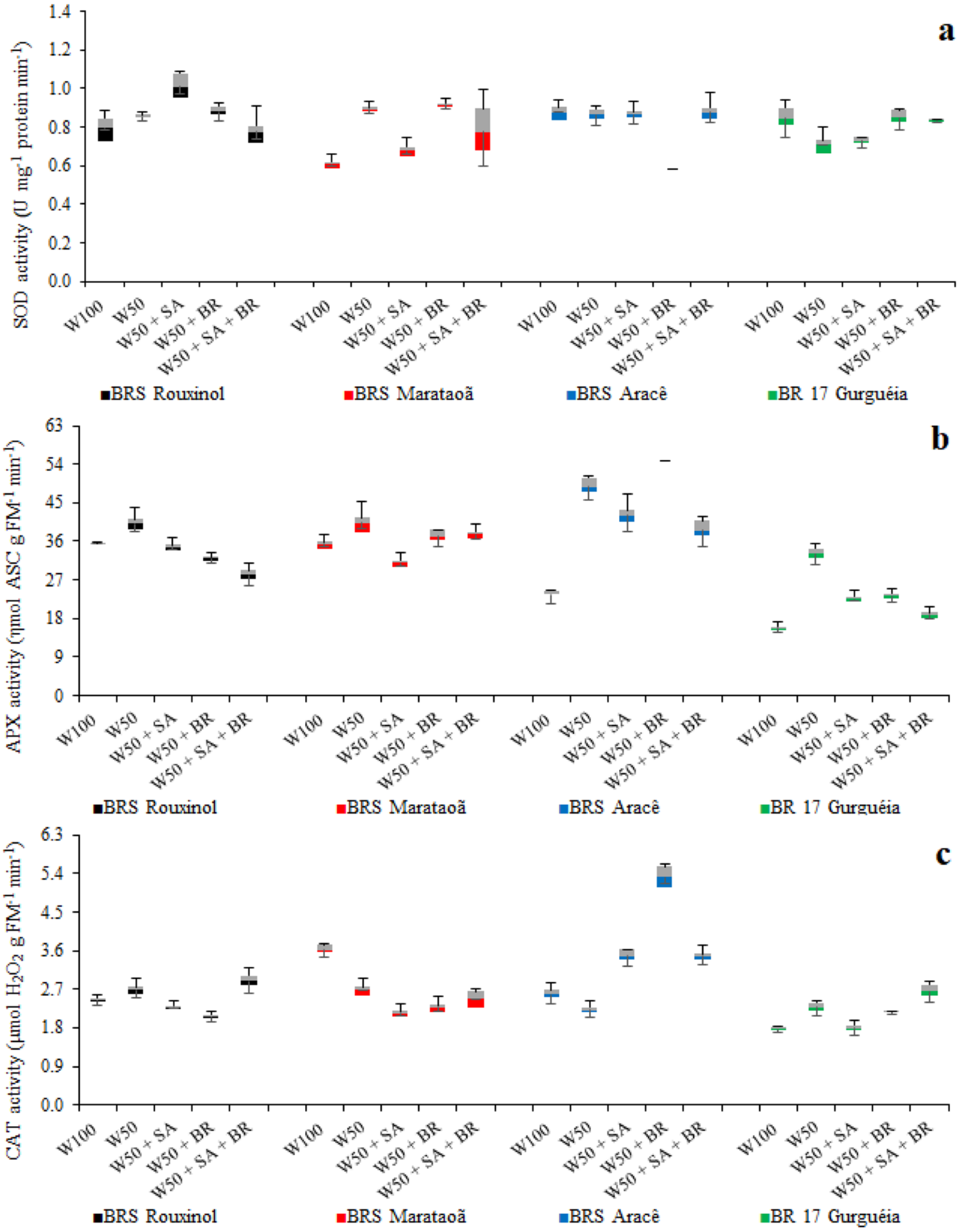

Fig. 3 Activities of superoxide dismutase (SOD) (**a**), ascorbate peroxidase (APX) (**b**) and catalase (CAT) (**c**) in leaves of cowpea genotypes at V9 stage subjected to different water availabilities, associated with salicylic acid application and *Bradyrhizobium* sp. ESA 17 inoculation. W100–100% replacement of crop evapotranspiration (control); W50–50% replacement of crop evapotranspiration (water stress); *SA* salicylic acid; *BR Bradyrhizobium* inoculation

(W50 + SA) led to leaf areas similar to those found in the W100 treatment for the genotypes BRS Marataoã, BRS Aracê and BR 17 Gurguéia. Additionally, after combined application of BR and SA (W50 + SA + BR), all genotypes showed leaf areas similar to those observed in the W100 treatment, except BRS Aracê (Fig. 4b).

Shoot dry matter accumulation under water deficit conditions was reduced in all genotypes, by 57%, 52% and 51% in BRS Rouxinol, BRS Marataoã and BRS Aracê, respectively, compared to the W100 irrigation depth. For BR 17 Gurguéia, despite the dry matter reductions of 46% in the treatments W50 in comparison to treatments W100, there was no significant difference (Figs. 4c, 5).

For BRS Rouxinol, the applications of SA + BR, combined, and of SA and BR, separately, under water deficit conditions, did not differ from the treatments W100 and W50, but increased the dry matter of the other genotypes by 19%, 9% and 50%, compared to the W50 treatment, respectively. For the genotype BRS Aracê, which showed the highest phytomass accumulation under W50 conditions, there was also no positive effect of the attenuators SA and BR, not differing from the W50 treatment. However, the treatments W50 + SA + BR for BRS Marataoã and W50 + BR for BR 17 Gurguéia, under water deficit conditions, increased dry matter accumulation by 16% and 116% compared to the W50 treatment (Fig. 4c).

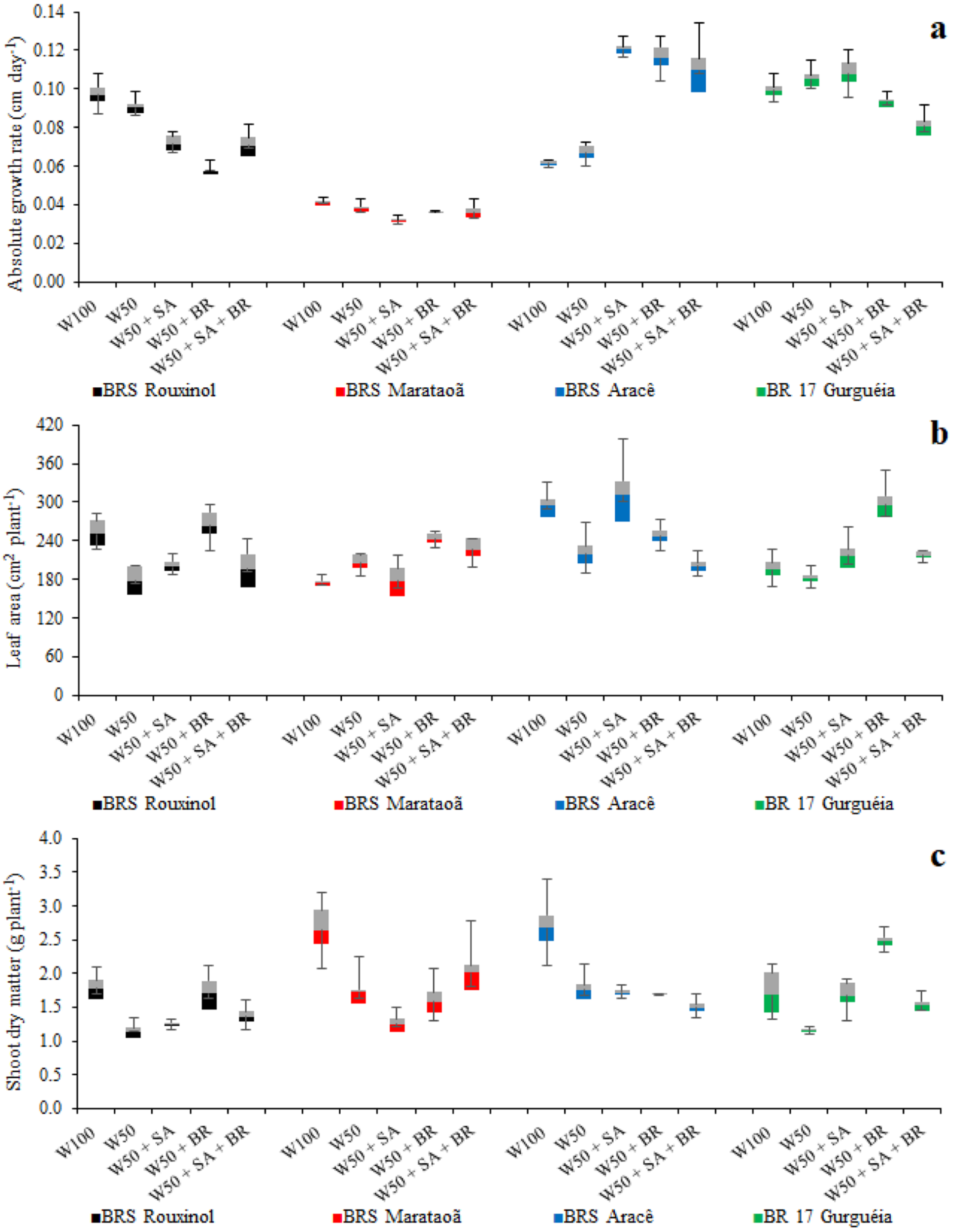

Fig. 4 Absolute growth rate (**a**), leaf area (**b**) and shoot dry matter (**c**) of cowpea genotypes at V9 stage subjected to different water availabilities, associated with salicylic acid application and *Bradyrhizobium* sp. ESA 17 inoculation. W100–100% replacement of crop evapotranspiration (control); W50–50% replacement of crop evapotranspiration (water stress); *SA* salicylic acid; *BR Bradyrhizobium* inoculation

Discussion

Salicylic acid and inoculation with *Bradyrhizobium* mitigate water stress and improve the activity of oxidizing enzymes in cowpea plants.

Under water deficit conditions, different types of damage occur in plants, including alterations in physiological conditions, especially those related to reduction in leaf water potential (Freitas et al. 2017). Such alteration in plant water balance leads to increase in leaf temperature, reductions in stomatal conductance, leaf area, total dry matter, transpiration, internal CO_2 concentrations and damage to photosynthetic processes, limiting grain filling and consequently the yield (Nascimento et al. 2011).

Leaf water potential shows that water stress was more severe in the genotypes BRS Marataoã and BR 17 Gurguéia than in BRS Rouxinol and BRS Aracê.

Inoculation with *Bradyrhizobium* was efficient to mitigate water stress only in the genotypes BRS Rouxinol and BRS Aracê, promoting improvements in water status. These genotypes showed higher tolerance to water stress than the others and, when inoculated with *Bradyrhizobium* under water deficit conditions, have a greater nutritional supply that favor their osmotic regulators, which induces regulation of water potential and promotes improvements in cell turgor, even under drought conditions (Kaushal and Wani 2016). In addition, rhizobia can trigger different metabolic pathways to ensure plant functioning and overcome drought periods

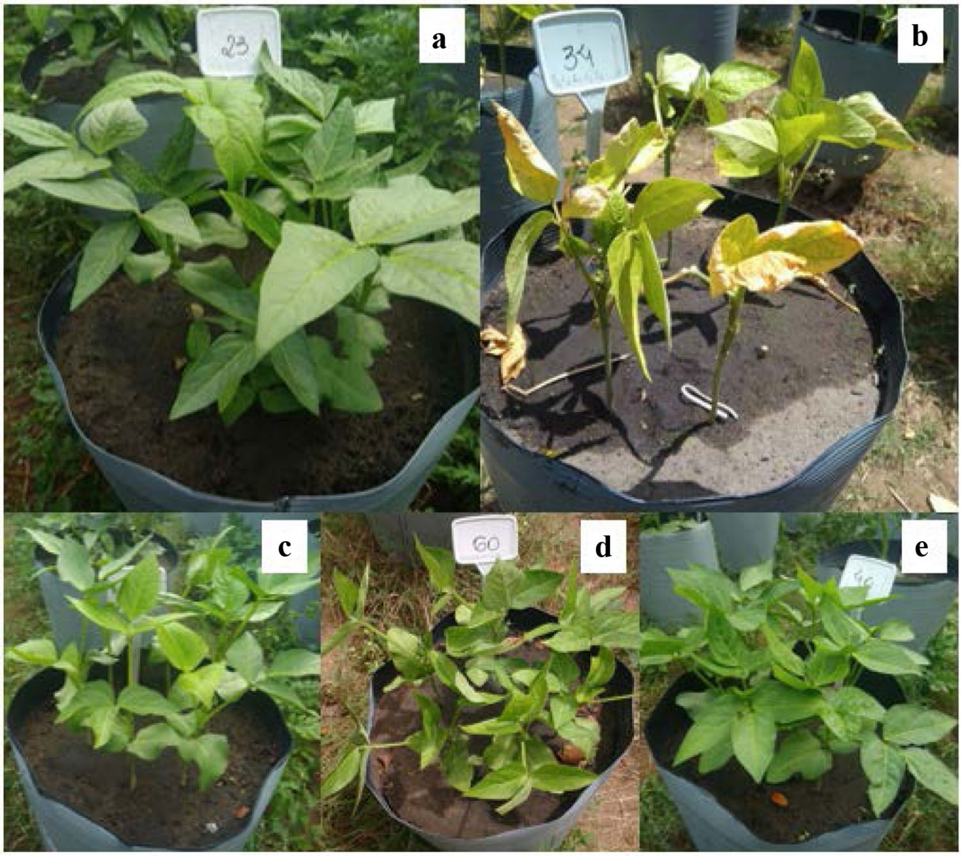

Fig. 5 Cowpea plants at V9 stage whit W100–100% replacement of crop evapotranspiration (control) (**a**); W50–50% replacement of crop evapotranspiration (water stress) (**b**); W50 + SA = water stress + salicylic acid (**c**); W50 + BR = water stress + *Bradyrhizobium* (**d**); W50 + SA + BR = water stress + salicylic acid + *Bradyrhizobium* (**e**)

as already reported for common bean (Suárez et al. 2008) and peanuts (Barbosa et al. 2018).

In the most sensitive genotypes, BRS Marataoã and BR 17 Gurguéia under water stress conditions, the inoculation (W50 + BR) was not promising, indicating that there is different symbiotic compatibility according to the level of tolerance of the cowpea genotype to water stress. In the treatment W50 + SA + BR, the effect of inoculation with *Bradyrhizobium* was efficient and enhanced the mitigation of water stress, compared to W50 + SA. Sole application of SA attenuated water stress in these genotypes and favored *Bradyrhizobium*, indicating that there is also different symbiotic compatibility between cowpea genotype and *Bradyrhizobium* according to the intensity of water stress.

The increased levels of proline observed in the present study, favored by *Bradyrhizobium* inoculation, were also identified by Hungria (2011) in plants inoculated with the genus *Rhizobium* and subjected to water deficit. In this study, there were improvements in water potential, shoot growth, biomass production and higher water supply in the apoplast. Pozzi-Tay (2017) observed that plants inoculated with rhizobacteria had higher proline levels when subjected to water deficit, and such increase may be related to the availability of nutrients to the plant, through an improvement in biological N fixation and other mechanisms.

Inoculation of *Bradyrhizobium* also caused beneficial effects on the maintenance of SOD, APX and CAT activities. Inoculation of rhizobacteria is an important tool to keep plants detoxified by breaking ROS, and an increase in these enzymes results in improvements in photosynthesis and may improve plant development (Gusain et al. 2015). Previous studies have indicated that the inoculation of *Bradyrhizobium* in peanuts (Barbosa et al. 2018) and *Rhizobium* inoculation in maize (Fukami et al. 2017) reduced the oxidative stress activities and gene expression, respectively, contrasting the results found in the present study, since the inoculation of *Bradyrhizobium* reduced the antioxidant activity (SOD, APX and CAT) only in genotypes with higher potential of tolerance (BRS Rouxinol and BRS Aracê); in genotypes sensitive to water stress (BRS Marataoã and BR 17 Gurguéia) the symbiosis was not efficient to reduce antioxidant activity, due to the severity of the stress.

Salicylic Acid and Inoculation with *Bradyrhizobium* Improve Osmoprotection in Cowpea Plants

Exogenous application of SA helped maintain water potential in the genotype BRS Marataoã, promoting better responses in relation to the W50 treatment, but not as effective as the others. Dutra et al. (2017), studying the effects of

SA on the antioxidative responses of cowpea subjected to water deficit, observed that SA increases the production of osmoprotectants, such as proline, which induced improvement in the regulation of leaf water potential in the studied plants, besides increasing the activity of antioxidant enzymes, in agreement with the results found in the present study.

Exogenous application of SA increases proline content, which also contributes to increasing antioxidant activity in some species, revealing the possible regulatory role of this acid at transcriptional and/or translational levels (Ghasemzadeh and Jaafar 2013). Additionally, Hussain et al. (2008), in a study with sunflower plants, observed that plants subjected to water deficit increased proline levels in their leaves and that SA application increased proline levels as a mechanism of defense against the stress, besides increasing the photosynthetic efficiency under water deficit conditions. The action mechanism of SA suggests that this acid is also responsible for increasing ROS concentrations in the first days of stress, leading to activation of the mechanism for cell detoxification, promoting tolerance to the stress (Horváth et al. 2007).

On the other hand, with the association of SA + BR, the responses were very promising in all genotypes. The increment in the production of osmoprotectants and also in the activity of the antioxidant enzymes in plants under water deficit, treated with SA (Dutra et al. 2017), and the increase in the levels of nutrients and osmoprotectant molecules, promoted by the presence of efficient rhizobia (Gusain et al. 2015), suggest a good joint action of these two factors in mitigating water deficit effects on water status, elevating the level of tolerance to drought in cowpea plants. The interaction is initially positive due to the osmoprotectant and antioxidant action of SA, which reduces water stress to levels that favor the symbiosis between plant and *Bradyrhizobium*, which leads to increase in the levels of nutrients and osmoprotectant molecules, enhancing the water stress mitigating action. This is seen in the sensitive genotypes (BRS Marataoã and BR 17 Gurguéia) because, under water stress conditions with inoculation of *Bradyrhizobium* in the absence of SA (W50 + BR), leaf water potential is not reestablished, confirming that there is different symbiotic compatibility between the cowpea genotype and *Bradyrhizobium* according to its tolerance and intensity of water stress.

The low activity of the enzymes observed in the present study may be related to the increase in proline content, because this amino acid can act in the mitigation of water deficit as it helps maintain photosynthetic and respiratory processes, reducing ROS production, which would partially justify the reduction in the activity of this peroxidase (Sharma et al. 2012).

For BRS Rouxinol, the tolerance to drought is probably so low that it restricts the capacity to absorb nutrients and establish symbiosis with N-fixing bacteria, under natural conditions. The simple fact that *Bradyrhizobium* inoculation improved plant nutrition was sufficient for this genotype to maintain osmotic/water homeostasis, since *Bradyrhizobium* activity in the roots provides N for the synthesis of amino acids (Tairo et al. 2017), confirmed in the present study by the increase in free proline contents in the leaves. In addition, the *Bradyrhizobium* sp. ESA 17 isolate can elicit other plant defense mechanisms to endure the drought conditions and promote higher tolerance in this genotype. It is important to keep in mind that the efficiency in the biological nitrogen fixation and other plant growth promotion traits, including induction of drought tolerance, in legume-rhizobia interaction is dependent on the plant and bacterial genotypes (Marinho et al. 2014). The ESA 17-BRS Rouxinol interaction should be further studied regarding the drought tolerance elicitation promoted by the rhizobia strain in this variety.

Salicylic Acid and Inoculation with *Bradyrhizobium* Mitigate Water Stress and Improve Plant Growth in Cowpea Plants

The maintenance of plant growth rate observed in all genotypes, even under water deficit conditions, suggests the action of mechanisms that enable some plant growth, indicating tolerance to the stress condition. However, the growth of BRS Aracê was only intensified by SA and BR, which is associated with the improvement. In this genotype, the synergistic effect of both stress alleviation treatments must rely on the reduction of osmotic and oxidative damage and on N nutrition (Kaushal and Wani 2016; Dutra et al. 2017).

For plants that were treated with SA and showed increments in their leaf area, it is possible to infer that the beneficial action of this hormone increases carbon assimilation, synthesizes metabolites and ensures the maintenance of water potential in the tissues, increasing plant photosynthetic capacity, which results in expansion of tissues (Karlidag et al. 2009; Farooq et al. 2010). In studies with mustard plants under water deficit, but treated with salicylic acid, SA alleviated the adverse effects of drought, causing increase in their leaf area, which may have resulted from the increase in photosynthetic efficiency (Nazar et al. 2015).

In relation to the reduction in biomass accumulation, the loss of cell water potential, observed in all genotypes subjected to water deficit in the present study, caused lower turgor in the cells, preventing their division, which retarded or precluded their normal growth (Carvalho et al. 2017). Nonetheless, N-fixing bacteria inoculation in genotypes with potential for tolerance, such as BRS Marataoã and BR 17 Gurguéia subjected to water deficit, stimulates photosynthetic processes and reduces negative effects of the stress, mainly due to the nitrite supply to the plant, which intensifies the fresh weight gain (Oliveira et al. 2017). In addition, plants subjected to water stress normally exhibit reduction of growth, but the presence of SA has beneficial effects on their growth characteristics, because it regulates several physiological processes such as photosynthesis and stomatal conductance, regulates the functions of chlorophylls (Liu et al. 2011) and improve carbon assimilation, which ensures the maintenance of water potential in the tissues (Farooq et al. 2010).

It is important to emphasize that these treatments were also promising in the reduction of leaf water potential in these genotypes (Table 1). However, despite being promising in reducing the effects of water stress on leaf water potential and growth, the above-mentioned attenuators were not efficient in mitigating the effects of water stress on the dry matter accumulation of BRS Rouxinol and BRS Aracê, compared to the W100 treatment.

In summary, water stress negatively affected leaf water potential and biochemical and morphological parameters of the cowpea genotypes. Sole application of SA was efficient in mitigating water deficit effects on the most sensitive genotype, BRS Marataoã. *Bradyrhizobium* inoculation favored the maintenance of water status in BRS Rouxinol and BRS Aracê, clearly in the former, in which the N supply through *Bradyrhizobium* promoted improvements in water and redox homeostasis and, consequently, in its growth. *Bradyrhizobium* inoculation associated with foliar application of SA was the most efficient strategy for BRS Marataoã, BRS Aracê and BR 17 Gurguéia, maintaining their values of leaf water potential, growth, proline content and activities of superoxide dismutase, ascorbate peroxidase and catalase similar to those observed in the control treatment.

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Compliance with Ethical Standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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