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Original Article

Salicylic acid mitigates damage caused by water deficit in forage sorghum[1]

Ácido salicílico mitiga os danos causados pelo déficit hídrico em sorgo forrageiro

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HIGHLIGHTS:

Salicylic acid (2.76 g L^{-1}) can be used to attenuate the effects of deficit irrigation depths.
Deficit irrigation depths delay growth characteristics of sorghum.
Salicylic acid favors the physiological variables of sorghum.

ABSTRACT: The sorghum crop is considered one of the most important worldwide due to its versatility. However, water stress can be considered a significant threat to its yield. The search for products that mitigate water stress is a crucial area of research in agriculture and water resources management. Thus, the objective in this study was to evaluate the feasibility of using salicylic acid as a water stress attenuator in sorghum. The experiment was conducted in a screened environment in a randomized block design with a 4 × 2 factorial scheme, with four replicates. Four levels of water (40, 60, 80, and 100% of evapotranspiration) and two concentrations of salicylic acid (0 and 2.76 g L^{-1}) were evaluated. Plant height, stem diameter, number of leaves, gas exchange, photosynthetic pigments, and chlorophyll a fluorescence were analyzed. Application of deficit irrigation depths reduced plant height, stem diameter, number of leaves, and total chlorophyll in sorghum. However, when these depths were associated with salicylic acid, it was observed that the damage was attenuated, especially in chlorophyll a and b. Furthermore, salicylic acid reduced leaf temperature and increased water use efficiency when applied alone. Thus, salicylic acid can be used to mitigate the effects of salt stress on sorghum plants.

Key words: *Sorghum bicolor* (L). Moench, abiotic stress, phytohormone

RESUMO: A cultura do sorgo é considerada uma das mais importantes do mundo devido a sua versatilidade de utilização. No entanto, o estresse hídrico pode ser considerado uma ameaça significativa para sua produtividade. A busca por produtos mitigadores do estresse hídrico é uma área de pesquisa crucial na agricultura e na gestão de recursos hídricos. Assim, o objetivo deste trabalho foi avaliar a viabilidade do uso do ácido salicílico como atenuante de estresse hídrico na cultura do sorgo. O experimento foi conduzido em ambiente telado, em delineamento em blocos ao acaso com esquema fatorial 4 × 2, com quatro repetições. Foram avaliados quatro níveis hídricos (40, 60, 80 e 100% da evapotranspiração) e duas concentrações de ácido salicílico (0 e 2,76 g L^{-1}). Foram analisados a altura das plantas, diâmetro do caule, número de folhas, trocas gasosas, pigmentos fotossintéticos e fluorescência da clorofila a. A aplicação de lâminas de irrigação deficitárias reduziu a altura da planta, diâmetro do colmo, número de folhas e clorofila total no sorgo. No entanto, quando essas lâminas foram associadas ao ácido salicílico, foi observado que houve atenuação dos danos, especialmente em relação a clorofila a e b. Além disso, o ácido salicílico quando aplicado isoladamente, contribuiu para redução da temperatura da folha e aumento da eficiência de uso da água. Assim, o ácido salicílico pode ser usado para mitigar os efeitos do estresse salino em plantas de sorgo.

Palavras-chave: *Sorghum bicolor* (L). Moench, estresse abiótico, fitohormônio

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Introduction

Forage sorghum [*Sorghum bicolor* (L). Moench], belonging to the Poaceae family, is among the main cereals in the world. Its recognized versatility extends from the use of its grains as human food and animal feed, as raw material for the production of various products, and extraction of sugar from its stalks to the numerous applications of its forage in ruminant nutrition (Albuquerque et al., 2021). The plant has a C4 metabolism, is adapted to short days, and has high photosynthetic rates. It needs temperatures above 21 °C for most of its genetic potential to thrive and to be more tolerant to water stress and excessive soil moisture than most other crops (Magalhães et al., 2003).

Stress can be defined as any environmental condition that prevents a plant from reaching its full genetic potential; one is water stress (Sun et al., 2020). When plant cells run out of water, cellular dehydration occurs, inducing the accumulation of abscisic acid (ABA), leading to stomatal closure, reducing gas exchange, and inhibiting photosynthesis (Singh & Roychoudhury, 2023).

The search for products that mitigate water stress is a crucial area of research in agriculture and water resources management. Among the plant growth regulators, salicylic acid (SA) stands out. It acts in the germination process, growth regulation, as a non-enzymatic antioxidant agent and in the activation of stress defense mechanisms (Lisboa et al., 2017; Li et al., 2022).

Gomes et al. (2018), when evaluating the effect of salicylic acid as a water stress attenuator in corn crop, observed an increase in chlorophyll levels and root mass in plants subjected to water deficit, suggesting a mitigating effect of this regulator. Therefore, the objective in this study was to evaluate the feasibility of using salicylic acid as a water stress attenuator in sorghum.

Material and Methods

The experiment was performed in a greenhouse located at the Universidade Federal Rural do Semi-Árido (UFERSA), in Mossoró, RN, Brazil (5° 12’ 11” S and 37° 19’ 25.7” W, altitude 13 m), conducted from October to December 2023. According to Köppen’s classification, the municipality of Mossoró is characterized as a semi-arid region with a hot and dry BSh climate (Alvares et al., 2013), with an estimated average annual rainfall of 555 mm, an average temperature of 27.8 °C, and an average relative humidity of the air of 63.25% (Climate-Data, 2023). During the experiment, the values of temperature and relative humidity were collected using a digital thermo-hygrometer and are presented in Figure 1.

The soil used was Ultisol (United States, 2014), classified as Argissolo Vermelho-Amarelo in the Brazilian Soil Classification System (Santos et al., 2018). The soil and organic compost used were collected in the didactic garden of UFERSA.

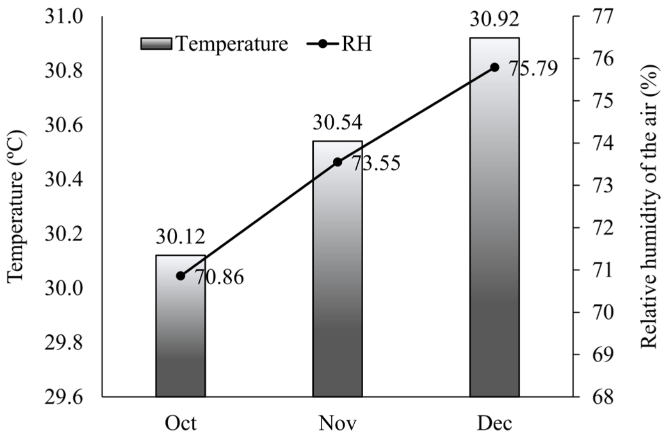


Figure 1. Temperature (°C) and relative humidity of the air (RH) (%) during the months of conducting the experiment

A soil sample was sent to the Soil, Water and Plant Analysis Laboratory (LASAP) of UFERSA for characterization of the chemical attributes (Table 1) according to the methodology described in the Manual of Methods of Soil Analysis of Embrapa (Teixeira et al., 2017).

The experiment was conducted in a randomized block design in a 4 × 2 factorial scheme, with four replicates. Four levels of water application (40, 60, 80, and 100% of ETo) and two concentrations of salicylic acid (0 and 2.76 g L^{-1}) were evaluated.

Each experimental unit consisted of a pot with 4 dm^3 of soil and organic compost in a 3:1 ratio (3 dm^3 of soil with 1 dm^3 of organic compost) with one sorghum plant, cultivar IPA SF-15, per pot, with four replicates, totaling 32 experimental plots.

Initially, the soil and organic compost were air-dried and sieved through a 4 × 4 mm mesh sieve. Subsequently, each pot was weighed. Water stress was determined through soil water retention tests, considering the actual ETo (evapotranspiration) of the soil. Initially, soil saturation was performed until drainage occurred. Then, the pots were sealed with a black plastic bag, and soil saturation was observed by weighing them after 24 and 48 hours.

After that, the amount of water retained in the soil after 48 hours was considered to represent field capacity (100% of actual ETo). With this value, the other water levels (40, 60 and 80%) were estimated (Table 2).

Three sorghum seeds of ‘IPA SF-15’ were sown per pot directly into the soil. This cultivar, developed by the Agronomic

Table 2. Arrangement of water depths applied to the soil for each water deficit treatment

Irrigation depths	(% ETo)	Water depth (g)
L1	100	800
L2	80	640
L3	60	480
L4	40	320

L1 - Irrigation depth 1 (100% ETo); L2 - Irrigation depth 2 (80% ETo); L3 - Irrigation depth 3 (60% ETo); L4 - Irrigation depth 4 (40% ETo)

Table 1. Chemical attributes of the soil before setting up the experiment

pH	EC	P	K^+	Na^+	Ca^{2+}	Mg^{2+}	EB	CEC	V	ESP
(water)	($dS\ m^{-1}$)	($mg\ dm^{-3}$)			($cmol_c\ dm^{-3}$)				(%)	
7.11	0.06	104.7	177.4	16.4	3.2	1.1	4.83	4.83	100	1.0

pH - Hydrogen potential; EC - Electrical conductivity; EB - Sum of exchangeable bases; CEC - Cation exchange capacity; V - Base saturation, ESP - Exchangeable sodium percentage

Institute of Pernambuco, is recommended for the Northeast region. It has an average height of 2.50 to 3.50 m, a cycle of 120 days, and can produce about 15 tons per hectare of dry matter and 60 tons per hectare of fresh matter (Oliveira et al., 2016).

During sowing and up to 15 days after germination, the soil in the pot was maintained at 80% field capacity with daily irrigation. After 15 days of germination, when the seedlings reached about 10 to 15 cm in height, thinning was carried out, leaving only one plant per pot. On the same day the thinning was performed, treatments with 48-hour intermittent irrigation began, and an attenuating agent was applied to the plants.

The attenuating agent was applied weekly with a manual sprayer. Tween 80® (0.25 mL L^{-1}) was added to the salicylic acid solution to improve absorption by the leaves. Evaluations of growth and physiological aspects were carried out 110 days after the application of the treatments (DAT).

Sorghum height was measured with a 3 m measuring tape, from the base to the highest leaf of the plant. Stem diameter was measured with a digital caliper, at 4 cm above the base of the plant. Number of leaves was obtained by manual counting.

Gas exchange measurements was performed between 8 a.m. and 11 a.m. using a portable infrared gas analyzer (IRGA - Model Gfs 3000 Walz). Artificial light with a photosynthetically active photon density of 1,200 μmol m^{-2} s^{-1} was used. The variables analyzed with this equipment were: net CO_2 assimilation rate (A, μmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}), instantaneous water use efficiency (WUE = A/E), intrinsic water use efficiency (iWUE = A/gs), leaf temperature (Tleaf - °C), and vapor pressure deficit (VPD, kPa).

Chlorophyll a, chlorophyll b, and total chlorophyll were measured using a portable chlorophyll meter (ClorofiLog, Falker-CFL1030) at two points of intermediate leaves of the branches, and the results were expressed in FCI (Falker Chlorophyll Index).

Chlorophyll fluorescence was also measured using a portable fluorometer (Sciences Inc. - model OS-30p, Hudson, USA), in intermediate leaves of the branches, pre-adapted to the dark for 30 min, using leaf clips. The following variables were analyzed: initial fluorescence (F_o), maximum fluorescence (F_m), variable fluorescence (F_v), PSII quantum efficiency (F_v/F_m), and F_v/F_o ratio.

The data were subjected to analysis of variance (ANOVA). In cases of significance, polynomial regression analysis was performed for the quantitative factor (irrigation depths), and the Tukey's test ($p \leq 0.05$) was performed to compare the means of the qualitative factor (salicylic acid). Statistical analyses were conducted using the SISVAR v. 5.6 program (Ferreira, 2011).

Results and Discussion

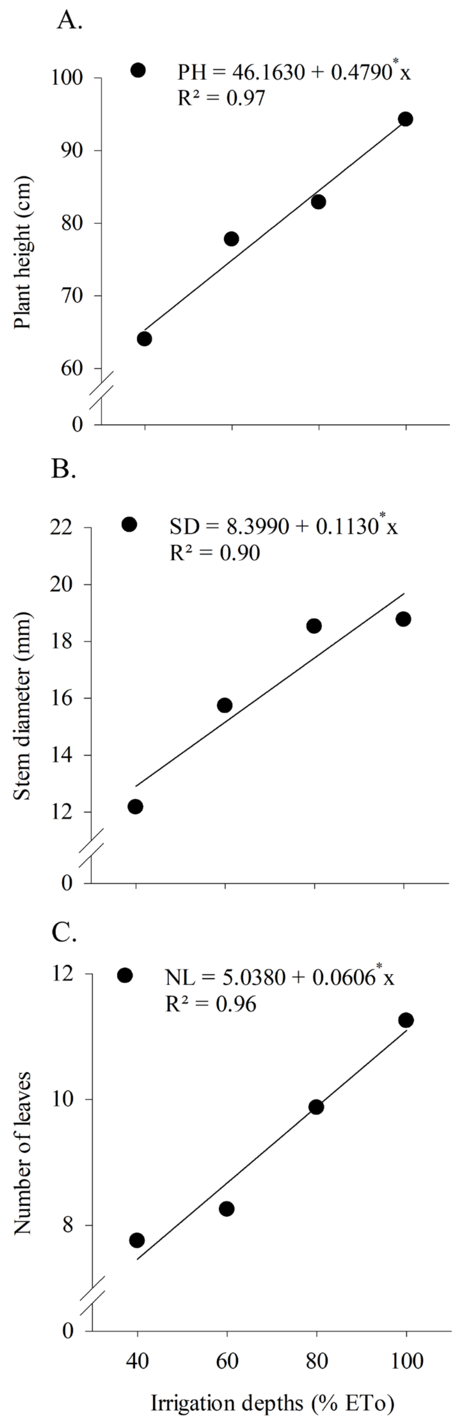
As observed in the results of the analysis of variance (ANOVA) for the growth characteristics of forage sorghum, the variables plant height (PH), stem diameter (SD), and number of leaves (NL) were significantly affected by the levels of water deficit (Table 3).

Irrigation depths with more significant water stress reduced plant biometric variables (Figure 2). When comparing the

Table 3. Summary of analysis of variance (ANOVA) for plant height (PH), stem diameter (SD), and number of leaves (NL) of sorghum plants as a function of irrigation depths and salicylic acid

Sources of variation	DF	Mean squares		
		PH	SD	NL
WD	3	1258.86^{**}	75.59^{**}	20.36^{*}
SA	1	69.03^{ns}	0.66^{ns}	1.53^{ns}
WD × SA	3	60.53^{ns}	3.46^{ns}	1.03^{ns}
Error	21	-	-	-
CV (%)		17.73	7.68	25.83

WD - Water deficit; SA – Salicylic acid; CV - Coefficient of variation; DF - Degree of freedom; ** - Significant at 0.01 level of probability by the F test; * - Significant at 0.05 level of probability by the F test; ns – Not significant



* - Significant at 0.05 level of probability by F test

Figure 2. Plant height (A), stem diameter (B), and number of leaves (C) of sorghum plants as a function of irrigation depths

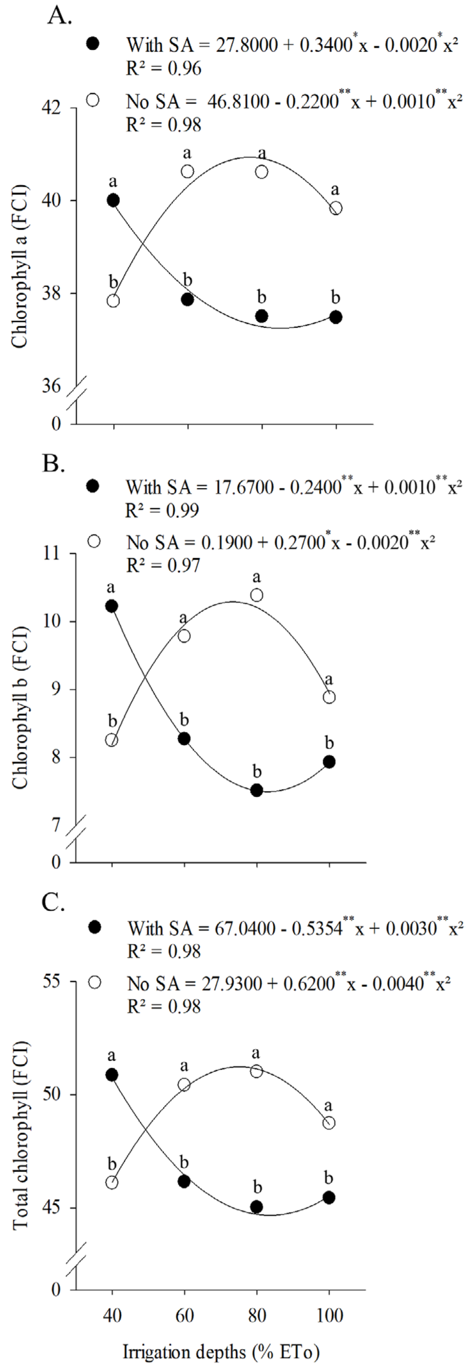
highest irrigation depth (100% ETo) with the highest water stress (40% ETo), there was a reduction of 32.10% in plant height (PH), 35.13% in stem diameter (SD), and 31.11% in number of leaves (NL). This behavior is associated with the reduction of water in the soil and the availability of nutrients associated with sorghum's survival mechanisms when subjected to extreme stress conditions.

According to Bhattacharya (2021), reducing the amount of water used in irrigation leads to reduced crop growth and yield. However, as observed in the present study and other studies such as Silva et al. (2023), applying depths between 70 and 80% of ETo provides acceptable production. It is justified by the reduction in water consumption, especially in arid and semi-arid regions.

The reduction in the amount of water in irrigation causes changes in the water potential of the soil and the behavior of the plants; under these conditions, sorghum can close its stomata to reduce water loss through leaf transpiration (Queiroz et al., 2023). With stomatal closure, the plant can reduce transpiration. However, it also reduces its photosynthesis and production of photoassimilates, which justifies the reduction of variables such as PH, SD, and NL (Ribeiro et al., 2023).

There was an interaction between the water levels and salicylic acid for chlorophyll a and b (Table 4). However, for total chlorophyll, a significant effect was observed only for the water levels. Regarding the variables initial fluorescence (F_o), maximum quantum yield of PSII (F_v/F_m), and the ratio between variable fluorescence and initial fluorescence (F_v/F_o), a significant response was observed to the water deficit factor. On the other hand, the maximum fluorescence (F_m) and variable fluorescence (F_v) showed a significant effect for both factors (water levels and salicylic acid), but individually. These results suggest that water deficit primarily affects photosystem II (PSII) efficiency and overall photosynthetic performance, as indicated by changes in F_v/F_m and F_v/F_o ratios. The interaction between water levels and salicylic acid on chlorophyll content indicates that salicylic acid may influence plant response to water stress by affecting chlorophyll biosynthesis or degradation (El-Bially et al., 2022). The independent effects of water levels and salicylic acid on F_m and F_v highlight their distinct roles in photochemical processes and stress responses.

Chlorophyll a, chlorophyll b, and total chlorophyll of sorghum, under water stress, showed similar responses to both factors (Figures 3A, B, and C). When salicylic acid was applied, the lower irrigation depth promoted increases of 6.72 and 28.88% in chlorophyll a and b levels, respectively, compared to the highest irrigation depth.



* and ** significant at 0.05 and 0.01 levels of probability, respectively, by F test. Means followed by the same letters do not differ from each other for treatment with SA and without SA, according to the Tukey's test ($p \leq 0.05$)

Figure 3. Chlorophyll a (A), chlorophyll b (B) and total chlorophyll (C) of sorghum plants as a function of irrigation depths and salicylic acid

Table 4. Summary of analysis of variance (ANOVA) for chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl T), initial fluorescence (F_o), maximum fluorescence (F_m), variable fluorescence (F_v), maximum PSII quantum yield (F_v/F_m), and the ratio between variable fluorescence and initial fluorescence (F_v/F_o) of sorghum plants as a function of irrigation depths and salicylic acid

Sources of variation	DF	Mean squares							
		Chl a	Chl b	Chl T	F_o	F_m	F_v	F_v/F_m	F_v/F_o
WD	3	0.58^{ns}	0.98^{ns}	3.04^{ns}	24.28^{*}	297.75^{**}	204.15^{**}	0.000273^{*}	0.056^{*}
SA	1	14.78^{ns}	5.63^{ns}	38.72^{ns}	13.78^{ns}	300.15^{*}	183.32^{*}	0.000010^{ns}	0.0038^{ns}
WD × SA	3	15.48^{*}	8.35^{*}	45.55^{*}	7.61^{ns}	62.54^{ns}	28.31^{ns}	0.000014^{ns}	0.0033^{ns}
Error	21	-	-	-	-	-	-	-	-
CV (%)		5.61	16.76	15.59	4.19	2.95	3.35	1.21	4.61

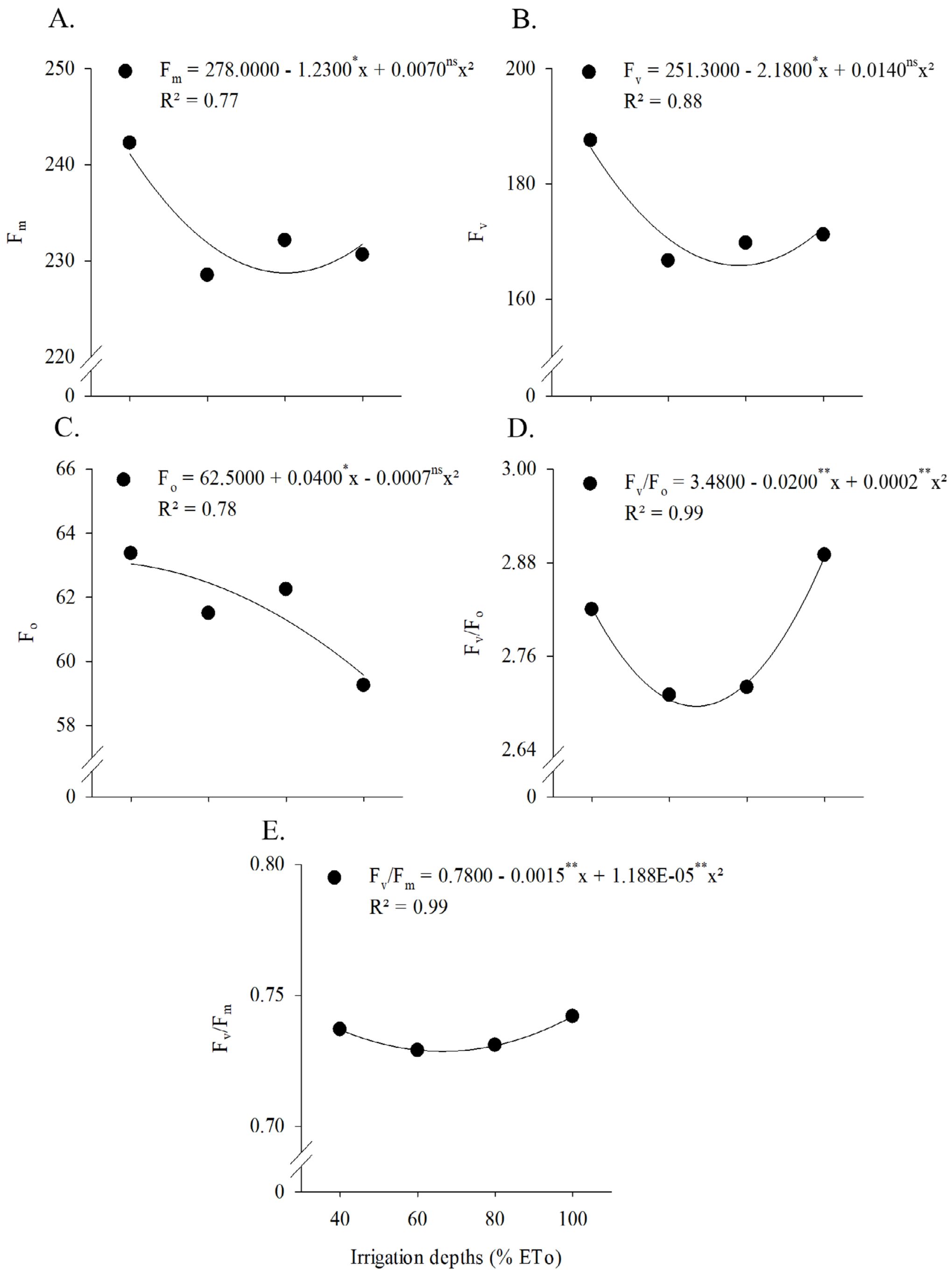
WD - Water deficit; SA – Salicylic acid; CV - Coefficient of variation; DF - Degree of freedom; ** - Significant at the level of 0.01 probability by the F test; * - Significant at the level of 0.05 probability by the F test; ns – Not significant

These results indicate that salicylic acid plays a role as a molecular signaling agent and non-enzymatic antioxidant that intervenes in water stress tolerance through the regulation of physiological processes, offering protection against biotic and abiotic stresses (Lefevere et al., 2020). Because of this, its contribution significantly reduced water stress, corresponding to a lower irrigation depth.

Sorghum plants that did not receive the attenuating agent obtained an increase in chlorophyll a, chlorophyll b, and total chlorophyll under the water depths of 60 to 80% ETo (Figures 3A, B and C). This behavior indicates that water restriction can have numerous consequences when plant species are subjected to this condition, with decreased leaf expansion, cellular and metabolic activities, stomatal closure, photosynthetic inhibition, leaf abscission, and cessation of plant growth (Taiz et al., 2024). In this context, sorghum plants were efficient under intermediate water deficit conditions, with lower depths capable of compromising chlorophyll production.

Reducing the chlorophyll index under deficit irrigation constitutes a plant defense mechanism to reduce the pressure of excess energy from PSI and PSII (Ayala et al., 2014). Mesquita et al. (2020) suggest that the chlorophyll index in cowpea plants under water restriction was reduced so as not to overload the photosynthetic apparatus, reducing the damage caused by stress.

Maximum fluorescence (F_m), variable fluorescence (F_v), and initial fluorescence (F_o) were reduced with increasing water levels (Figures 4A, B, and C). The ratio between variable



*, ** and ^{ns} significant at 0.05 and 0.01 levels of probability and non-significant, respectively, by F test

Figure 4. Maximum fluorescence – F_m (A), variable fluorescence – F_v (B), initial fluorescence – F_o (C), ratio between variable fluorescence and initial fluorescence - F_v/F_o (D), and maximum quantum yield of PSII - F_v/F_m (E) of sorghum plants as a function of irrigation depths

fluorescence and initial fluorescence (F_v/F_o) and the maximum quantum yield of PSII (F_v/F_m) (Figures 4D and E) showed a quadratic increase as a function of the increase in soil water availability.

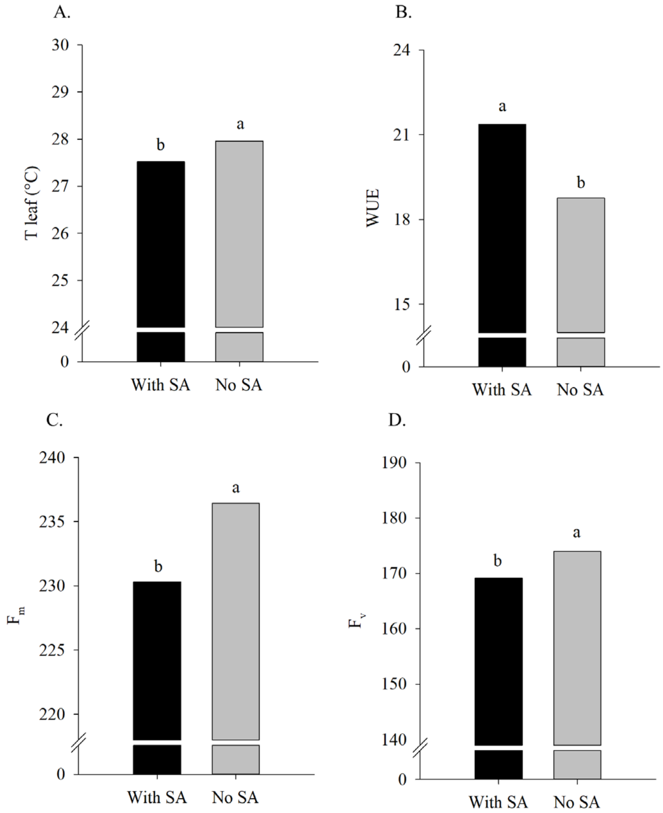
The highest F_m, F_v, and F_o values were obtained at the 40% ETo depth, being 242.25, 187.56, and 63.37, respectively. Compared with the highest water depth applied (100% ETo), these values showed reductions of 5.04, 9.58, and 6.95%, respectively. These results indicate that the stress from the water deficit may have caused the reduction in F_m, F_v, and F_o values (Medyouni et al., 2021).

On the other hand, the highest values of F_v/F_o and F_v/F_m were observed with the application of the highest irrigation depth (100% ETo) (Figures 4D, 4E). Compared with the lowest irrigation depth, these values showed increases of 2.42 and 0.67% in F_v/F_o and F_v/F_m, respectively. According to Lichtenthaler et al. (2005), the reduction in F_v/F_o and F_v/F_m values can be attributed to a photochemical decline in PSII due to a possible disturbance or damage to the photosynthetic apparatus. This behavior is caused by the stress suffered by the plants when irrigated with deficit depths.

Leaf temperature (Tleaf) was significantly affected by the factors, water deficit and salicylic acid, which were evaluated individually (Table 5). As for the vapor pressure deficit (VPD), there was a significant difference only for the water deficit factor, while for the water use efficiency (WUE), the significance was observed only for the salicylic acid factor.

When salicylic acid was applied, Tleaf showed a lower value in sorghum plants (Figure 5A). This behavior can be attributed to salicylic acid's regulatory role in physiological processes, providing protection against stress. The reduction in leaf temperature indicates that salicylic acid may improve the ability of plants to dissipate heat, possibly through the regulation of internal mechanisms such as antioxidant activity and stabilization of cell membranes (Zahra et al., 2023). This protective effect may be crucial for maintaining photosynthetic efficiency and reducing oxidative stress during drought stress conditions.

WUE increased under application of salicylic acid (Figure 5B). This result is consistent with what Agami et al. (2019) observed with application of salicylic acid (0.1 mM) to wheat plants under water deficit. In this study, it was observed that water stress induced a decrease in WUE. However, applying salicylic acid could relieve stress due to water deficit, resulting in higher WUE values. As it is a standard method to assess the water balance in plant leaves when water is insufficient, WUE is essential to maintain cells and tissues in a healthy state and induces metabolic activity (Slabbert & Krüger, 2014).



Means followed by the same letters do not differ from each other according to the Tukey's test ($p \le 0.05$)

Figure 5. Leaf temperature (Tleaf - A), water use efficiency (WUE - B), maximum fluorescence (F_m – C), and variable fluorescence (F_v - D) of sorghum plants as a function of the presence (With SA) or absence (No SA) of salicylic acid

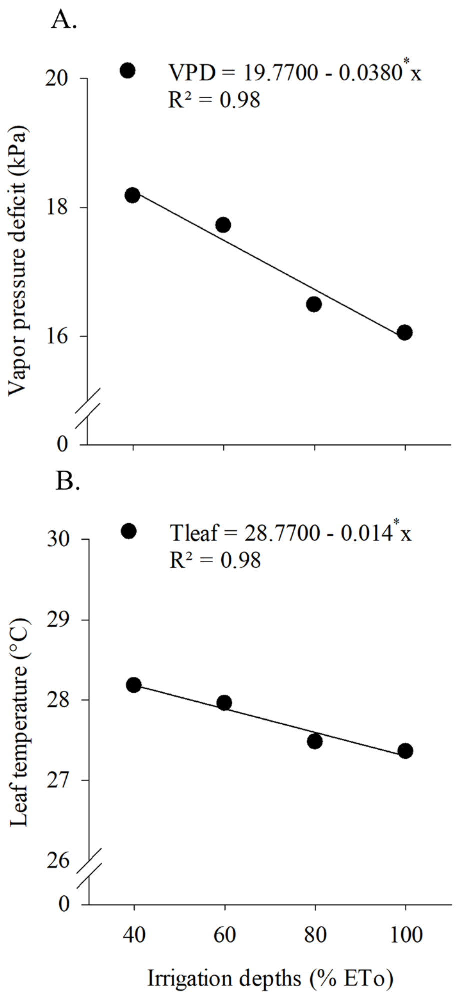
Maximum fluorescence (F_m) and variable fluorescence (F_v) were lower in sorghum plants when associated with the use of salicylic acid (Figure 5C and D). The decrease in F_m and F_v indicated the interruption of photochemical activities in PSII and electron transport. These results reinforce the idea that water deficit leads to the impairment of the reaction centers, although the disturbances are reversible and do not compromise the photosynthetic apparatus.

Vapor pressure deficit (VPD) and leaf temperature (Tleaf) showed a reduction in their values with the increase in the irrigation depth (Figures 6A and B). The reduction in leaf temperature associated with the application of the highest water depth indicates that the presence of water cools the leaf.

Table 5. Summary of analysis of variance (ANOVA) for leaf temperature (Tleaf), transpiration rate (E), vapor pressure deficit (VPD), stomatal conductance (gs), CO_2 assimilation rate (A), instantaneous water use efficiency (WUE), and intrinsic water use efficiency (iWUE) of sorghum plants as a function of irrigation depths and salicylic acid

Sources of variation	DF	Mean squares						
		Tleaf	E	VPD	gs	A	WUE	iWUE
WD	3	1.21^{**}	0.14^{ns}	8.09^{**}	1083.51^{ns}	7.31^{ns}	24.55^{ns}	0.0176^{ns}
SA	1	1.53^{**}	0.74^{ns}	3.08^{ns}	2475.08^{ns}	10.53^{ns}	171.63^{*}	0.030^{ns}
WD × SA	3	0.08^{ns}	0.13^{ns}	1.02^{ns}	649.00^{ns}	10.95^{ns}	1.74^{ns}	0.0006^{ns}
Error	21	-	-	-	-	-	-	-
CV (%)		1.6	41.49	5.95	41.49	25.93	26.14	27.5

WD - Water deficit; SA – Salicylic acid; CV - Coefficient of variation; DF - Degree of freedom; ** - Significant at 0.01 level of probability by the F test; * - Significant at 0.05 level of probability by the F test; ns – Not significant



* - Significant at 0.05 by F test

Figure 6. Vapor pressure deficit (A) and leaf temperature (B) of sorghum plants as a function of different irrigation depths

Leaf temperature can be considered a relevant indicator to identify plants that are experiencing water stress. According to Taiz et al. (2024), high temperatures in the leaves contribute to stomatal closure. With the closure of the stomata, the plant gets overheated, impairing its metabolism, a behavior similar to that observed when water depth equivalent to 40% ETo was applied. This overheating occurs because stomatal closure limits transpiration (Ribeiro et al., 2020; Ribeiro et al., 2022), a key mechanism for heat dissipation in plants. Reduced transpiration increases leaf temperature and reduces CO_2 assimilation, negatively impacting photosynthesis and plant growth.

Conclusions

1. High water deficit (40 and 60% of ETo) reduced height, stem diameter, number of leaves, total chlorophyll, vapor pressure deficit, and leaf temperature in sorghum plants.

2. Application of salicylic acid (2.76 g L^{-1}) positively influenced the chlorophyll a and b variables, making sorghum plants respond more efficiently even under water stress conditions.

3. Salicylic acid has been shown to be effective in reducing leaf temperature and increasing water use efficiency in sorghum plants.

Contribution of authors: R. M. O. da S. Marcelino worked on research and methodology, data acquisition, data analysis, and writing of the manuscript – original draft. F. M. da S. Morais worked on research and methodology, data acquisition, data analysis, and manuscript. F. N. Ferreira worked on research and methodology, data acquisition, data analysis, and manuscript. L. de S. Alves worked on research and methodology, data acquisition, data analysis, and manuscript. E. L. Moura worked on research and methodology, data acquisition, data analysis, and manuscript. A. K. de S. Melo worked on research and methodology, data acquisition, data analysis, and manuscript. J. F. de Medeiros worked on research and methodology. A. G. de L. Souto worked on research, methodology, and data acquisition. A. M. dos S. Pessoa worked on research, methodology, and data analysis. J. E. da S. Ribeiro served as research advisor and worked on the conceptualization of the problem, literature review, data acquisition, improvements, and corrections to the manuscript - review & editing.

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