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Article

Unraveling the Mechanisms of Salicylic Acid and Rehydration and the Physiological Benefits in Pepper Plants Grown under Water Deficit

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Abstract: The present study aims to unravel the mechanisms of salicylic acid and rehydration and the physiological benefits in pepper plants grown under water deficit. 3x4 factorial scheme adopted, with three water regimes (irrigated, dry and rehydration) and four SA concentrations, namely: 0 (control), 0.5, 1 and 1.5 mM. Evaluated leaf moisture percentage, leaf water potential, chlorophylls (*a* and *b*), carotenoids, stomatal conductance, chlorophyll *a* fluorescence and H₂O₂ concentration at different times throughout the day and water conditions (irrigated, dry and rehydration) and SA applications (without the addition of the regulator (0) and with SA addition at concentrations equal to 0.5, 1 and 1.5 mM). Exogenous SA application favored stomatal conductance responses (*g_s*) and modified fluorescence parameters (*F_m*, *F_o*, *F_m'*, *F_o'*, *F_v/F_m*, *qP*, *ETR* and *NPQ*) of sweet pepper plants subjected to drought followed by rehydration. SA concentration of 0.5 mM allowed a more expressive recovery of *g_s* indices, effective quantum yield of photosystem II, with emphasis on excess energy (E) and maximum quantum yield (FV/FM) over time in plants subjected to drought and rehydration. We discovered that the use of SA, especially in association with rehydration, by modulating the variables *g_s* and chlorophyll *a* fluorescence, prevents damage to membranes and consequently mitigates water deficit in pepper plants.

Keywords: vegetal regulator; water deficit; photosynthetic pigments; stomatal conductance; chlorophyll *a* fluorescence; hydrogen peroxide

1. Introduction

Temporary water deficit influences physiological, biochemical, metabolic and morphological processes, interfering with plant growth and development, and consequently plant productivity [1,2]. Water deficiency in these processes involves complexity and plants respond to stress through mechanisms that avoid or lead to adaptation to these effects [3,4].

In plants under water deficit, the formation of chlorophyll can be inhibited, due to the increased production of hydrogen peroxide, which causes damage to membranes and degradation of pigments

[5]. In periods of drought, lack of water can alter the photosynthetic activities of plants [6], modifying stomatal conductance (g_s) and restricting the entry of CO_2 into the substomatal chamber [7,8].

The reduction in CO_2 assimilation promoted by stomatal closure contributes to the imbalance between photochemical activity in photosystem II (PSII) and the requirement for electrons for photosynthesis, promoting photoinhibitory damage to the PSII reaction center [9,10], causing deviation of absorbed light to other processes, such as thermal dissipation to protect the photosynthetic apparatus [11,12], especially under stress conditions due to water scarcity.

Under water stress conditions, resistance inducers have been applied, which act in plant protection, improving plant defense mechanisms. Among protection inductors, salicylic acid (SA) stands out, an endogenous signaling molecule involved in physiological processes and in the induction of biosynthesis reactions for the development of protection systems [13,14,15].

SA has been used to modulate the physiological and biochemical response of plants subjected to drought, salinity, low and high temperatures [16,17]. Among physiological responses, SA increases net photosynthesis [18], interfering both in the activity of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBP) [19], and regulating sweating under dry conditions, which reduces water loss and maintains turgor, leading to maintenance of photosynthesis and productivity [20,21].

Thus, the attenuating effect of SA under drought conditions may be associated with its role in photosynthesis [22,23] and in stomatal regulation [24], which, in turn, act as pathways eliminating reactive oxygen species and providing protection to the photosynthetic apparatus under stress conditions [25,26].

Specifically, in the cultivation of bell pepper (*Capsicum annum* L.), water is an essential factor and its scarcity causes production losses [27,28]. In this context, SA application may represent a promising source for inducing drought resistance mechanisms in pepper and several other crops of agricultural interest, including vegetables, which are generally susceptible to water stress. 'Melina' hybrid pepper, suitable for cultivation in tropical regions, has high acceptability by producers; however, it is subject to adverse weather conditions, such as cycles consisting of lack of water, followed by rain, which can lead to plant non-reestablishment due to the presence of successive stress cycles. Although the physiological responses of plants to SA under water stress are known, specifically in pepper plants, there is a lack of research on this topic. Thus, we believe that rehydration and exogenous SA application strengthen the physiological responses of plants, favoring carbon dioxide assimilation and the production of pigments, resulting in less damage to the photosynthetic apparatus and increase in plant defense when subjected to a drought period.

Thus, the hypothesis of this research is that exogenous SA application together with rehydration improves leaf water potential, pigment and carotenoid synthesis, stomatal conductance and chlorophyll *a* fluorescence throughout the day and it is crucial to attenuate effects arising from the production of hydrogen peroxide in young sweet pepper plants subjected to drought.

If the hypothesis of this study is accepted, it will make it possible to advance strategies for optimizing the use of SA to combat water deficit with global implications given the increase in the frequency of drought with climate change, which affects pepper production in different growing regions.

This study aims to unravel the mechanisms of salicylic acid and rehydration and the physiological benefits in pepper plants grown under water deficit on based on pigment content, leaf water potential, stomatal conductance and chlorophyll *a* fluorescence throughout the day, as well as hydrogen peroxide content in young pepper plants subjected to drought.

2. Results

The moisture percentage in leaves was different throughout the day only at 12:00 am and 02:00 pm, with higher values for leaves of plants in the irrigated treatment, an expected result, since plants under this condition have greater water availability in the soil. However, there was no effect of salicylic acid (SA) concentrations on the moisture percentage in leaves (Table 1).

Table 1. Leaf moisture percentage throughout the day in the Irrigated and Dry treatments, at 7 days after application of treatments (DAAT) and at 12 DAAT days after application of rehydration. Two-way ANOVA (two water conditions x four SA-salicylic acid concentrations). Average data.

Treatment	08:00 am	10:00 am	12:00 Am	02:00 pm	04:00 Pm
7 DAAT					
Irrigated (%)	74.000	70.400	74.244 a	70.150 a	66.619
Dry (%)	73.600	70.700	70.219 b	64.919 b	63.675
Significance					
Water condition	n.s.	n.s.	P<0.01	P<0.05	n.s.
Concentration SA	n.s.	n.s.	n.s.	n.s.	n.s.
Water condition X SA	n.s.	n.s.	n.s.	n.s.	n.s.
12 DAAT					
Irrigated (%)	69.875	74.681	74.181	69.037	68.119
Rehydration (%)	68.894	74.475	73.825	68.85	65.131
Significance					
Water condition	n.s.	n.s.	n.s.	n.s.	n.s.
Concentration SA	n.s.	n.s.	n.s.	n.s.	n.s.
Water condition X SA	n.s.	n.s.	n.s.	n.s.	n.s.

Regarding plants submitted to irrigated treatment and rehydration of plants submitted to drought 12 days after application of treatments (DAAT), no statistical difference was observed either in relation to the period of the day or in relation to SA concentrations. However, it is possible to verify the recovery of plants subjected to water suspension followed by rehydration, at 12:00am - 02:00 pm, seven days after being subjected to drought (Table 1).

The water potential did not vary in plants in the irrigated and dry treatments, with no effect of SA application being observed (Table 2).

Chlorophylls *a*, *b* and carotenoids were not influenced by water suspension, not differing from plants not subjected to water deficit (Table 2). However, when the highest SA concentration (1.5 mM) was applied, there was reduction in chlorophyll *a* and carotenoid contents (Table 2), reflecting reduction in the hydrogen peroxide levels (H₂O₂) (Figure 5), suggesting that this concentration is excessive for the plants defense mechanism.

Table 2. Water potential of shoots, chlorophyll *a*, *b* and carotenoid content of plants submitted to Irrigation and Dry treatments at 7 days after application of treatments (DAAT) and at 12 DAAT days after application of rehydration. Two-way ANOVA (two water conditions x four SA-salicylic acid concentrations). Average data.

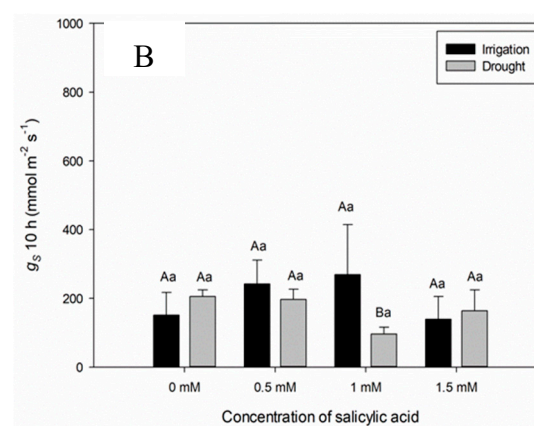
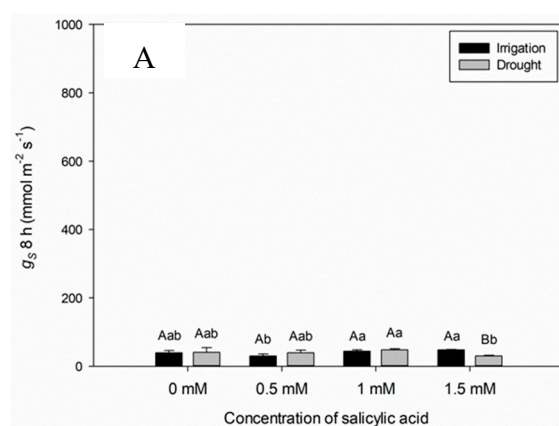
7 DAAT				
Treatment	Aboveground water potential	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Carotenoid <i>s</i>
Irrigated	0.289	5.643	1.530	2.269
Dry (%)	0.297	5.620	1.539	2.434
Concentration SA				
SA	Aboveground water potential	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Carotenoid <i>s</i>
0mM	0.285	6.424 a	1.670	2.564 a
0.5mM	0.275	5.442 ab	1.470	2.307 ab
1mM	0.315	5.524 ab	1.563	2.357 ab

1.5mM	0.296	5.135 b	1.434	2.177 b
Significance				
Water condition	n.s.	n.s.	n.s.	n.s.
Concentration SA	n.s.	P<0.05	n.s.	P<0.05
Water condition X SA	n.s.	n.s.	n.s.	n.s.
12 DAAT				
Treatment	Aboveground water potential	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Carotenoid <i>s</i>
Irrigated	0.244	5.416	1.526	2.331
Rehydration	0.271	5.906	1.587	2.496
Concentration SA	Aboveground water potential	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Carotenoid <i>s</i>
0mM	0.310	6.227	1.918	2.520
0.5mM	0.256	5.311	1.299	2.332
1mM	0.225	5.489	1.500	2.372
1.5mM	0.238	5.617	1.510	2.430
Significance				
Water condition	n.s.	n.s.	n.s.	n.s.
Concentration SA	n.s.	n.s.	n.s.	n.s.
Water condition X SA	n.s.	n.s.	n.s.	n.s.

Plants in the irrigated and rehydrated treatment and those subjected to SA concentrations showed no difference in water potential, chlorophyll *a*, *b* and carotenoids (Table 2).

When analyzing the stomatal conductance (g_s) at seven DAAT, significant difference between plants of the Irrigation and Irrigation Suspension treatments was observed (Figure 1D). Plants in the irrigated treatment showed higher conductance at 12:00 am (Figure 1C). This behavior is due to the stomatal opening provided by light for CO₂ assimilation and photosynthesis. The g_s values of plants under irrigation suspension showed significant reduction in comparison with plants kept irrigated, since, under water deficit conditions, the low availability of water contributes to stomatal closure and reduction of g_s values (Figures 1C and D).

The g_s values varied in plants submitted to different SA concentrations, both in the irrigated and in those submitted to water suspension, being able to recommend the lowest SA concentration (1 mM), which contributes to the increase in g_s values (Figures 2 B,C and D).



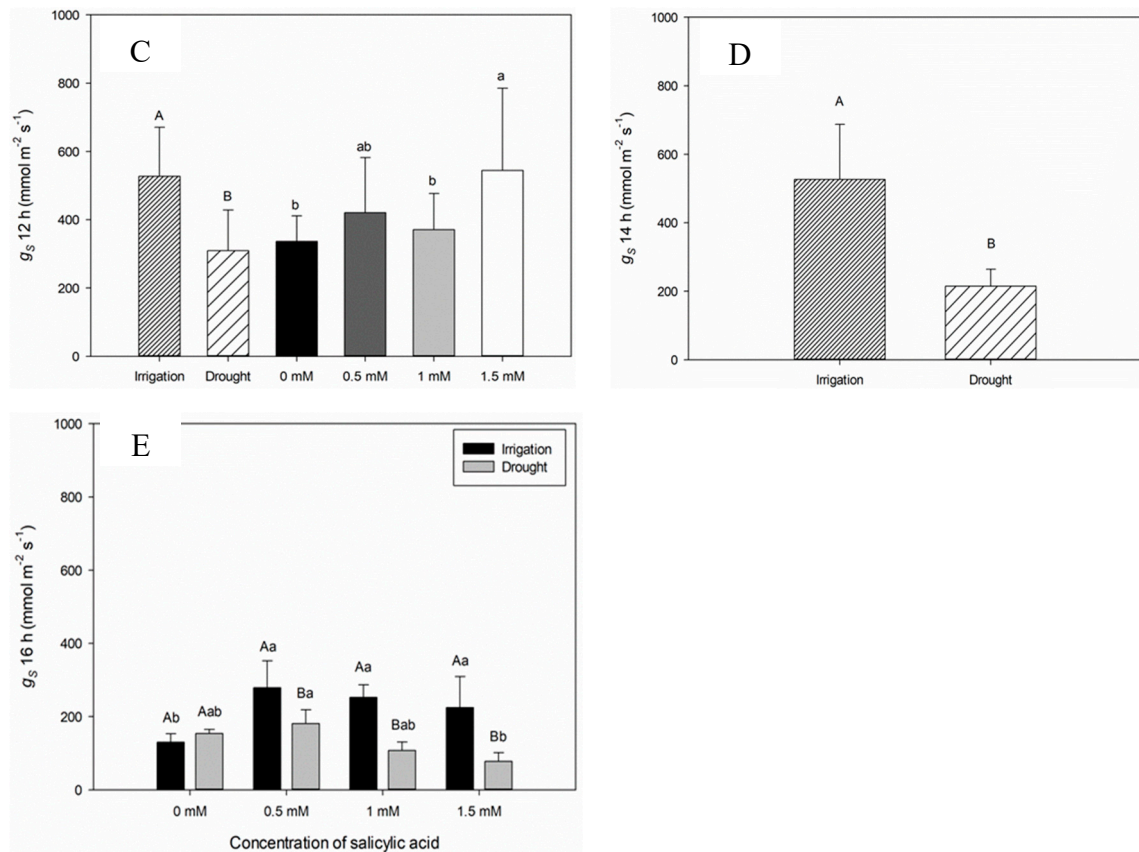
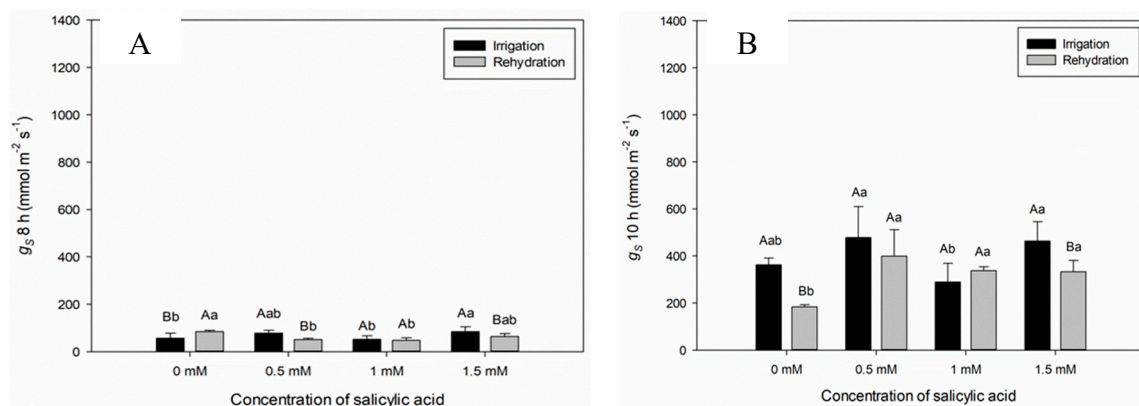


Figure 1. Stomatal conductance (g_s) over time (8, 10, 12, 14 and 16 h) for seven days after the beginning of treatments. Black bars represent Irrigation treatment, white bars represent Irrigation without treatment. Letters show difference between treatments, by the 5% Tukey's test. Values represent means \pm standard deviation of 4 replicates. Capital letters compare means of water conditions and lowercase letters compare means of salicylic acid concentrations.

The g_s values varied in plants submitted to different SA concentrations, and in the SA concentration of 1 mM, no statistical difference was verified between plants submitted to irrigation and rehydration treatments (Figures 2 B,D and E), being able to highlight the SA concentration of 1 mM under rehydration condition (Figures 2 B,C and D).



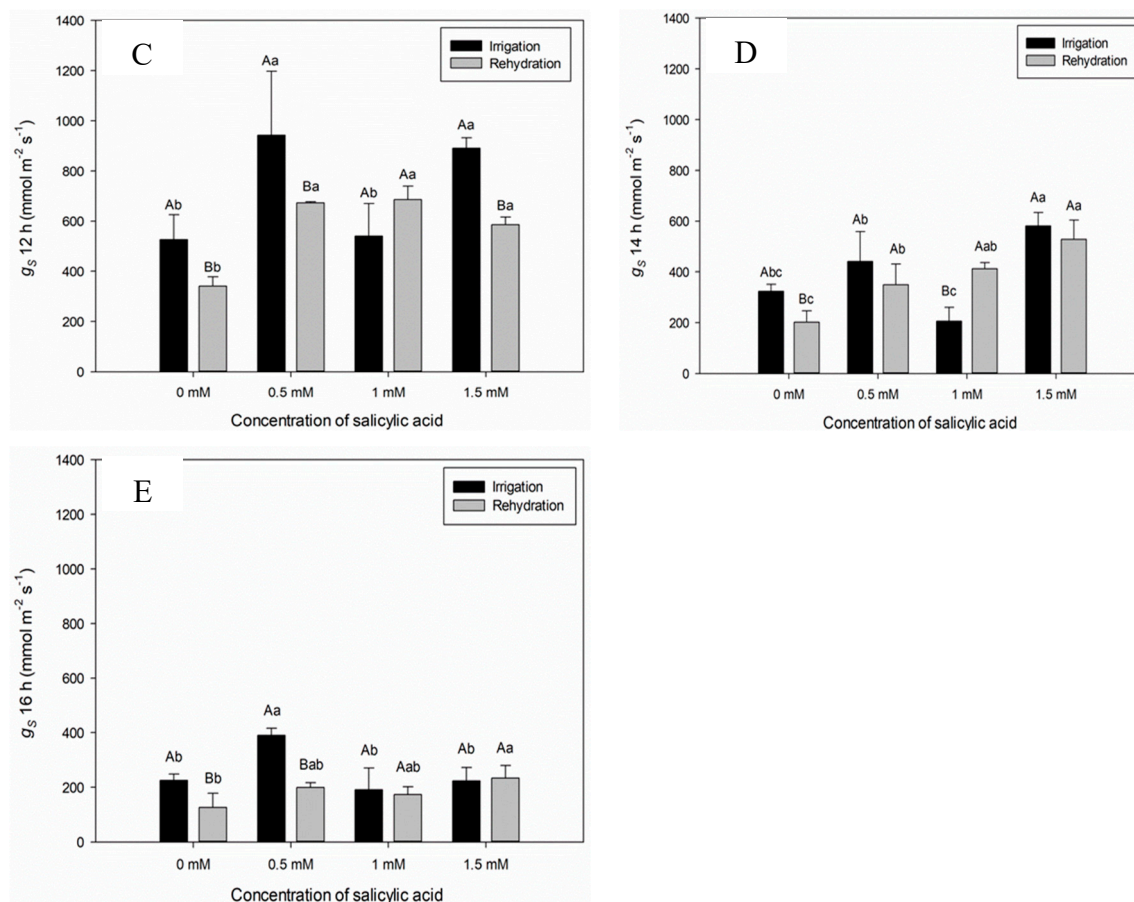


Figure 2. Stomatal conductance (g_s) over time (8, 10, 12, 14 and 16 h) during twelve days after the beginning of treatments. Black bars represent Irrigation treatment, white bars represent Rehydration treatment. Letters show the difference between treatments, by the 5% Tukey's test. Values represent means \pm standard deviation of 4 replicates. Capital letters compare means of water conditions and lowercase letters compare means of salicylic acid concentrations.

In general, effective quantum yield (ΦPSII), transport efficiency, maximum quantum yield (FV/FM), photochemical quenching (qP), non-photochemical quenching (NPQ), dissipation (D) and excess (E) of electrons of plants subjected to irrigation suspension showed the same behavior as plants irrigated at salicylic acid concentration of 1 mM (Figures 3 A,B,E,F and G).

The maximum quantum yield (FV/FM) in sweet pepper plants at seven DAAT did not differ in relation to the irrigated treatment and with irrigation suspension (Figure 3E). The Fv/Fm ratio is used to indicate photoinhibitory damage in plants subjected to water stress. It was observed that the water suspension to which sweet pepper plants were subjected was not enough to cause photoinhibitory damage (Figure 3E). The same behavior was observed for both dissipation (D) and excess (E) (Figures 3F and G).

Non-photochemical quenching (NPQ) of plants submitted to irrigation suspension seven days after the application of treatments showed reduction in irrigated plants and with SA concentration (1.5 mM) resulting in less energy dissipation in the form of heat in plants under irrigation suspension (Figure 3D).

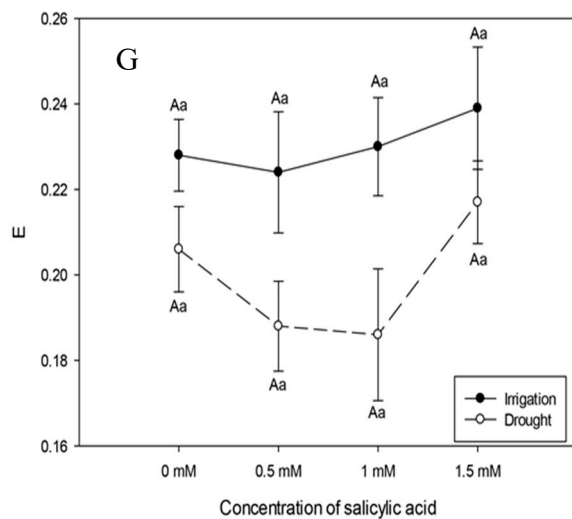
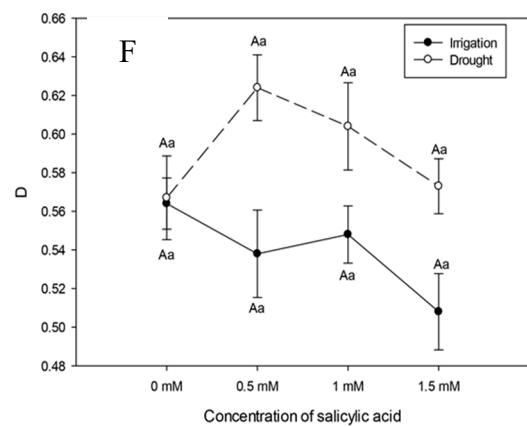
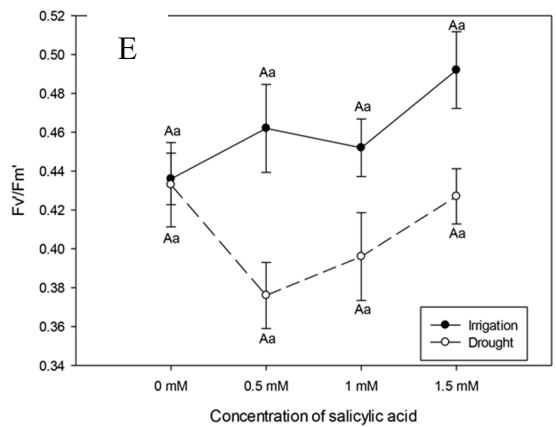
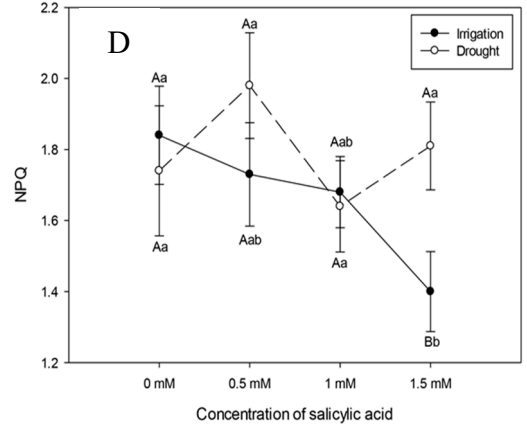
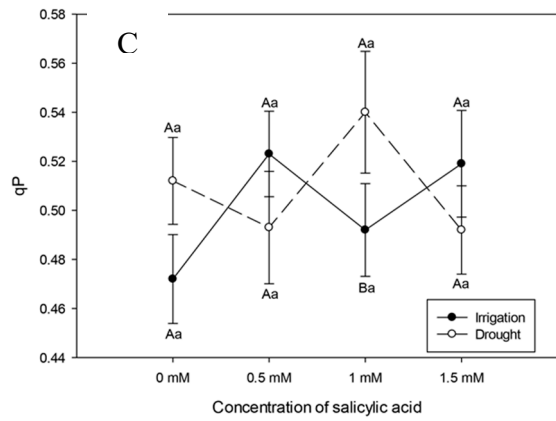
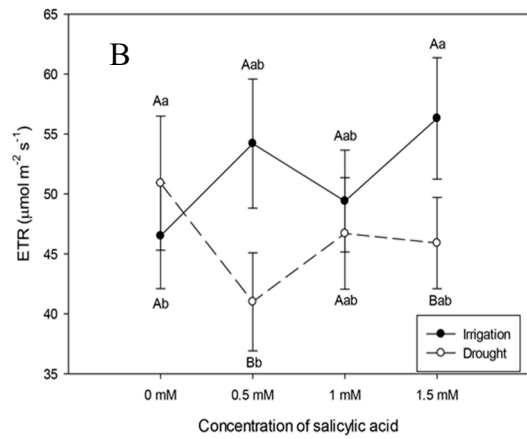
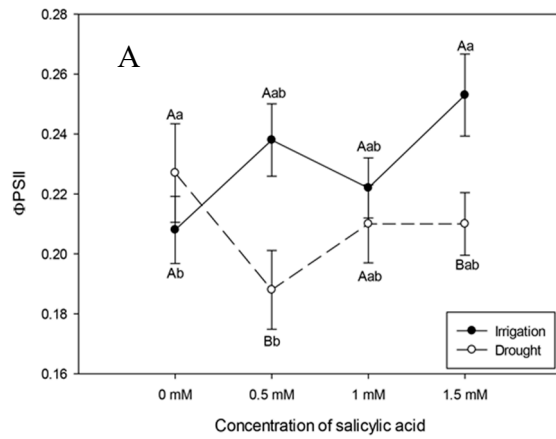


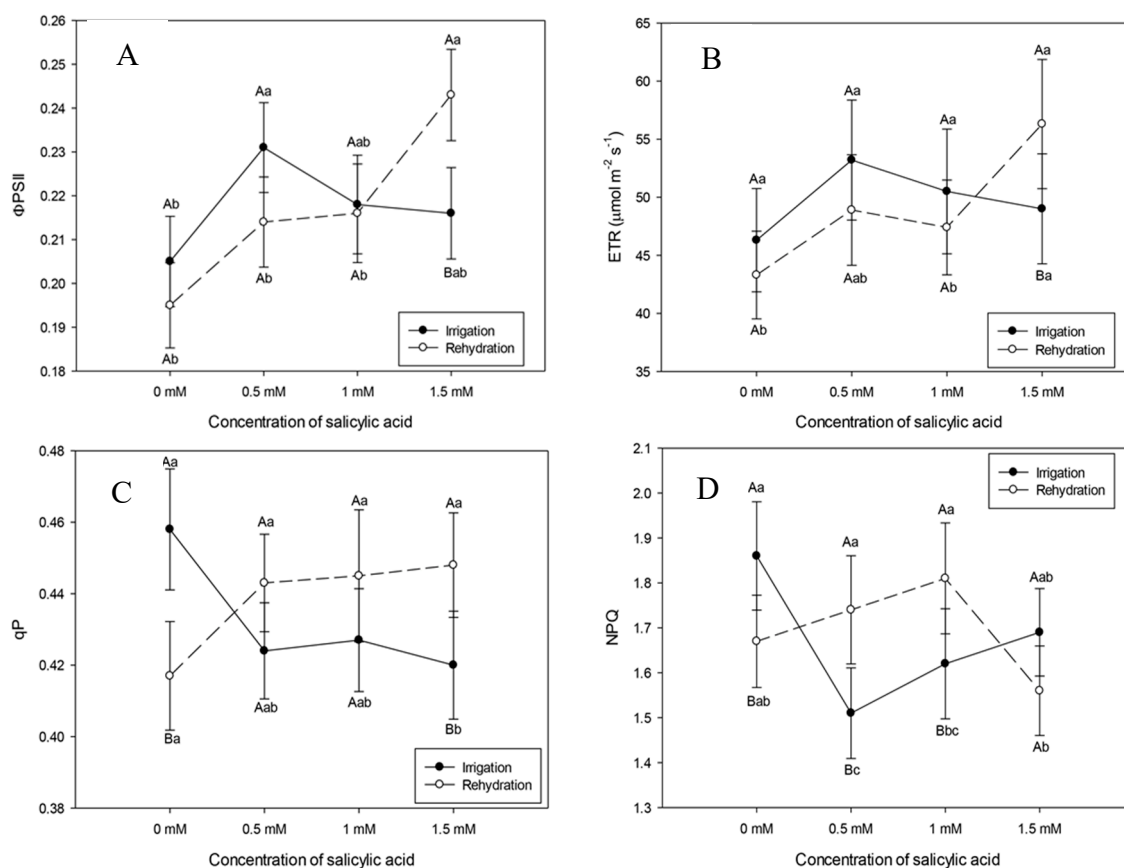
Figure 3. - Effective quantum yield (Φ PSII), electron transport efficiency (ETR), maximum quantum yield (FV/FM), photochemical quenching (qP), non-photochemical quenching (NPQ), dissipation (D) and excess (E) seven days after the beginning of treatments. Black bars represent Irrigation treatment, white bars represent Rehydration treatment. Letters show the difference between treatments, by the 5% Tukey's test. Values represent means \pm standard deviation of 4 replicates. Capital letters compare means of water conditions and lowercase letters compare means of salicylic acid concentrations.

Plants subjected to irrigation suspension, rehydrated and at the highest SA concentration (1.5 mM) increased the effective quantum yield (Φ PSII) and the electron transport rate (ETR) (Figures 4A and B).

Photochemical quenching (qP) showed reduction in irrigated plants submitted to the highest SA concentration (1.5 mM) (Figure 4C). Even with this concentration, rehydrated plants increased qP (Figure 4B). This result interferes with the photosynthetic carbon metabolism. Based on the results above, it could be inferred that the SA concentration of 1.5 mM helps to successfully complete the electron transport.

Irrigated plants showed reduction in non-photochemical quenching (NPQ), that is, decrease in energy dissipation in the form of heat with SA concentrations of 0.5 and 1 Mm (Figure 4D).

There was no statistical difference in the maximum quantum yield between irrigated and rehydrated plants with values ranging, respectively, from 0.68 to 0.74, possibly demonstrating a non-stressful condition, since the FV/FM ratio is used as an indicator of stress conditions on the photosynthetic apparatus (Figure 4E).



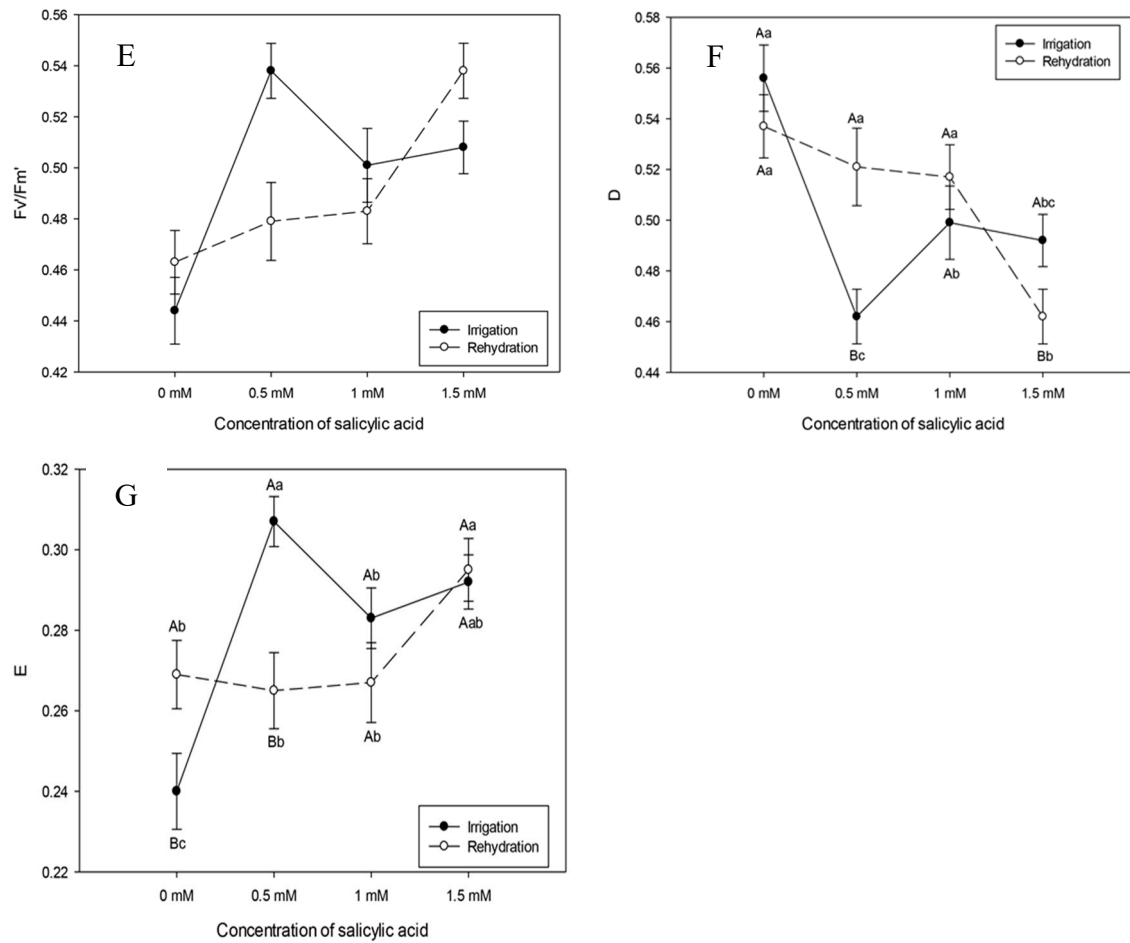


Figure 4. Effective quantum yield (Φ PSII), electron transport efficiency (ETR), maximum quantum yield (FV/FM), photochemical quenching (qP), non-photochemical quenching (NPQ), dissipation (D) and excess (E) twelve days after the beginning of treatments. Black bars represent Irrigation treatment, white bars represent Rehydration treatment. Letters show the difference between treatments by the 5% Tukey's test. Values represent means \pm standard deviation of 4 replicates. Capital letters compare means of water conditions and lowercase letters compare means of salicylic acid concentrations.

Figure 5A shows that plants without water deficit had higher H_2O_2 concentration in SA concentrations of 0 and 1 mM, while rehydrated plants showed higher H_2O_2 concentrations with SA concentrations of 0.5 and 1.5 mM (Figure 5B). SA application of 0.5 mM when applied exogenously acts by raising the level of reactive oxygen species under rehydration condition.

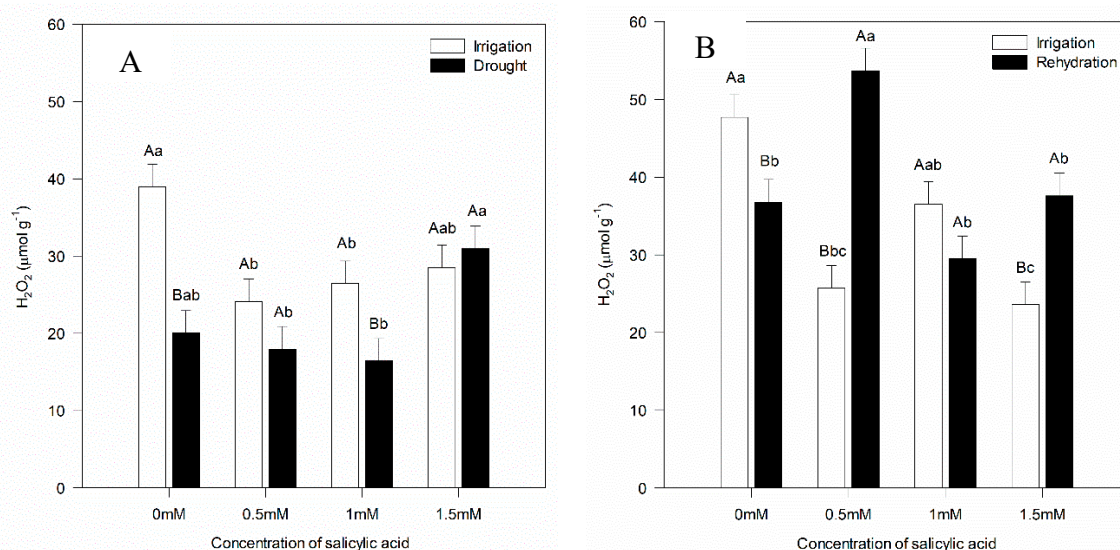


Figure 5. Hydrogen peroxide concentrations seven and twelve days after the beginning of treatments. Black bars represent Irrigation treatment, white bars represent Rehydration treatment. Letters show the difference between treatments by the 5% Tukey's test. Values represent means \pm standard deviation of 4 replicates. Capital letters compare means of water conditions and lowercase letters compare means of salicylic acid concentrations.

3. Discussion

The drop in the leaf moisture percentage from 74.2% to 70.2% at 12:00 pm and from 70.1% to 64.9% at 02:00 pm for plants under irrigation suspension reflected in the decrease of g_s values during these periods throughout the day (Table 1, Figure 1). In addition to suspending irrigation, environmental variation must be taken into account, which influences the water potential gradient between soil, plant and atmosphere. Thus, the lower water availability in the soil hinders its flow via the xylem, resulting in less water in leaves, especially at warmer times and with lower relative humidity, as observed between 12:00 am and 02:00 pm. Plants subjected to water deficit close their stomata to reduce water loss through transpiration. Sweet pepper plants kept with higher water potential in soil and leaves (Irrigation treatment) showed higher stomatal conductance values (g_s) when compared to those submitted to gradual increase in water stress (Irrigation Suspension treatment) (Table 1, Figure 1). Exogenous salicylic acid (SA) application acts as an important inducer in improving gas exchange indices, increasing g_s values, similarly to transpiration, reducing the internal CO_2 concentration, through the elevation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBP), reflecting in lower water use efficiency [29]. SA application at concentration of 0.5 mM contributes to the maintenance of g_s values in plants subjected to irrigation suspension (Figure 1). Xu and Tian [30] reported that SA acts as a secondary messenger involved in signal transduction in response to stress, inducing reactions that promote plant protection. Singh and Yasha [31] reinforce that plants treated with SA generally have higher relative water content and higher activity of the ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme.

Both the moisture percentage in leaves and the water potential did not differ between irrigated and rehydrated plants or those subjected to SA concentrations, demonstrating that the recovery of plant hydration does not depend on the use of the plant regulator (Table 1). Non-application (0.0 mM) and the highest SA concentration (1.5 mM) reduced g_s values (Figure 1). Probably, this concentration (1.5 mM) may have acted as a stressor. However, SA concentration equal to 1.0 mM contributed to the maintenance of g_s values in rehydrated plants similar to plants not subjected to stress (Figure 2). As high SA concentrations (1.5 mM) can harm physiological processes, it is recommended the application of the plant regulator at low concentrations, which can promote the recovery of plants subjected to stress [32,33,34]. Research shows that beneficial effects under stress

conditions are possible at SA concentrations ranging from 0.05 to 0.5 mM [35,36]. Waseem *et al.* [37] reported that exogenous SA application can induce resistance to drought in wheat plants.

Although the beneficial effects of SA are described in literature, plant responses to the exogenous application of this resistance inducer are still controversial [38,39] and the authors reinforce that exogenous SA application can negatively influence the plant's defense processes, especially at high concentrations, as observed in the present research. Furthermore, the effective SA concentration strongly depends on the plant species, environmental conditions and stress level.

The pigment results revealed that the application of the highest SA concentration (1.5 mM) reduced chlorophyll *a* and carotenoid contents, regardless of stress (Table 2). SA application does not affect pigment production [40]. However, this result is different from that obtained by Sayyari *et al.* [41], who observed that under drought conditions, photosynthetic pigments decreased considerably, while SA increased the carotenoid content in basil plants.

Under dry conditions, SA concentration of 1.5 mM increased the H₂O₂ content, in relation to the other concentrations (Figure 5A). In rehydration, higher H₂O₂ content was verified when SA concentration of 0.5 mM was applied (Figure 5B). Sahu *et al.* [42] observed that SA application (50 μ M) in wheat plants did not change the H₂O₂ content. In the same study, the authors also observed that the highest SA concentration (1000 μ M) increased the H₂O₂ level. Exogenous SA application acts as a potential inducer of the defense response, raising H₂O₂ levels in plants [43], which was confirmed in the present study, in which plants not subjected to water stress had higher H₂O₂ concentrations than plants subjected to water deficit and those treated with 1 mM SA (Figure 5A). Increase in H₂O₂ content was also observed in the presence of SA concentration of 0.5 mM in plants submitted to drought and rehydration. Janda *et al.* [44] reinforce that SA can cause oxidative stress to plants, partly due to H₂O₂ accumulation.

In Figure 3, it can be seen that the increase in SA concentrations resulted in decrease in maximum and effective quantum yields of PSII photochemistry and electron transport efficiency, respectively, Fv/Fm, Φ PSII and ETR, in plants subjected to drought, corroborating results observed by Poo'r and Tari [45] in tomato plants, in which SA application of 10⁻³ M resulted in decrease in maximum and effective quantum yields of the PSII photochemistry, respectively, Fv/Fm and Φ PSII, and in the parameter of photochemical extinction in guard cells. However, in rehydrated plants, SA application increased Fv/Fm and Φ PSII, demonstrating recovery of the photochemical apparatus (Figure 4). Maximum quantum yield (FV/FM) is widely used to detect disturbances induced by stress in the photosynthetic apparatus [46,47].

Plants under dry conditions and rehydrated with SA application of 0.5 mM showed higher non-photochemical quenching (NPQ) values, demonstrating protection against damage resulting from water stress (Figure 4). Janda *et al.* [48] found that tobacco plants that received SA application had limited energy dissipation capacity. Cao *et al.* [49] reinforce that NPQ contributed to protection against photodamage in tomato plants subjected to water stress.

It was also observed that plants subjected to drought and rehydrated with SA application of 0.5 mM showed greater photoprotective capacity (Figure 4), acting as an important factor in mitigating the effects of water stress. In addition, this demonstrates that despite the increase in H₂O₂ levels, regardless of treatment and concentration, respectively, plants have defense capacity under drought and rehydration conditions (Figures 5, 3 and 4).

Exogenous SA application increases the tolerance of plants to abiotic stress, including water stress [50]. War *et al.* [51] observed that SA application of 2.0 mM causes phytotoxicity and reduced peroxidase activity. However, the authors suggest SA concentration of 1.5 mM as inducer of plant defense mechanisms. The research managed to define the best management for the use of SA and should effectively contribute to reducing drought damage without risk to the environment and at a relatively low cost for the dose used and will have a strong impact on the sustainable cultivation of this vegetable that is grown in different regions of the world. The future perspective is that new studies will be carried out at the metabolomics level to better understand the genes that are activated by SA in improving the drought tolerance of crops.

4. Material and Methods

4.1. Experiment location

The experiment was carried out in greenhouse at the São Paulo State University, Botucatu, Brazil. The average temperature of the hottest month is greater than 22.0°C and that of the coldest month is 17.5°C, with average annual temperature of 21°C.

4.2. Environmental conditions

Temperature, relative humidity and air vapor pressure deficit measured using porometer (Decagon Devices) between 8:00 am and 4:00 pm are shown in Figure 5, and correspond to the averages of forty-eight readings performed at 7 am and 4 pm, 22 days after application of treatments on sweet pepper seedlings with water regime and salicylic acid (SA) variation.

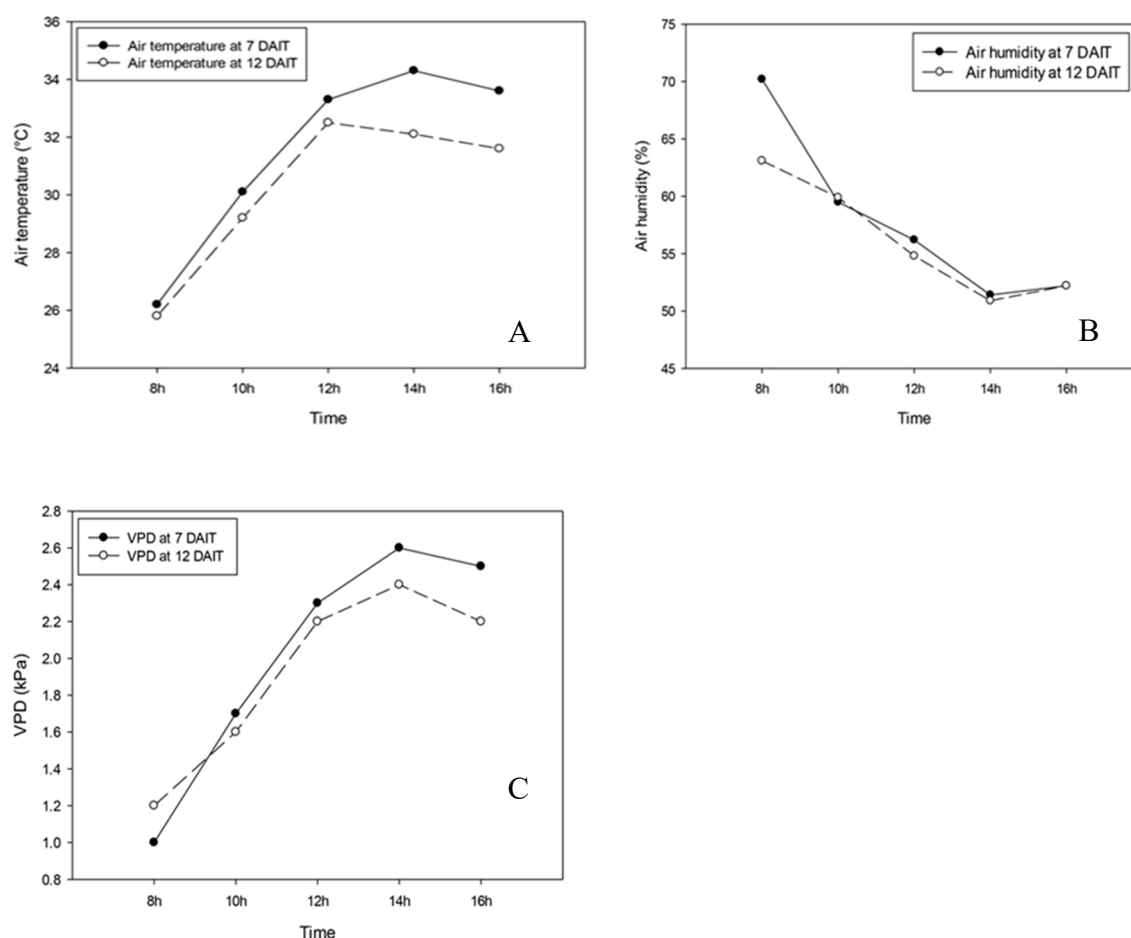


Figure 5. Temperature, relative humidity and air vapor pressure deficit. Average of 48 readings performed 7 and 22 days after application of treatments with water regime and salicylic acid variation.

4.3. Driving seedlings

The sowing of hybrid 'Melina' bell pepper (*Capsicum annum* L.) was carried out in 128-cell polystyrene trays and after 45 days of emergence, seedlings with 8 cm in height and six pairs of definitive leaves were transplanted to plastic bags with volume of 400 mL, containing soil as substrate, with the following chemical characteristics: pH: 5.8; P: 84.0 mg dm⁻³; K: 1.9 mmolc dm⁻³; Ca: 21 mmolc dm⁻³; Mg: 7.0 mmolc dm⁻³; Al³⁺: 0.8mmolc dm⁻³; organic matter: 12 g dm⁻³; H⁺+Al³⁺: 13.0 mmolc dm⁻³; S: 2.0 mg dm⁻³; B: 0.27 mg dm⁻³; Cu: 7.0 mg dm⁻³; Fe: 54.0 mg dm⁻³; Mn: 6.7 mg dm⁻³; Zn: 3.5 mg dm⁻³; SB: 30.0; CTC: 42.0 mmolc dm⁻³; V: 70%. Daily irrigation was carried out for 13 days, keeping seedlings in plastic bags at field capacity. For this purpose, all bags were irrigated to maximum water holding capacity (100%) and kept without irrigation overnight to release excess

water. During this period, the surfaces of bags were sealed with aluminum foil to prevent evaporation. The following morning, bags were weighed and their masses were used at the maximum water holding capacity (100%). The weighing of bags (g) throughout the experiment compared with their masses at maximum holding capacity (100%) was used to calculate the water content in the substrate (CAS) using the following formula: $CAS = (M_{\text{Treatment}} / M_{100\%}) * 100$, according to Varone et al. [52]. This calculation allowed controlling and replenishing water to keep bags containing plants at their maximum holding capacity, which procedure was daily performed.

After 13 days of acclimatization, seedlings were subjected to treatments with water regime and SA variations.

4.4. Water Regime

Seedlings were subjected to three water regimes and, therefore, they were maintained at field capacity, in water deficit and in water deficit and rehydration. Changes in field capacity were monitored according to Thameur *et al.* [53]. Seedlings subjected to water deficit were not irrigated until leaves showed signs of wilting, when they showed lower water potential, stomatal conductance and water percentage. Water deficit was maintained for seven days before seedling evaluation. Then, rehydration was performed and seedlings were evaluated after four days at field capacity.

4.5. Salicylic acid

Seedlings were also subjected to SA variation. Thus, they were cultivated without water stress, drying and rehydration and without the addition of regulator (0) and with addition of regulator at concentrations of 0.5, 1 and 1.5 mM. SA was applied twice, half at 13 days after transplanting seedlings into plastic bags and the other, seven days after the first application.

4.6. Experimental design

Seedlings were cultivated in treatments with different water regimes and SA variation. The experimental design was randomized blocks, in a 3x4 factorial scheme, three water regimes and four SA concentrations, with 20 plants per treatment and four replicates.

4.7. Chlorophyll *a*, *b* and total carotenoid content

To determine the chlorophyll (*a* and *b*) and total carotenoid content, leaf discs from seedlings were collected and placed in test tubes containing 1mL of dimethylformamide reagent. Test tubes were covered with aluminum foil and stored in refrigeration at 4°C for 24 hours, after which the entire solution volume was transferred to a cuvette and reading was performed in spectrophotometer at wavelengths of 480, 646, 8 and 663.8 nm for determination of chlorophyll *a* and *b* and total carotenoid content. Pigments were determined according to Lee *et al.* [54].

Chlorophyll *a*: $12 \times \text{Absorbance at } 663.8 \text{ nm} - 3.11 \times \text{Absorbance at } 646.8 \text{ nm}$.

Chlorophyll *b*: $20.78 \times \text{Absorbance at } 646.8 \text{ nm} - 4.88 \times \text{Absorbance at } 663.8 \text{ nm}$.

Total carotenoids: $(1000 \times \text{Absorbance at } 480 - 1.12 \times \text{chlorophyll } a - 34.07 \times \text{chlorophyll } b) / 245$.

4.8. Leaf water potential (Ψ_w)

Leaf water potential (Ψ_w) was determined between 5:00 am and 6:00 am, using Scholander pressure pump [55] PMS Instrument, model 600. Seedling stem fragments were separated and immediately placed in the pressure chamber, whose measurement was performed after immediate pressure application and liquid exudation [56].

4.9. Stomatal conductance

To evaluate stomatal conductance ($\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), Porometer (Decagon Devices) was used. Readings were performed on completely expanded leaves of the middle part of seedlings at 8:00 am, 10:00 am, 12:00 pm, 2:00 pm and 4:00 pm.

4.10. Chlorophyll *a* fluorescence

Fluorescence was evaluated using pulse-modulated fluorometer (JUNIOR-PAM, Walz®). Evaluations were performed at 5:00 am, 12:00 pm and 5:00 pm. With the exception of evaluations carried out at 5:00 am, leaves were acclimatized for a period of 30 minutes in the dark with aluminum foil. Then, saturation pulse of 10000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ of photosynthetically active photon flux density (DFFFA) with 0.6s was applied to obtain F_m (maximum dark-adapted fluorescence) and F_m' (maximum light-adapted fluorescence). In addition to maximum light- and dark-adapted leaf fluorescence, F_o (minimum dark-adapted fluorescence) and F_o' (minimum light-adapted fluorescence) were also obtained. Between each saturation pulse, actinic light pulse of 1150 $\mu\text{mol m}^{-2}\text{s}^{-1}$ of DFFFA of 15 seconds duration was performed. Using F_m , F_o , F_m' and F_o' , maximum quantum yield (F_v/F_m) was calculated, which reflects the ability of PSII to oxido-reduce the primary QA acceptor (quinone A) [57], yield effective quantum (ΦPSII), which corresponds to the energy fraction that is photochemically converted into photosystem II (PSII) [58], photochemical quenching (qP), which reflects the photosynthetic carbon metabolism [59], non-photochemical quenching (NPQ), which corresponds to photoprotection mechanisms [60], electron transport rate, which corresponds to the transport of electrons between photosystems, considering that 84% of light is absorbed by chlorophyll, with 50% of photons activating photosystem II chlorophyll and 50% photosystem I [61]. F_m , F_o measurements and F_v/F_m , ΦPSII , qP , ETR and NPQ determinations were represented by the mean of two leaves.

4.11. Determination of the hydrogen peroxide (H_2O_2) content

To determine the hydrogen peroxide (H_2O_2) content, the methodology by Alexieva *et al.* [62] was adopted. Samples of 0.1 - 0.25 g were homogenized in liquid nitrogen and 1mL of TCA 0.1% was added followed by vortexing. Subsequently, samples were centrifuged at 12,000 g for 15 minutes at 4°C.

For the reaction, 0.5 mL of extract + 0.5 mL of 0.1 M Phosphate Buffer (pH 7.0) + 2.0 mL of 1 M KI were used, with extract and reaction buffers kept in the dark for 1 hour. Readings were performed in spectrophotometer at 390 nm and the H_2O_2 concentration was calculated considering the curve (0-100 μM), measuring the ratio between H_2O_2 content and the dry mass of samples.

4.12. Statistical analysis

Physiological and biochemical variables were submitted to analysis of variance and means compared by the Tukey test at 5% significance level [63].

5. Conclusions

Therefore, exogenous salicylic acid (SA) application minimizes the effects of water stress, especially in plants subjected to drought followed by rehydration.

In general, the lowest SA concentration allowed the maintenance of mechanisms in plants subjected to drought and recovery of rehydrated plants, evidenced by stomatal conductance, effective quantum yield, excess energy dissipation and maximum quantum yield data, in addition to minimizing possible damage of membranes.

Thus, photosynthetic pigments, stomatal conductance and fluorescence provide information for further studies in the elucidation of mechanisms of plants when subjected to drought followed by exogenous SA application and rehydration; above all, antioxidant system responses and membrane damage. SA can be more safely adopted as a technology in arid and semiarid regions, especially when applied with irrigation water.

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review and editing, E.O.O., R.d.M.P., and C.S.F.B.; visualization, F.C.d.M.G., C.V.C., E.O.O., R.d.M.P. and C.S.F.B.; project administration, F.C.d.M.G., C.V.C. and C.S.F.B.; term, C.S.F.B., resources, L.F.R.d.A., C.S.F.B. and J.D.R.; supervision, C.S.F.B. All authors have read and agreed to the published version of the manuscript.

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