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Research paper

Impact of exogenous application of salicylic acid on the drought-stress tolerance in pepper *(Capsicum annuum* L.)

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Abstract
To investigate the effect of drought stress on the physiological and chemical properties of the sweet pepper and the impact of the foliar application of salicylic acid (SA) on alleviating the negative effects of this stress, a factorial experiment was carried out using a completely randomized design in the greenhouse conditions. Drought stress levels were 100% field capacity (as control), moderate stress (60% field capacity), and severe stress (30% field capacity). Foliar SA was also applied at three concentrations of 0 (as control), 0.5, and 1 mM when the plants were at the four-leaf stage. The results showed that drought reduced shoot and root fresh weight and dry weight, relative leaf water content, fruit length and diameter, chlorophyll index, and leaf area, and increased electrical conductivity, antioxidant capacity, total phenolic content, ascorbate, polyphenol oxidase, and ascorbate peroxidase. After foliar of application SA, electrical conductivity decreased and other above-mentioned characteristics increased, however, the increase in shoot dry weight, root fresh weight, and chlorophyll index was not significant. From the results of this experiment, it can be concluded that SA alleviates the negative effects of drought stress in pepper.

Keywords: Ascorbate peroxidase; Chlorophyll; Electrical conductivity; Field capacity; Polyphenol oxidase; Total phenolic content

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Introduction

Drought is a limiting environmental factor that restricts crop growth, development (Shen *et al.* 2014), and yield (Farooq *et al.* 2009). The drought decreases the relative water content (RWC), transpiration rate, and water potential of plant leaves (Siddique *et al.* 2001). It also causes oxidative stress by increasing the reactive oxygen species (ROS), such as hydroxyl radicals (•OH), superoxide anion radicals (O2−), singlet oxygen (1O2), and hydrogen peroxide (H2O2) (Munné-Bosch and Penuelas 2003), which leads to peroxidation of membrane lipids and degradation of nucleic acids and proteins (Farooq *et al.* 2009). Drought damages the photosynthetic system by impairing the photosynthetic machinery (Wahid and Rasul 2005). The plants have enzymatic antioxidant systems such as ascorbate peroxidase (APX) (Mittler 2002) and polyphenol oxidase (PPO) (Boeckx *et al.* 2015). Cell membranes are prime and sensitive sites in cells (Rajasekaran and Blake, 1999). The drought stress causes membrane disruption and leads to increasing electrolyte leakage (Wang and Huang 2004). The plant's antioxidant capacity under stress conditions may not be sufficient to reduce the harmful effects of oxidative stress, so plants produce signaling molecules such as jasmonic acid, ethylene, and salicylic acid (SA) (Kadioğlu *et al.* 2011).

Plant growth regulators (PGRs) are inexpensive and easy-to-use compounds. They can enhance water consumption efficiency and improve the adaptability of plants to drought. SA as a signaling molecule and a PGR is known to participate in regulating plant immunity (Koo *et al.* 2020). External application of SA improves seed germination (Cutt and Klessig 1992), transmission and absorption of ions (Harper and Balke 1981), photosynthesis, and various enzyme activities (Hayat *et al.* 2010). Hussein *et al*. (2007) stated that SA contributes to an enhancement in growth rate and photosynthesis in the maize plant. In another study (Habibi 2012), SA enhanced photosynthetic activity and stomatal conductance in barley under drought stress conditions. Kadioğlu *et al.* (2011) indicated that salicylic acid modulates plant response and leads to more tolerance to drought stress. Salicylic acid plays a key role in cell membrane stability (Belkadhi *et al.* 2015), reduction of electrolyte leakage from cell membranes (Hayat *et al*. 2010), and regulating growth in plants under abiotic stress (Khan *et al.* 2015). Furthermore, salicylic acid increases antioxidant enzymes activity such as APX and PPO and reduces the harmful effects of ROS (Qados 2015).

Sweet pepper (*Capsicum annuum* L.) is a rich source of essential vitamins and minerals such as potassium. Also, pepper fruit contains high levels of antioxidants and beneficial substances such as vitamin C, carotenoids, and phenolic compounds (Bosland and Votova 2000).

We aimed to study the external application of SA on drought-induced oxidative damage in sweet pepper.

Materials and Methods

Experimental procedure

The experiment was arranged as factorial using a completely randomized design with three replications. Three levels of drought stress (field capacity or control, 60% and 30% of field capacity as moderate and severe stress, respectively), and three concentrations of SA [sprayed with distilled water (control), 0.5, and 1 mM] were randomly assigned to the experimental units. Seeds were obtained from the Faculty of Agriculture, Ilam University, Iran. Before sowing the seeds, they were soaked in 1% sodium hypochlorite for 10 minutes, then washed with tap water for 1 minute, and sowed in 23 × 20 cm plastic pots. The pots were filled with a mixture of sand, garden soil, and leaf mold in equal amounts, each weighing 7 kg. The soil was analyzed to determine its properties (Table 1).

The relative humidity and temperature during the growth period were 60-70% and 18/25 °C (day/ night), respectively. SA was sprayed on the upper and lower leaves at the fourth leaf stage. Tween-20 was used as a surfactant. SA was applied three days before and two weeks after the start of the drought treatment. At the beginning of the plant growth, all pots were watered until the field capacity was reached. The experiment, lasted approximately four months, three days after the foliar spray until sampling. The pots were weighed daily until the end of the experiment.

Table 1. Physico-chemical properties of the experimental soil

Properties	Units	Values
Moisture content	(%)	32
P	(ppm)	3.47
K	(ppm)	33.63
pH		7.3
Sand	(%)	22
Clay	(%)	11
Silt	(%)	67
Soil texture		Silty loam
EC	(ds/m)	0.7
Organic carbon	(%)	0.42
Total N	(%)	0.04

To measure the physicochemical characteristics, leaf samples were taken from pepper seedlings at the green stage (80% maturity), then the samples were immediately placed in liquid nitrogen and stored at -80 °C.

Growth characters

Leaf area index (LAI) was measured by the method of Phimchan *et al.* (2012). Leaf chlorophyll index was measured by SPAD 502 on the last developed leaves of pepper plants (Bianco *et al.* 2017). The shoots' fresh weight (FW) of pepper plants was measured with a digital scale, and then the shoots were dried in an oven at 70° C and their dry weight (DW) was obtained. After separating the roots of bell peppers, they were thoroughly washed several times with distilled water and then were measured with a digital analytical scale. Then the roots were dried in an oven at 70 °C (Khazaei and Estaji 2020). Morphological characteristics of pepper fruits such as length and diameter of the fruits were measured considering their maximum diameter and length.

Leaf RWC

To determine the RWC of the leaves, fully developed leaves of the pepper seedlings were weighed. Then, the leaves were put in the test tubes containing distilled water and were left at room temperature for 3 hours and then their turgid weight (TW) was measured. To obtain the DW of the leaves, they were placed in an oven at 70 ° C for 48 hours. The following equation was used to calculate the RWC of pepper leaves:

$$\text{RWC (\%)} = \text{FW-DW/TW-DW} \times 100$$

Electrical conductivity

Electrical conductivity was measured according to Deshmukh *et al.* (1991). The fresh pepper leaves were washed with distilled water and transferred to test tubes containing 10 ml of distilled water. These test tubes were kept at 30 °C for 30 min to measure the initial electrical conductivity of the solution (Ec_1). For calculating the secondary electric conductivity (Ec_2), the tubes were heated for 15 min at 100 °C in a bath of water. Then, the electrical conductivity of the samples was calculated using the following formula:

EL (%) = (EC_1/EC_2) × 100.

Non-enzymatic antioxidants

To measure ascorbic acid (AsA) in the pepper leaves, about 0.5 g of the pepper leaf samples were put in a mortar containing liquid nitrogen. Then, they were homogenized in cold TCA (trichloroacetic acid, 1% w/v) by pestle and centrifuged at 12000 × g at 4 °C for 20 min. The supernatant was mixed with ascorbate oxidase and 50 µl of K_2HPO_4 buffer at pH 7.0. Eventually, the absorption was recorded at 265 nm (Luwe *et al.* 1993).

To determine the total phenol content, about 0.4 g of the pepper leaf sample was crushed in a 5 ml methanol/hydrochloric acid mixture and then centrifuged at 15000 × g for 20 min at 4 °C. Following this, a mixture containing distilled water and Folin-Ciocalteu reagent (1:10) was mixed with 200 µl of centrifuge supernatant. Then 8 ml of 7.5 Na_2CO_3 was added to the previous solution and placed in a dark place for 30 min. Then, the solution was centrifuged at 5000 × g for 5 min and the absorbance was measured at 765 nm. The total phenol content was calculated through the gallic acid calibration curve (Kähkönen *et al.* 1999).

DPPH was measured to determine the antioxidant activity of the pepper samples by the method of Abe *et al.* (1998). About 0.2 g of the fresh leaf samples were placed in a mortar and crushed by a mortar handle with 2 ml of ethanol at 4 °C. About half of the extract was mixed with 0.25 ml of 0.5 mM DPPH and 0.5 ml of 100 mm acetate buffer at pH 5.5 and placed at room temperature for 30 minutes. The absorption was read at 517 nm. Antioxidant capacity was calculated using the following formula:

$$\text{Antioxidant capacity (\%)} = \left(1 - \frac{A\ Sample\ (517nm)}{A\ Control\ (517\ nm)}\right) \times 100$$

The activity of antioxidant enzymes

The pepper leaf samples were added to a mortar containing 50 ml of sodium phosphate buffer and were crushed. Then, they were centrifuged at 10 °C with 16000 × g. The resulting supernatant was used to determine the activity of APX and PPO antioxidant enzymes.

To measure the PPO activity, 100 µl of the enzyme extract was added to the reaction mixture consisting of 900 µl of catechol and 40 µl of 0.01 M sodium phosphate buffer at pH 6.8. The absorbance was read at 400 nm at 25 °C.

For determining the APX activity, the reaction mixture contained 0.5 mM ascorbate at 0.1 mL, 1% H_2O_2 at 0.2 mL, 50 mM K_2HPO_4 buffer at 2.5 mL, and 0.1 mL EDTA, which was one-tenth of that. The absorbance of the sample was recorded at 290 nm (Nakano and Asada 1992).

Statistical analysis

After analysis of variance, means were compared by Duncan's multiple range test was at the 5% probability level. SPSS software was used to analyze the data. Excel software was used to draw the graphs.

Results

Growth characters

Drought stress and SA had a significant effect on the fresh and dry weight of the shoots and roots, length and diameter of fruit, chlorophyll index, and LAI (Tables 2 and 3). The results showed that with increasing drought stress, fresh and dry weight of the shoots and roots, length and diameter of fruit, LAI, and chlorophyll index decreased. As illustrated in Tables 2 and 3, foliar application of SA significantly increased the above-mentioned traits noted. Furthermore, the interaction of drought with SA on the fresh weight of the shoots and roots was significant (Figure 1a, 1b), however, the interaction was the "change-in-magnitude" type.

RWC

RWC content of the pepper seedlings decreased significantly under drought stress (Table 3). Foliar application of SA significantly decreased the unfavorable effects of drought stress and thereby increased RWC (Table 2). For this trait, there was also a significant interaction between drought stress and SA (Figure 1d), however, it was a "change-in-magnitude" type.

Table 2. Means of morphological traits as affected by salicylic acid and drought stress in pepper

Factors	FWSH (g)	DWSH (g)	FWR (g)	DWR (g)	FD (mm)	FL (mm)
Salicylic acid						
0 mM (control)	13.92c	2.90a	17.12a	4.12c	1.37b	1.68b
0.5 mM	17.90b	3.27a	20.46a	7.28b	1.50b	1.69b
1 mM	22.59a	3.72a	22.86a	10.60a	1.92a	1.94a
Drought stress						
0 (control)	29.98a	4.29a	27.36a	11.72a	9.38a	7.22a
60%	14.50b	3.55a	19.09b	6.58b	2.14b	2.86b
30%	9.93c	2.05b	13.99b	3.70c	1.76c	1.68c

The Means with the same letters in each column for each factor indicate no significant difference at the 5% probability level using Duncan's multiple range test; FWSH, DWSH, FWR, DWR, FD, and FL were the abbreviations of shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, fruit diameter, and fruit length, respectively

Table 3. Means of physiological traits as affected by salicylic acid and drought stress in pepper

Factors	LAI (cm^2)	CHLI	RWC (%)	EC (%)
Salicylic acid				
0mM (control)	12.26b	63.98a	37.94b	10.97a
0.5 mM	12.32b	65.36a	45.51a	7.87b
1 mM	14.06a	68.42a	46.40a	5.58c
Drought stress				
0 (control)	13.97a	74.97a	54.80a	6.44c
60%	13.10a	64.93ab	40.88b	7.40b
30%	11.56b	57.86b	34.17c	

Means with the same letters in each column for each factor indicate no significant difference at the 5% probability level using Duncan's multiple range test; LAI, CHLI, RWC, and EC were the abbreviations of leaf area index, chlorophyll index, relative water content, and electrical conductivity, respectively

Electrical conductivity

Results showed that drought stress and SA had a significant effect on electrolyte leakage. Drought stress increased electrolyte leakage into the intercellular space. Leaf spray of SA significantly decreased the electrical conductivity in the stressed seedlings (Table 3). Also, the interaction of SA with drought stress was significant. By increasing the drought stress, electric conductivity content increased, and after the use of SA decreased (Figure 1c).

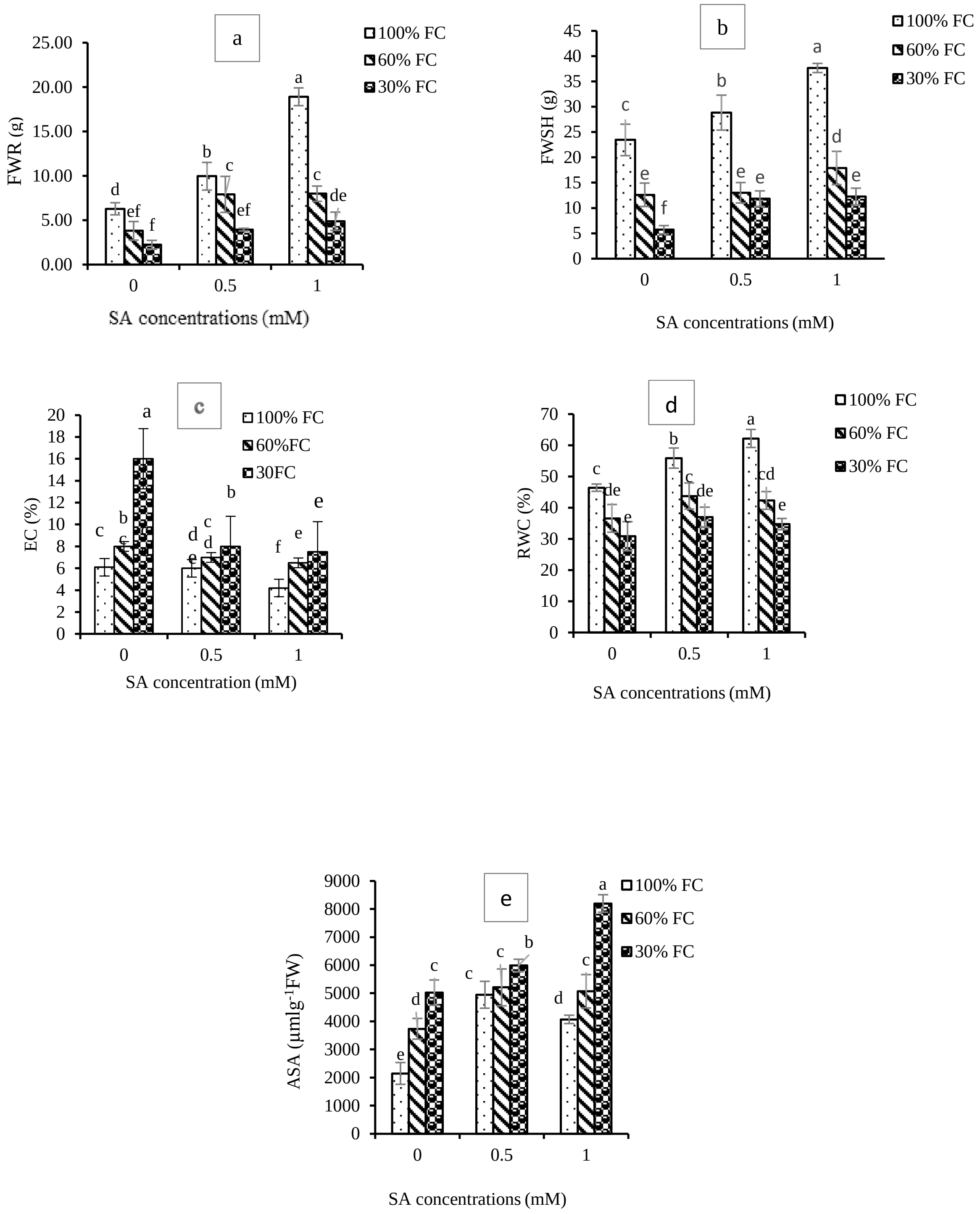

Figure 1. Effects of foliar application of salicylic acid (SA) and drought stress on some f pepper seedlings traits; a) FWSH, b) DWR, c) EC, d) RWC, and e) AsA were the abbreviations of fresh weight of shoot, dry weight of root, electrical conductivity, relative water content, and ascorbic acid, respectively

AsA

Different concentrations of SA significantly enhanced the amount of AsA in the pepper seedlings under drought and control conditions (Table 4, Figure 1e) and this increase was greater in the plants treated with SA under drought stress with the 30% field capacity. Interaction of drought stress with SA was also significant but the highest amount of AsA belonged to the drought stress of 30% field capacity (Figure 1e).

Total phenolic content

The drought had a significant effect on the total phenolic content of the pepper plants at 60% field capacity (Table 4). After applying different concentrations of SA, total phenolic content was significantly increased as compared to the control conditions (Table 4). The interaction of drought with SA concerning total phenolic content was significant and the highest amount of total phenol content was observed in the pepper plants at the 30% field capacity and the SA concentration of 1 mM (Figure 2a).

Antioxidant capacity

The antioxidant activity was remarkably enhanced in the pepper seedlings by imposing drought stress as compared to the control conditions (Table 4). Also, the application of foliar SA significantly increased the antioxidant activity (Table 4). The interaction of drought stress with concerning antioxidant activity was significant. There was no significant difference between drought stress levels at 0 and 0.5 mM of SA but antioxidant activity increased significantly with increasing drought at the SA concentration of 1 mM (Figure 2b).

Table 4. Means of biochemical characteristics as affected by salicylic acid and drought stress in pepper

Factors	AC (%)	TPC (mg of GAE g^{-1}FW)	ASA (µmolg^{-1}FW)	PPO (unit g^{-1} min^{-1})	APX (unit mg^{-1}protein)
Salicylic acid					
0mM (control)	15.49c	1.76c	3.63b	37.88c	28.86c
0.5 mM	37.99b	4.22b	5.38a	44.00b	54.93b
1 mM	47.14a	5.48a	5.77a	59.83a	77.83a
Drought stress					
0 (control)	26.40c	3.32b	3.72c	39.07c	42.83c
60%	32.27b	3.45b	4.67b	47.85b	53.94b
30%	41.95a	4.69a	6.40a	54.79a	64.85a

Means with the same letters in each column for each factor indicate no significant difference at the 5% probability level using Duncan’s multiple range test; AC, TPC, ASA, PPO, and APX were the abbreviations of antioxidant capacity, total phenolic content, ascorbic acid, polyphenol oxidase, and ascorbate peroxidase, respectively

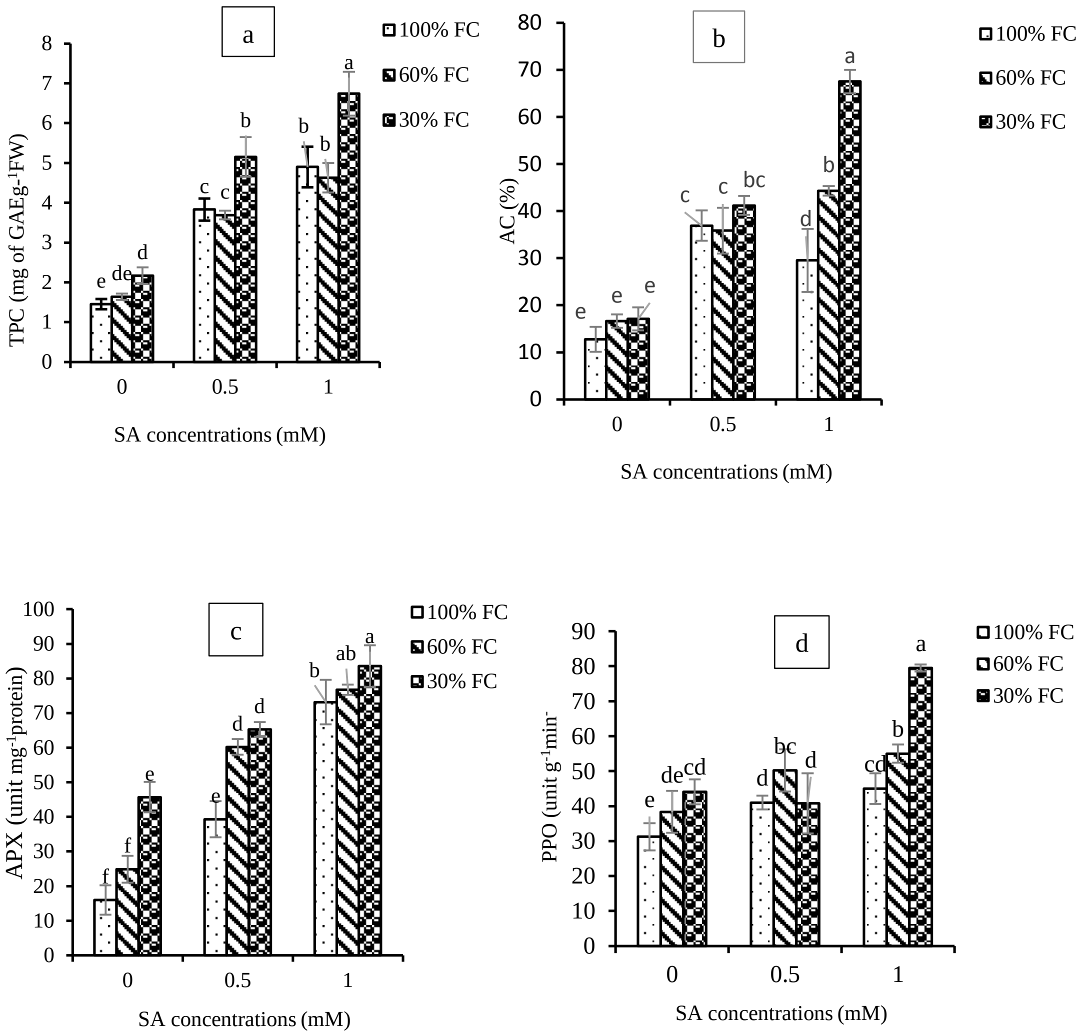

Figure 2. Effects of foliar application of salicylic acid (SA) and drought stress on some pepper seedlings' traits; a) TPC b) AC, c) APX, and d) PPO, were the abbreviations of total phenolic content, antioxidant capacity, ascorbate peroxidase, and polyphenol oxidase, respectively

Antioxidant enzyme activities

To inhibit ROS in the pepper seedlings under drought stress, their antioxidant system was activated and the enzymes such as PPO and APX were significantly increased under drought conditions. The SA treatment also increased the amount of these enzymes as compared to the control (Table 4). The interaction of drought with SA concerning antioxidant enzymes was significant and the highest amount of PPO and APX was obtained in the pepper plants at the SA concentration of 1 mM and the 30% field capacity and the (Figure 2c, 2d).

Discussion

Drought is one of the main factors restricting the growth and physiological processes of plants (Siddique *et al.* 2001; Still and Pill 2004; Wang and Huang 2004; Wahid and Rasul 2005; Farooq *et al.* 2009; Shen *et al.* 2014). Types of plant species and genotypes, duration and severity of the drought stress, and the plant's developmental stage play a role in drought stress response. On the other hand, plant growth regulators can be used to maintain proper water balance in the plant under drought conditions (Fahad *et al*. 2017).

In the present experiment, drought decreased growth characteristics such as fruit length, fruit diameter, and shoot and root growth of pepper seedlings, while SA generally increased them under drought stress (Table 2). The decrease in growth is regarded as the plant response to moisture depletion (Efeoglu *et al*. 2009) and severe water deficit affects cell elongation (Nonami 1998). However, improvement of the growth of the pepper plants by the SA application indicates its involvement in the stress tolerance and alleviating the adverse effects of drought (Senaratna *et al.* 2000). A study by Fariduddin *et al*. (2003) showed that the external spray of SA enhanced the growth rate in *Brassica juncea* plants. Other studies indicated that SA was involved in the enhancement of nutrient uptake (Yildirim *et al*. 2009), induction of root formation (Shen *et al*. 2014), and increased cell division in the apical region of the root meristem eventually resulting in the increment of plant growth (Sakhabutdinova *et al*. 2003).

Severe drought stress reduced LAI significantly (Table 3). This decrease in leaf area most likely resulted from the lower light interception and a consequent decrease in photosynthesis (Yordanov *et al*. 2003). However, SA increased LAI significantly at the concentration of 1 mM (Table 3). Senaratna *et al*. (2000) indicated that the application of salicylic acid in bean and tomato plants could induce stress tolerance which is consistent with our findings on pepper seedlings.

The results of our study showed that chlorophyll content decreased significantly in the pepper seedlings under severe drought stress (Table 3). Razavizadeh *et al*. (2004) also reported a decrease in chlorophyll content under drought stress and accumulation of phenolic compounds, flavonoids, and anthocyanin in the *Carum copticum* plants. The decrease in the chlorophyll content by imposing the drought stress was also observed by Sánchez-Blanco *et al*. (2004) in the *Rosmarinus officinalis* plants. Although the chlorophyll content increased with the application of SA, the change was not significant (Table 3). However, Tang *et al*. (2017) reported that salicylic acid spray increased chlorophyll content under drought conditions.

In this study, RWC decreased on pepper seedlings under drought stress conditions but the SA leaf spray in these plants increased RWC (Table 3). RWC is a measure of assessing the physiological status of water in stressed plants. Several studies have shown that SA can lead to an increase in RWC under drought stress conditions (Kadioğlu *et al*. 2011; Alam *et al*. 2013; Ying *et al*. 2013).

The amount of electrolyte leakage increased with the increase in drought stress (Table 3). Electrolyte leakage is the indication of damage to the cell membrane (Kraus and Fletcher 1994). The disruption of the cell membrane under environmental stress can be explained by the increase in permeability and ion leakage from the membrane (Gupta *et al*. 2000; Guo *et al*. 2006). In this study, SA reduced the electrolyte leakage of the pepper plants (Table 3). Korkmaz *et al*. (2007) and Ying *et al*. (2013) also reported a reduction in electrolyte leakage by imposing the SA treatment. Some studies stated that the decrease in electrolyte leakage using SA can be related to the amelioration of the antioxidant defense system under drought stress (Liu *et al.* 2016; Abbaspour and Ehsanpour 2016).

Our study indicated that AsA content significantly increased under drought stress and exogenous SA played a significant role in enhancing non-enzymatic components such as AsA (Table 4). Drought stress not only slows the growth of plants but also increases the concentration of some metabolites such as AsA (Shan *et al*. 2011). Zhou *et al*. (2009) indicated that the use of SA resulted in the increased AsA content under mercury toxicity and prevented oxidative stress in roots of alfalfa. Increased AsA content plays an effective role in scavenging ROS via the APX reaction (Caverzan *et al*. 2019).

Phenols content increased due to imposing both drought and SA treatments (Tables 4). Khalil *et al*. (2018) also showed that total polyphenol content in *Thymus vulgaris* L. plants increased compared to control under salicylic acid as well as drought stress. The phenols' content is an indicator of assessing environmental stress tolerance and improving plant metabolism (Sharma *et al*. 2019). Polyphenols can tolerate stress in plants through protection from oxidative damage (Agati and Tattini 2010).

Our observations showed that the amount of antioxidant capacity increased with increasing SA concentration and drought levels in the pepper plants (Table 4). The application of appropriate concentrations of SA reduces the harmful effects of oxidative stress by improving antioxidant capacity as well as the synthesis of protective compounds in plants (Hayat *et al.* 2007). Abbaspour and Ehsanpour (2016) showed that plants exposed to drought stress exhibited greater total antioxidant capacity at higher PEG concentrations. Furthermore, salicylic acid increased total antioxidant capacity in the control and drought-tolerant plants. These findings are consistent with our results in this experiment.

Drought stress and salicylic acid treatments increased the amount of APX and PPO enzymes in pepper plants (Table 4). Antioxidant enzymes are important components in ROS elimination (Navrot *et al*. 2007; Qados 2015). Foliar spray of maize plants with SA increased the activity of APX antioxidant enzyme under cadmium stress (Krantev *et al*. 2008). Kang *et al*. (2003) indicated that the increased activity of APX antioxidant enzyme in cold conditions was due to the increase in H_2O_2 which increased plant tolerance to the cold stress. Siddika *et al*. (2015) observed that the activity of PPO enzyme in wheat was enhanced under high-temperature stress and reduced the oxidative damage in *Basella alba*. Thus, PPO can be regarded as an index of adaptation to adverse environmental conditions.

Conclusion

The results of the current study on pepper showed that SA treatment plays an essential role in plant drought tolerance. Foliar application of SA by inducing an antioxidant system in pepper seedlings reduced the harmful effects of drought conditions and improved plant growth.

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Conflict of interest

The authors declare that they have no conflict of interest.

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تاثیر کاربرد خارجی اسید سالیسیلیک بر تحمل به تنش خشکی در گیاه فلفل دلمه‌ای (*Capsicum annuum* L.)

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چکیده

به منظور بررسی تاثیر تنش خشکی بر ویژگی‌های فیزیولوژیکی و شیمیایی فلفل دلمه‌ای و اثر تیمار اسپری اسید سالیسیلیک بر کاهش اثرات منفی این تنش، آزمایشی در قالب فاکتوریل بر پایه طرح کاملاً تصادفی در گلخانه انجام شد. سطوح مختلف تنش خشکی شامل ۳۰٪ ظرفیت زراعی (تنش شدید)، ۶۰٪ ظرفیت زراعی (تنش متوسط) و شرایط غیر تنش (شاهد) بودند. استعمال تیمار اسپری اسید سالیسیلیک نیز در سه سطح صفر (شاهد)، ۰/۵ و ۱ میلی مولار در مرحله چهار برگی صورت گرفت. نتایج نشان داد که تنش خشکی سبب کاهش وزن تر و خشک شاخساره و ریشه، میزان آب نسبی برگ، طول و قطر میوه، میزان کلروفیل و سطح برگ و افزایش هدایت الکتریکی، ظرفیت آنتی اکسیدانی، میزان فنول کل، آسکوربات، پلی فنول اکسیداز و آسکوربات پروکسیداز شد. در این آزمایش بعد از اسپری برگی با اسید سالیسیلیک، هدایت الکتریکی کاهش و سایر متغیرهای بالا افزایش یافت ولی افزایش شاخص کلروفیل. وزن خشک شاخساره و وزن تر ریشه معنی‌دار نبود. با توجه به نتایج حاصل، مشخص شد که اسید سالیسیلیک سبب تعدیل اثرات منفی تنش خشکی در گیاه فلفل دلمه‌ای می‌شود.

واژه‌های کلیدی: آسکوربات پروکسیداز؛ پلی فنول اکسیداز؛ ظرفیت زراعی؛ فنول کل؛ کلروفیل؛ هدایت الکتریکی